Embyronic Nicotine Exposure and Its Impact on Spinal Neuron Development in Zebrafish

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EMBRYONIC NICOTINE EXPOSURE AND ITS IMPACT ON SPINAL NEURON DEVELOPMENT IN ZEBRAFISH

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by

Latoya Telameca Paul
B.S., Southern University, 2003
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LIST OF ABBREVIATIONS

aat- anti-acetylated tubulin
ACh- acetylcholine
AChE- acetylcholine esterase
AD- Alzheimer’s disease
ADHD- attention deficit hyperactivity disorder
CaP- caudal primary motoneuron
ChAT- choline acetyltransferase
CNS- central nervous system
COPA- commissural primary ascending neuron
CPG- central pattern generator
DHβE- dihydro-beta-erythroidine
DLF- dorsal longitudinal fasculi
DPF- days post fertilization
DRG- dorsal root ganglia
GFP- green fluorescent protein
HPF- hours post fertilization
ICC- immunocytochemistry
IHC- immunohistochemistry
MiP- middle primary motoneuron
mAChR- muscarinic acetylcholine receptor
nAChR- nicotinic acetylcholine receptor
PBS- phosphate buffer saline
PBST- phosphate buffer saline containing 20% Tween 20
PCD- programmed cell death
PKC- protein kinase C
pmn- primary motoneuron
PNS- peripheral nervous system
RB-Rohon-Beard
RoP- rostral primary motoneuron
SIDS- Sudden Infant Death Syndrome
smn- secondary motoneuron
TUNEL- terminal deoxynucleotidyl transferase dUTP nick end labeling
ABSTRACT

Nicotine, a drug of abuse, has been shown to have deleterious outcomes on nervous system development in vertebrates. Using zebrafish behavior as a diagnostic tool, we aimed to understand the effects of embryonic nicotine exposure on spinal neuron development. We initially quantified nicotine incorporation into zebrafish embryos. We found that only a fraction (~4 µM) of the waterborne concentration of nicotine (30 µM) was incorporated into embryos. However, this amount was enough to initiate a swim-like behavior. Also, when embryos were exposed to epibatidine and ABT 418, potent α4/β2 nAChRs agonists, the embryos exhibited a robust swim-like behavioral response similar to that of nicotine. In addition, we previously showed that nicotine can evoke a swim-like behavior in the absence of any brainstem input, suggesting that nicotine mediates its actions via a spinal mechanism. We then performed immunohistochemistry (IHC) utilizing the mAB290 antibody and a zebrafish specific β2 antibody and found that Rohon-Beard (RB) neurons express the β2 subunit of nAChRs. Thus, we hypothesized that nicotine would affect RB development. To test this, we first systematically characterized RB neurons in embryonic zebrafish using known RB cellular markers zn12, anti-hu, and anti-acetylated tubulin (aat). Early on, RB neurons form two rows in dorsal spinal cord then migrate medially towards the midline to form a linear row. We also examined the distribution of aat immunoreactivity in the peripheral processes of RB neurons. It has been reported that the distribution of immunoreactivity in the processes of RB neurons is regulated during development and can be used as an indicator of programmed cell death (PCD) (Svoboda et al, 2001). Afterwards, we evaluated the consequences of chronic nicotine exposure on RB neuron development. Altered distribution of aat in RB peripheral processes is a reliable marker for RB PCD. The distribution of att in nicotine exposed embryos revealed a more continuous distribution of aat when compared to stage-matched controls. Also, RB migration to the midline
as well as morphology appeared to be delayed. We conclude that chronic embryonic nicotine exposure can modulate RB PCD via nicotine binding to nAChRs expressed on RB neurons, thus playing a neuroprotective role on RB development.

Lastly, we accidently discovered a novel zebrafish specific primary motoneuron marker. Upon using the \( \beta_2 \) nAChR antibody, we noticed that not only RB neurons were labeled, but also primary motoneurons. To determine the stability and potential uses of this \( \beta_2 \) nAChR antibody, we systematically characterized \( \beta_2 \) antibody labeling in zebrafish ranging in age from 30 hpf to 12 dpf. We conclude that the \( \beta_2 \) antibody specifically labels primary motoneurons, namely CaP and MiP consistently during development. Therefore, we are the first to describe and show a potential primary motoneuron marker that can be used for \textit{in vivo} double labeling studies during development.
CHAPTER 1
INTRODUCTION

1.1 Nicotine

Nicotine is a plant alkaloid derived from the *Solanaceae* plant family. It is collected from the dried leaves and stems of the *Nicotiana tabacum* and *Nicotiana rustica* plants and can metabolize to form many metabolites including cotinine and nicotine-1’-N-oxide. As a naturally occurring clear liquid, nicotine is known to change to a brown-like color when burned and develop a tobacco-like odor when exposed to air. Nicotine was first discovered in the early 1800s and since then there has been a vast array of research to understand how it exert its effects on the brain and body. Originally used as an insecticide, nicotine is now one of the most commonly used addictive drugs in North America. For many years now, the use of tobacco products has been the primary method of nicotine consumption. Reports issued by the Surgeon General have concluded that cigarettes and other types of tobacco related products are highly addictive and that nicotine is the main chemical in tobacco that causes this addiction. On average, one cigarette contains about 10-25 milligrams of nicotine of which 1 to 2 milligrams are actually consumed in smokers.

Nicotine is a water and lipid soluble chemical which can be readily absorbed through the skin into the bloodstream. This drug specifically targets organs of the peripheral (PNS) and central nervous systems (CNS) and can be delivered *via* a variety of routes including: insufflation, smoking, direct inhalation, oral consumption, mastication, intravenous methods, or transdermal applications. Depending upon the method of nicotine consumption, nicotine can rapidly reach high levels in the bloodstream and subsequently in the brain given its ability to exceed and pass the blood-brain barrier. Nicotine actions on the brain produce various behavioral effects. Primarily, nicotine initiates the release of the neurotransmitter dopamine which in turn
activates the reward circuitry of the brain that regulates the feelings of pleasure and well being. Dopamine is one of the main neurotransmitters functionally involved in the brain. Research has shown that nicotine can increase the levels of dopamine within the reward pathway, thus the reason for is powerful addictive properties. Studies have shown that there is a dose dependent relationship involved in the behavioral and tolerance effects mediated by nicotine. Depending on the dosages, nicotine can behave either as a stimulant (low levels of nicotine) or a relaxant (high levels of nicotine) when administered through tobacco products.

According to the reports from the United States Department of Health and Human Services, nicotine readily crosses the placenta and has been found in amniotic fluid and the umbilical cord blood of newborns. Nicotine can also be found in the breast milk, in cervical mucus secretions, and in the breast fluid of non-lactating women who smoke (McCann, 1992; Dahlström et al., 1990; Petrakis et al., 1978). These findings have been linked to a number of developmental abnormalities seen in infants born to women who smoked while pregnant. In fact, research has shown that high doses of nicotine during the first trimester can produce teratogenic effects on the developing fetus including neurological disorders, cognitive and behavioral deficits, and morbidity (Law et al, 2003). Ongoing exposure to nicotine contributes to a number of clinical effects. For instance, nicotine has an overall effect on the cardiovascular system which is manifested as tachycardia, blood pressure elevations, brachycardia, and subsequently, sudden cardiac death (Feng et al., 2010). Nicotine also exerts its effects on the respiratory system by inducing the development of emphysema, respiratory arrest, bronchoconstriction (Maritz, 1997; Beck et al., 1986). Reports show that prenatal exposure to nicotine during the last trimester reduces fetal breathing movements. This data suggests that the nicotine affect on the respiratory system is most likely the main contributing factor in Sudden Infant Death Syndrome (SIDS; Fuller et al., 2009; Campos et al., 2009; Eugenin et al, 2008).
The actions of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs) specifically expressed in the CNS. Acetylcholine receptors in the brain (neuronal nAChR) have more of an affinity for nicotine compared to receptors in the muscle. Studies have shown that nicotine at low dosages can increase the level of both the activity and expression levels of these receptors (Huang and Winzer-Serhan, 2006; Falk et al., 2005). Long-term nicotine exposure upregulates subtype specific nAChRs in the brain including the $\alpha_4/\beta_2$ and $\alpha6/\beta_2$ nAChRs (Harkness and Millar, 2002). The ability of nicotine to modulate nAChR expression suggests that it may be able to alter normal vertebrate development.

Nicotine has been shown to have both neuro-toxic and neuro-protective effects on the CNS. The functional selectivity of nicotine depends on timing, conditions, and the specific nAChR subtypes expressed (Huang et al., 2007). For instance, recent studies have shown that chronic exposure to nicotine inhibits ACh-dependent angiogenesis, whereas acute nicotine stimulates angiogenesis (Konishi et al., 2010). In addition, researchers have shown that chronic nicotine exposure increases the function and expression of the $\alpha_4/\beta_2$ nAChRs in the dopaminergic pathway (Xiao et al., 2009), thus suggesting a possible link to Parkinson’s disease. Various studies have shown that chronic and acute nicotine exposure can have harmful effects on development by disrupting axonal pathfinding and spinal neuron development, as well as altering normal behavior and growth in vertebrates (Menelaou et al., 2009; Parker and Connaughton, 2007; Svoboda et al., 2002). Furthermore, nicotine has also been shown to have selective affects on non-neuronal cell types. Studies show that nicotine exposure can either induce cell death or promote the survival of lymphocytes and other non-neuronal cells (Oloris et al., 2009).
1.2 Cholinergic Receptors: Nicotinic Acetylcholine Receptors (nAChRs)

There are two types of acetylcholine receptors, muscarinic (mAChR) and nicotinic receptors (nAChR). Muscarinic receptors are G protein-coupled acetylcholine receptors expressed by cardiac and smooth muscle, glands, and post-ganglionic neurons within the sympathetic nervous system. They function by mediating synaptic transmission via a second messenger pathway. There are five subtypes of muscarinic receptors that have been identified; namely M1-M5. These subtypes vary in function which corresponds to the diversity of outcomes mediated by mAChRs.

Nicotinic receptors are ligand gated ion channels that are formed in a pentamer comprised of five subunits uniformly positioned around a central pore region. These nicotinic receptor subunits belong to a multigene family and are composed of either homomeric or heteromeric combinations of $\alpha_2$-$\alpha_{10}$ and $\beta_2$-$\beta_4$ subunits that “randomly” assemble to form a diverse group of receptors carrying specific functional properties. Reconstitution experiments have shown that functional nAChRs can be obtained from a minimum of two subunits ($\alpha_4$ and $\beta_2$) forming heteromeric receptors and a single subunit ($\alpha_7$) forming homomeric receptors. In addition, these experiments demonstrated that homomeric receptors $\alpha_7$-$\alpha_9$, but not heteromeric receptors could be blocked by low concentrations of $\alpha$-bungarotoxin ($\alpha$Bgt), a snake venom toxin (Elgoyhen et al., 2001). Heterologous expression studies have shown that both the $\alpha$ and $\beta$ subunits determine the functional qualities of the subunit combination. These studies have demonstrated that the expression of $\beta_2$ subunits in combination with various $\alpha_2$-$4$ subtypes form functional receptors that vary in their pharmacological properties. However, the “random” assortment of subunits could also form non-functional nAChRs and are referred to as regulatory subunits. For example, the $\beta_1$ and $\alpha_5$ subunits are unable to form a functional receptor when
solely expressed, but can only form a receptor if expressed together with an alpha/beta pair (i.e. \( \alpha_2-\alpha_4 \) and \( \beta_2 \) or \( \beta_4 \); Groot-Kormelink et al., 1998; Ramirez-Latorre et al., 1996).

Currently, 12 vertebrate nAChR subunits have been identified (Li and Burmeister, 2009). Of these, the \( \alpha_2-\alpha_7 \) and \( \beta_2-\beta_4 \) subunits have been cloned in humans and some have also been cloned in mammals and zebrafish. The remaining nAChR subunit genes have been identified in the rat and chick genome (Charpantier et al., 1998; Le Novere et al, 1996). nAChR subunits are separated into two functional classes, the muscle type and the neuronal type. The neuronal type consists of the \( \alpha_2-\alpha_{10} \) and \( \beta_2-\beta_4 \) subunits whereas, the muscle type consists of the \( \alpha_1, \beta_1, \delta, \gamma, \) and \( \varepsilon \) (embryonic form) subunits. Additionally, the receptors in the muscle are located at the neuromuscular junction where they mediate excitatory postsynaptic potentials (EPSP) by increasing the permeability of sodium and potassium ions in the postsynaptic cell of open, ligand-bound channels. Receptor subtypes located in autonomic ganglia include \( \alpha_3 \) and \( \beta_4 \) and their function is similar to the muscle type. Also, subunits expressed in the CNS (mainly \( \alpha_4/\beta_2, \alpha_3/\beta_4, \) and \( \alpha_7 \) homomeric) particularly in the brain play a role in post and pre-synaptic transmission. There are various chemicals that function as either agonists or antagonists of nAChRs. The former includes acetylcholine, nicotine, and epibatidine (\( \alpha_4/\beta_2 \) specific) to name a few, and the latter includes \( \alpha \)-bungarotoxin, \( \alpha \)-conotoxin, tubocurarine, mecamylamine.

The agonist binding site of neuronal nAChRs is composed of amino acid residues of both the alpha and beta subunits of heteromeric receptors or two alphas in homomeric receptors which allow nAChRs agonists, such as acetylcholine and nicotine and more competitive antagonists such as DH\( \beta \)E and d-tubocurarine to bind (Li and Burmeister, 2009). When nAChR agonists bind to the receptor, the binding causes a conformational change of the subunit triggering the channel to open and allow the movement of positively charged ions across it. Typically, \( \text{Na}^+ \) and
Ca^{++} ions enter the cell as K^{+} ions move out of the cell. On the other hand, nAChR antagonists compete for the binding site of agonists resulting in the inhibition of the pharmacological actions of the agonist or antagonist. The differentiation of binding sites on nAChRs contributes to the functional diversity of nAChRs (Arias, 1997).

Continuous or repeated application of a stimulus can result in loss of responsiveness of that receptor. This phenomenon is referred to as receptor desensitization. This term can be further marked by an increased affinity of the receptor for its agonist causing a conformational change within the receptor to an inactive state (Ochoa et al., 1989). Interestingly, at low doses, certain nAChRs antagonists can evoke nicotinic responses in the form of desensitization similar to that of agonists. In fact, certain drugs have shown to have a desensitizing effect on nAChRs without initiating an agonist-like action (Xiao et al., 2006). Also, desensitization has been suggested to be mediated by specific nAChR subtypes. Studies show that desensitization following the application of acetylcholine significantly increases in Xenopus oocytes expressing $\beta_2$ nAChRs compared to other subtypes being expressed (Bohler et al., 2001). These findings suggest that the $\beta_2$ subunit of nAChR, particularly the diversity of the extracellular domain of the $\beta_2$ subunit, plays a key role in how the receptor responds to a stimulus. The mechanism of desensitization remains to be fully understood. However, previous studies suggest that the underlying properties of desensitization may be important for the regulation of synaptic efficiency and responses to cholinergic substances (Giniatullin et al., 2005). Studies show that desensitization of nAChRs can be mediated by prolonged exposure to nicotine during smoking or increased acetylcholine when chronically treating Alzheimers disease (AD) with cholinesterase blockers (Wang and Sun, 2005). Understanding the precise mechanism involved in the desensitization of nAChR could potentially lead to discovery of therapeutic targets (Buccafusco et al., 2009).
Nicotinic acetylcholine receptors are expressed very early during vertebrate development. The presence of both nAChRs and agonist, acetylcholine during the early stages of development implies that nAChRs are a vital component of normal vertebrate development. It has been shown that nAChR proteins and gene transcripts can be detected as early as 4-5 weeks of gestation in the CNS of humans (Hellström-Lindahl et al., 1998). In zebrafish, acetylcholinesterase (AChE), the enzyme that catalyzes ACh released in the synaptic cleft is present during somitogenesis (Behra et al., 2002). Also, choline acetyltransferase (ChAT), the synthetic enzyme for ACh, was detected at the neural plate stage in presumptive crest (Smith et al., 1979).

Many developmental abnormalities in the CNS have been linked to the functional properties of nAChRs in the brain and spinal cord of vertebrate models. It is known that multiple nAChR subunits are involved in cognitive function (Bencherif and Schmitt, 2002; Levin, 2002). Also, the activation of α4/β2 and α7 nAChRs in the brainstem can stimulate the release of vasopressin and indirectly effect cardiovascular functions (Moore et al., 2008). Given the complexity of nAChRs and their subunit compositions, defining the extent of subtype specificity in relation to therapeutic advances remains an ongoing process. However, studies have shown a link between subtype specific nAChR activation in the brain region and certain neurological diseases such as Parkinson’s disease and Alzheimer’s disease. In these studies, nAChRs mediate the neuroprotective function of agonist, nicotine (Picciotto and Zoli, 2008).

1.3 The Zebrafish Model in Biological Research

The zebrafish (Danio rerio) is a small tropical freshwater fish known for its popularity in home aquariums. In the past three-four decades, zebrafish has emerged as a powerful model system to study developmental toxicology, biology, and genetics. Zebrafish has a number of advantages that make it an excellent model for use in laboratories: simple quantifiable behaviors, a sequenced genome, rapid generation and development time, large transparent embryos, develop
outside of the mother, well-characterized developmental profile, and require low-cost maintenance. All of these features allow for experimental manipulation and observation, simple staining protocols, and the direct application of experimental drugs.

Zebrafish development occurs very rapidly. Compared to human nervous system development (approximately 5 weeks), development of the CNS in zebrafish can occur in a matter of hours. Initially characterized by the Charles Kimmel group at the University of Oregon, zebrafish development can be systematically outlined as six organized stages of maturation. The zygote phase occurs within the first hour of development, where embryos undergo a series of fertilization steps leading up to the cleavage stage. During the zygote period, the one cell stage is very obvious. This is the stage where most injection techniques are performed in order gain the optimal effect.

Following the zygote phase, cells enter a division period referred to as the cleavage stage. Cells continue to divide rapidly entering into the blastula phase then gastrulation between 5 and 10 hours of development. By 12 hours post fertilization (hpf), embryos have begun to undergo neurulation and a noticeable anterior/posterior axis can be observed. During the 10-24 hpf period, embryos began to increase in size and length and become segmented. Following this phase are the pharyngula and hatching stages (24-72 hpf), where embryos began to hatch from there chorions, become anatomically organized, and exhibit behavior responses to tactile stimuli. Zebrafish ranging from 0-60 hpf are commonly referenced to be in the embryonic stages. Whereas, by 72 hpf, zebrafish are said to be in the larval stages of development.

The early work that established zebrafish as an ideal model system in developmental biology was pioneered by George Streisinger and Charles Kimmel. This work has paved the way for genetic and molecular research of neuronal development in the embryonic zebrafish. Much of our understanding of the role of gene products in biological systems is made possible through the
use of mutant organisms. Until recently, large scale mutagenic screening approaches were impossible for vertebrates. However, large scale mutagenesis screening in zebrafish performed by the laboratory of Christiane Nüsslein-Volhard of the Max-Planck Institute led to the discovery of ~166 motility mutants (Granato et al., 1996). Among these were the touch insensitive mutant macho (mao) which exhibited alterations in Rohon-Beard neuronal function (Ribera and Nüsslein-Volhard, 1998), the paralytic mutant sofa potato which lacks skeletal muscle nAChRs, and the unplugged and diwanka mutants both of which have developmental and motoneuron axonal pathfinding abnormalities. Their work has paved the way for the further identification of other locomotor mutants such as the narrowminded (nrn) mutants which display altered development of neural crest structures and lack of RB neurons and slow muscle fibers (Artinger et al., 1999). In addition, the zebrafish ache mutant which abolishes AChE enzymatic activity in the embryo has been shown to cause defects in motility and neuromuscular development leading to premature death of primary sensory neurons (Behra et al., 2002).

Throughout the years, zebrafish has emerged into a suitable model organism to study a wide range of clinically relevant diseases that plague our communities. Around the mid 1900s, zebrafish were introduced as a potential model to study vertebrate development. Since that time, the zebrafish community has developed a number of techniques involving genetic manipulations and developmental profiling which aid in the understanding of many clinical unknowns. Currently, zebrafish are widely used as a model of human diseases including cardiovascular disease (Chico et al., 2008), neurodegenerative disease (Best and Alderton, 2008), and carcinogenesis (Stern and Zon, 2003). Also, many studies utilize the zebrafish model to study aspects of vision and retinal development (Kassen et al., 2008; Collery et al., 2006).
1.4 Spinal Neuron Development in Zebrafish

The development of spinal neurons in zebrafish has been characterized throughout the years (Bernhardt et al., 1990; Kuwada et al., 1990). The spinal cord of zebrafish can be divided into three distinctive regions along the dorsal-ventral axis (dorsal, medial, and ventral). Primary neurons within spinal cord can be further separated into three distinctive groups: sensory neurons, interneurons, and motoneurons. The most dorsal region of spinal cord contains a population of sensory neurons called Rohon-Beard (RB) neurons. RB neurons are mechanosensory neurons that are derived from the same neural plate domain that produces neural crest cells of the trunk region. There is some controversy as to whether or not RB neurons are neural crest cells, but to date no clear evidence has been provided to call them as neural crest cells. However, RB neurons and neural crest cells share a close relationship in that they both express levels of the L2/HNK-1, a cell adhesion carbohydrate, are both populations of migratory cells, both originate from the lateral neural plate domain, and both differentiate by a mechanism involving the transcription factor prdm1, a transcription factor that plays a role in cell fate differentiation (Kucena et al., 2006; Sobkow et al., 2006; Hernandez-Lagunas et al., 2005; Morikawa et al., 2001; Metcalfe et al., 1990). Studies have also shown that RB neurons distinctly differ from neural crest cells in their level of bHLH gene expression, which aids in the differentiation of RB neurons (Lewis et al., 2003). The first study of RB neurons dates back over half a century ago in amphibians. That work provided thorough in vivo characterizations of RB morphology, life-span, and function (Stephens, 1968; Suter, 1966; Hughes, 1957; Coghill, 1914). Based on these studies, we know that RB neurons are dorsally located in spinal cord with approximately four RB cells present within each spinal hemi segment. Similar to amphibians, RB neurons in zebrafish have ascending axons that terminate in the hindbrain region and descending axons that span across approximately ten segments. In addition, RB neurons extend
peripheral processes out into the skin over the underlying musculature where they respond to touch stimuli (Reyes et al., 2004; Bernhardt et al., 1990). RB longitudinal axons are positioned ventral to their cell bodies where they form the dorsal longitudinal fasciculus (dlf). Also, they have large cell bodies, giant nuclei, and die early during development.

RB cell death in amphibians has been shown to be related to the development of dorsal root ganglia (drg) or a change in thyroxine levels during the metamorphic climax. Dorsal root ganglia are neural crest derivatives that replace the function of RB neurons at later stages of development (An et al., 2002). Exactly how and why RB neurons die is still poorly understood.

In amphibians, most of RB neuron precursors are born between the blastula stage (2-4 hpf) and 24 hpf and become differentiated RB neurons during gastrulation (Rossi et al., 2009; Lamborghini, 1980). In zebrafish, RB neurons have been detected as early as 15 hpf in in vivo studies. At 19 hpf, RB cells begin to die via a programmed cell death process and reach peak levels of cell death around 26-34 hpf (Williams et al., 2000).

The process by which RB neurons die may involve an increase in the levels of endogenous ACh (Behra et al., 2002), the regulation of neural activity (Svoboda et al., 2001), caspase-dependent mechanisms and the expression of neutrophins (Williams et al., 2002), and reduced expression of cyclin-dependent kinase 5 (cdk5) (Kanungo et al, 2008). RB neurons undergo specific morphological changes that are indicative of their demise (Reyes et al., 2004).

RB neurons have been claimed to be a homogeneous neuronal population because certain molecular markers, namely anti-Hu, aat, and zn-12 label RB neurons. However, recent studies have shown that certain molecular markers are only expressed by a subset of RB neurons. In addition, RB neurons have been found to express different sodium channel subtypes and protein kinase C (PKC) isoforms suggesting that RB neurons are a heterogeneous neuronal population (Patten et al., 2007; Pineda et al., 2005).
RB neurons do not receive synaptic input from any other neuronal population. However, they are presynaptic to commissural primary ascending (CoPA) neurons within spinal cord (Downes and Granato, 2006) as well as hindbrain Mauthner Cells. CoPA interneurons are dorsally located in spinal cord and extend axons to contralateral spinal cord. They are easily identified by their characteristic T-shape; formed by the rostral and caudal processes projecting from their cell body and a vertically projecting, their ventral axon (Figure 1.1).

Located ventral in spinal cord are two classes of motoneurons, primary and secondary. Each class of motoneuron is identified based on their cell number, position, axonal trajectory, and time of development (Westerfield et al., 1986). Although they develop early on, they are present and functional even into adulthood. Primary motoneurons (pmn) mainly consist of three types: Caudal primary (CaP), Middle primary (MiP), and Rostral primary (RoP) (Figure 1.1). They are born early during development and are characterized by their large cell bodies, thick axons, and broad fields of innervation. They only innervate fast muscle fibers. The first pmn to extend an axon from spinal cord is the CaP motoneuron. This is followed by MiP motoneurons, which is the second pmn to extend an axon ventrally side by side with CaP. However, once the ventrally directed MiP axons reach the “choice point”, they retract and project their axons dorsally to innervate dorsal muscle fibers. RoP is the third pmn that follows the CaP and MiP axons to the horizontal myoseptum where it innervates muscle fibers. These three pmn follow two pathways of development: the common pathway and the cell-specific pathway. The common pathway states that all three pmn extend their axons between the exit point within spinal cord and the horizontal myoseptum. The cell-specific pathway states that the axons of each pmn will extend down unique individual paths. Although these three pmn follow similar paths, their development is independent (Pike and Eisen, 1990). There is also a transient population of pmn called the Variable primary (VaP) motoneuron which is equivalent to the CaP motoneuron.
There is not much known about VaP motoneurons except for the fact that they die off during the early stages of development. Moreover, pmn express the same LIM gene, but later in development, they began to express different combinations of LIM genes. These differences in expression can regulate the differentiation of pmn (Hutchinson et al., 2007; Appel et al., 1995). Secondary motoneurons (smn) differ from pmn in that they either innervate fast or slow muscle fibers, thus they can play a role in both slow and fast swimming. Compared to primary motoneurons, smn are more numerous, have smaller somata, thinner axons, smaller fields of innervation, and are born later in development. Typically, pmn are born during gastrulation later followed by smn development.

Motoneuron axonal pathfinding is complex and is regulated by a variety of different factors. Earlier studies suggested that guidance cues in the environment, either on the surface of the adaxial cells, within the extracellular matrix, or from the periphery (diffusible cues) influence axonal pathfinding (Zeller et al., 2002; Ott et al., 2001). However, pmn axons are still able to select their appropriate paths regardless of where they are positioned (Gatchalian and Eisen, 1992). Ablation studies have also shown that to a certain degree, pmn axons may guide the axonal pathways of smn to the periphery (Pike et al., 1992).

1.5 The Significance of This Work

The use of tobacco related products during pregnancy is an ongoing addiction among women in the United States and some developing countries. According to the 2004 Pregnancy Risk Assessment and Monitoring System (PRAMS) report, approximately 13% of women smoked while pregnant. These reports showed that younger, less educated, non-Hispanic, white women, and American Indian women had the most substance use during pregnancy than older, more educated, women. In fact, data from NIDA revealed that more than 17% of pregnant
women between the ages of 15 and 44 smoked, with a higher rate observed between the ages of 15 and 17.

Figure 1.1 Primary neurons in zebrafish. A representative cartoon illustration of primary spinal neuron (RB; blue), interneuron (CoPA; yellow), and primary motoneurons (CaP, MiP, and RoP; green, orange, and purple). RB and CoPA neurons extend their processes into the dorsal longitudinal fasciculus (dlf). Primary motoneurons are positioned medially in spinal cord near the ventral longitudinal fasciculus (vlf) and project their axons independently in specific regions of the musculature.

Prenatal tobacco exposure has been associated with many deleterious and long-term health effects. Studies have shown that women who smoke during pregnancy have an increased risk of experiencing premature rupture of membranes, placental abruption, and placenta previa during the course of pregnancy. In addition, smoking during pregnancy can increase the risk of miscarriages, still birth, premature delivery, and sudden infant death syndrome (SIDS). Also, babies born to tobacco users are more likely to be born with low birth weight (~25% reduction or
less than 5.5 pounds), a cleft lip or palate, and long-term cognitive and behavioral deficits (Falk et al., 2005 and DiFranza and Lew, 1995). Moreover, recent studies have shown that mother’s smoking while pregnant can lead to reduced fetal lung function and growth causing respiratory illnesses (asthma and bronchitis), reduced fetal blood flow resulting in shorter body length and decreased cranial size. Infants born to smoking mothers are more prone to develop cardiovascular disease, become addicted to tobacco products if the child begins to smoke later in life, and develop significant neurobehavioral defects including problems with vision, gastrointestinal function, and CNS function (Law et al., 2003). These risks are dramatically increased if a pregnant woman consumes more than one pack a day (20-25 cigarettes per pack) of tobacco-related products. Also, studies show that there is a dose-dependent relationship reflected in the birth weight of an infant born to a smoking mother- the more the mother smokes, the greater the reduction of infant birth weight.

Given that there are many tobacco related health disorders and diseases, research on the developmental toxicity of tobacco smoke constituents has increased dramatically. It has become globally accepted that nicotine, a main ingredient in tobacco products, is associated with the developmental abnormalities observed in babies born to smoking mothers. Nicotine can readily cross the placenta and reach higher concentrations inside the developing fetus than in the mother. The fetus is at risk of being exposed to toxic levels of nicotine by the accumulation of nicotine in fetal blood, amniotic fluid, and breast milk. Exposure to nicotine from maternal serum has been linked to many of the health effects mentioned previously including long-term neurological and behavioral dysfunctions such as Attention Deficit Hyperactivity Disorder (ADHD) (Paz et al., 2006). Nicotine has also been shown to constrict blood vessels of the umbilical cord and uterus causing low levels of oxygen to reach the fetus.
It is widely accepted that nicotine mediates its actions \textit{via} AChRs. The presence of nAChR expression early in development suggests that they play an important role in development. Past studies showed that prenatal exposure to tobacco related products effects the expression level of subtype specific nAChRs in the brain of human fetuses (Falk et al., 2005) suggesting that early exposure to nicotine disrupts the development of the cholinergic system in the brain. Also, prenatal nicotine exposure upregulates $\alpha7$ nAChR expression levels in the lungs of fetal monkeys suggesting that nicotine can effect normal pulmonary development and function through specific nicotinic receptor subtypes (Harmanjatinder et al., 2001).

Since a significant number of women continue to smoke tobacco related products during pregnancy, the number of fetuses exposed to nicotine continues to grow. Therefore, aiming to understand the mechanisms of nicotine and its actions on nAChRs during fetal development is clinically relevant.

The work presented in this thesis was done utilizing zebrafish as the model system to study aspects of nervous system development \textit{in vivo}. Given the molecular, genomic, and handling capability of this model in conjunction with optimal imaging software, we are able to study CNS development in ways never reported before. In saying this, our work signifies the ability to link behavioral studies with cellular mechanisms in order to gain insight into vital developmental processes related to clinical disease states such as human nicotine toxicity.

Specifically, we made 3 fundamental contributions to the fields of zebrafish developmental biology and neuro-toxicology. Under Chapter 3, we provide a thorough anatomical characterization of what we call a model cell within a model organism, the zebrafish RB neuron. We focused on these cells because they were found to express nAChRs early in embryogenesis. This anatomical information could potentially lay a foundation for other researchers who wish to study aspects of vertebrate neuronal programmed cell death \textit{in vivo}. 

The anatomical characterization of RB neurons in chapter 3 allowed me to address a fundamental question: Does nicotine exposure influence the development of neurons which possess nicotinic acetylcholine receptors, and if so, how? In the context of RB neurons, we showed that nicotine exposure can actually be neuro-protective. The exposure did not speed up RB cell death, it actually delayed it. This phenomenon was revealed in part, because RB neurons are a transient discrete population of neurons which were easy to study in an in vivo context.

Lastly, in the course of my studies, we used various antibodies to label RB neurons for anatomical purposes. We found that one of those, a polyclonal antibody known as anti-β2 nAChR, which our research group designed, may be the best antibody marker available to study primary motoneuron axonal trajectories in zebrafish. The characterization and utility of that antibody is presented under Chapter 5.
CHAPTER 2
CHOLINERGIC MODULATION OF EARLY EMBRYONIC MOTOR BEHAVIOR IN ZEBRAFISH

2.1 Introduction

The Zebrafish (Danio rerio) is an ideal model system for studying embryonic nervous system development. For more than a decade now, the zebrafish model was used to study neural circuits and their interactions within the CNS to produce simple motor behaviors (Fetcho et al., 2008). Recently, the zebrafish has been established as an excellent model vertebrate for examining chemical and drug toxicity in the context of human diseases (Hill et al, 2005). The convergence of motor behavior, chemical toxicity, and developmental biology, allows for an advantage in studying the effects of chemical toxicity in the context of the whole animal. In this context, we have been studying the consequences of nicotine exposure on zebrafish locomotive behavior. We found that acute exposure to nicotine can induce significant changes in early embryonic motor behaviors. These nicotine-induced changes are manifested as increases in the musculature bend rates of the embryo. This overt change in motor activity during early embryogenesis can potentially be harmful to the developing organism.

Zebrafish behavior can be distinguished by three levels of movement: spontaneous activity, touch responses, and swimming. Spontaneous activity is characterized by arrhythmic alternating contractions or bends of the trunk musculature in young embryos. This activity is known to have a “rise and fall” type of pattern in that it initiates at 17 hpf (10-15 bends/min), peaks around 19-22 hpf (15-25 bends/min), and gradually declines by 24-27 hpf (4-8 bends/min). Removing the embryos from their protective chorion has been shown to increase the frequency of these contractions without affecting the onset and peak activity (Saint-Amant and Drapeau,

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By 24-27 hpf, young embryos begin to respond to tactile stimuli to the head and tail. The swimming behavior in zebrafish can be described as the forward movement of the embryo by at least one full body length. The age of the embryo in conjunction with the region of the stimulus (head or tail) determined the frequency and amplitude of swimming. Older embryos swim more vigorously when touched on the tail (Saint-Amant and Drapeau, 1998).

Vertebrate motor outputs are produced by the use of excitatory neurotransmitters acetylcholine and glutamate by spinal neurons. Application of ACh can modulate the rhythmic motor output in the spinal cord of lampreys (Quinlan et al., 2004). In Xenopus laevis motoneurons excite interneurons with ACh, thus providing the drive to sustain swimming (Roberts and Perrins, 1995). In spinal cords of embryonic mouse and chick, the frequency of the spontaneous motor activity can be reduced by nAChR antagonists (Myers et al., 2005; Milner and Landmesser, 1999). These findings suggest that nAChRs are present in the spinal circuitry of vertebrates where they are involved in producing early locomotor behaviors.

The spinal circuitry that produces locomotion in zebrafish resembles those circuits that generate motor behaviors in other swimming vertebrates. In zebrafish, nAChRs are likely expressed by cells within spinal circuits that produce locomotive behaviors. In fact, we have previously shown that nicotine, a nAChR agonist can elicit a motor output in the absence of brainstem mechanisms further implying that nAChRs are present in the spinal circuits that generate the motor output. In this chapter, we show that acute nicotine exposure can modulate the embryonic motor output as early as 18 hpf. When nicotine is applied exogenously, the early motor output alternates in a left–right–left synchronous fashion. The ability of nAChR agonist to activate early embryonic motor behaviors at the level of the spinal cord in addition to producing early rhythmic locomotive activity implies that nicotine is activating nAChRs within an embryonic central pattern generator. In addition, we wanted to know what specific nAChRs
played a role in the nicotine mediated behavior response. By exposing embryos to two potent α₄/β₂ nAChRs agonists, epibatidine (an alkaloid found on the skin of the poisonous frog, Epipedobates tricolor) and ABT 418 (a clinical drug developed by Abbott laboratories used to treat individuals with ADHD and Alzheimer’s disease), we were able to evoke a “swim-like” behavior response that mimicked the nicotine response. These results suggested that nicotine likely mediates its actions through α₄ or β₂ containing nAChRs. This prediction was confirmed based on immunohistochemical analyses which revealed that the β₂ nAChR subunit was indeed expressed on RB neurons. Also, we characterize the actual amount of nicotine incorporation in the embryo from the waterborne concentration. We show that only a small fraction of nicotine is taken up by the embryo to elicit a behavioral response.

2.2 Materials and Methods

**Zebrafish Maintenance**

Embryos were collected from natural spawnings of adult zebrafish according to the Zebrafish Book (Westerfield, 1995). The embryos used in this study were obtained from wildtype (Ekwill Waterlife Resources, Gibsonton, Fla.) and transgenic [Tg (isl3: GFP); expresses GFP in RB neurons] zebrafish lines. Adult fish were housed at 28.2°C on a 14 h light/10 h dark cycle. Fertilized eggs were collected from paired or grouped spawnings, rinsed several times, and transferred to a Petri dish containing embryo medium prior to experiments. All embryos were raised at ~28°C until 12-13 hpf and afterwards were raised in laboratory conditions at ~25-26 °C unless otherwise noted.

**Drug Administration**

The (-) - nicotine used in this study was purchased from Sigma (St. Louis, Missouri, USA, catalog # N3876-5 ml). Nicotine stock solutions were made in distilled water and then diluted in embryo medium to obtain final concentrations ranging between one and 30 μM. In
some exposure paradigms, the nicotine effect on motor output was significantly reduced. We hypothesized that the potency of the main nicotine stock was decreasing over time. We examined this possibility by exposing 23-24 hpf embryos to 30 μM nicotine made from either the “clear” nicotine stock or the “tinted/yellowish” nicotine stock (refer to Figure 2.2). Embryos exposed to nicotine made from the “clear” nicotine stock had a robust motor output. Whereas, embryos exposed to nicotine from the “tinted/yellowish” nicotine stock had a significantly reduced motor output. Therefore, we conclude that due to light and air sensitivity of the nicotine stock, the quality and potency of nicotine can be affected. This could lead to experimental variability.

Tritiated nicotine (−)-[N-methyl-3H] nicotine) was purchased from Perkin Elmer Life and Analytical Sciences (Wellesley, MA, USA), with a specific activity of 60.0 Ci/mmol in ethanol and stored at -20 ºC. The activity of [3H]-nicotine was measured with high efficiency via liquid scintillation counting (Tri Carb 2900TR, Perkin Elmer). Quench correction with the tSIE/AEC indicator was used to determine radioactivity. In each experiment, radioisotope (0.2 μCi/L-(−) [N-methyl-3H]) was added to a known concentration of the “cold” nicotine (Sigma) stock solution. This final cocktail is referred to as [3H]-nicotine.

Epibatidine and ABT 418 stocks were made in distilled water daily and stored at 4ºC. Prior to experiments, initial stocks were diluted in embryo medium to concentrations ranging between 50-2000 nM for epibatidine and 100 μM for ABT 418.

**Behavior Analysis**

Embryos (18-29 hpf) were placed in 50-100 mm diameter Petri dishes containing embryo medium and videotaped using a Kohu video camera mounted to a Zeiss SV6 dissecting microscope. The embryos’ motor output (bends of spinal musculature) were recorded in control embryo medium for 3-20 min. Embryos were then transferred to another Petri dishing containing nicotine (5-30 μM) made with embryo medium and the motor output was recorded again. In
some cases, the embryos were transferred to a third Petri dish containing control embryo medium for an extended wash period and their motor output was recorded. The motor behavior was quantified by manually counting the tail bends of each embryo using a clicker and a time log worksheet. Experiments were performed on embryos while in their chorions. All behavioral recordings were performed in laboratory conditions (~25-26°C).

**Immunohistochemistry-Rohon-Beard and nAChR Expression**

Whole mount immunohistochemistry was performed using a modified version of our previous published protocol (Pineda et al., 2006; Svoboda et al., 2002; 2001). Tg (isl3: GFP) zebrafish embryos were first fixed in 4% paraformaldehyde overnight at 4 °C and then stored in PBST (PBS containing 0.1% Tween 20). After permeablisation, they were incubated overnight at 4 °C in either the β2 nAChR zebrafish specific primary polyclonal antibody (referred to has β2 for now on; Welsh et al., 2009) or mAb 290 (monoclonal antibody 290). Primary antibodies were prepared in NCS block at dilutions of 1:250 and 1:500. The next day, embryos were washed in PBST for 60-90 minutes and incubated for an additional 60-90 minutes in a fluorescent secondary antibody conjugated to Alexa 546 (Molecular Probes, Eugene Oregon) at 1:5000 and 1:1000 dilutions. After incubation, embryos were washed again in PBST for 60-90 minutes and prepared for image acquisition. Images were captured using a Zeiss Axiovert 200 M inverted microscope (Thornwood, NY) equipped with epifluorescence, a Zeiss ApoTome and an ORCA-ER digital camera (Hammamatsu, Japan).

**Accumulation of [³H]-nicotine**

The uptake of [³H]-nicotine was measured in embryos ranging in age from 22 to 24 hpf. All embryos were exposed to varying waterborne concentrations (1, 5, 10, 15, and 30 μM) of [³H]-nicotine. After incubations, the embryos were transferred from Petri dishes to 100 mm nylon filters (BD Falcon, Franklin Lakes, NJ, USA) and washed using a filtration apparatus. By
placing embryos in these filters, easy removal from fluxing solution and minimal handling stress was achieved.

**Flux Methodology**

**Influx**- Embryos were placed in Petri dishes containing radioisotope and fluxed for 5-10 minutes depending on the experiment. In some experiments, the behavior of the embryo was simultaneously monitored during the flux period using a Kohu video camera mounted to a Zeiss Stemi 2000C dissecting microscope and videotaped. Following the flux period, embryos were transferred to nylon filters within a Petri dish and rinsed up to 5 times with embryo medium. They were then immediately transferred to 1.5 ml centrifuge tubes for further processing (2-3 embryos per tube).

**Efflux**- Following the flux period (refer to Influx methodology), embryos were divided and placed into individual Petri dishes (minimum of 6 embryos per dish) containing 3 ml of embryo medium (wash solution) and then removed in 30 minute intervals. At the end of each wash interval, embryos were transferred to nylon filters on a filtration apparatus and briefly rinsed with embryo medium. Afterwards, they were transferred to 1.5 ml centrifuge tubes (2-3 embryos per tube) for further processing.

**Sample Processing**

For all experiments, water samples were taken at the start and end of the flux period for monitoring of waterborne [³H]-nicotine. Following radioisotope fluxing, embryos were transferred to a filtration apparatus and washed with embryo medium to remove external unincorporated isotope. Embryos were then transferred to centrifuge tubes and weighed using a micro-balance. Afterwards, embryos were digested overnight in 1N trace-metal grade HNO₃ acid in an incubator at 60ºC. The next day, digests were centrifuged to collect supernatants. Ultima Gold scintillant (Perkin Elmer) at 5 ml was added to the supernatants and radioactivity levels
were then measured by liquid scintillation counting. Specific activities were used to convert radioactivity into nmol values. These absolute uptake rates were then expressed per milligram of wet weight.

**Statistics**

All values are presented as means ± standard error of the means (SEM). Analysis of Variance (one-way repeated measures analysis of variance with Holm Sidak Correction) or Student T-test (SigmaStat 3.5) were performed to test for significance, which was assigned if the p value was < 0.05.

**2.3 Results**

**Exogenous Cholinergic Agonists Activate a Motor Output in Zebrafish Embryos**

Zebrafish embryos demonstrate a repertoire of locomotive behaviors throughout embryonic development. The earliest behavior, spontaneous bends of the musculature, occurs as early as 17 hpf. Robust responses to tactile stimulation appear at 27 hpf. However, if young embryos (21 hpf) are repeatedly stimulated with a probe, an increase in bends of the musculature can occur.

Systematic evaluations of embryonic bend rates of embryos in the chorion are scarce in the literature. The work of Sipple (1998) simultaneously monitored embryos in the chorion over time while using computer software to quantify the bend rates. In that work, the embryonic muscle bend rates peaked at 19-20 hpf as reported by Saint-Amant and Drapeau, but the maximum peak number of bends occurred at approximately 22 bends per minute. In this study, embryos were videotaped each hour from 17-25 h at 28°C and their motor behavior was analyzed. Here we show that the muscle bend rates peak at around 19-20 hpf with an average bend rate of approximately 26 bends per minute. For embryos raised at 25-26°C, the bend rates still peaked at 19-20 hpf, but the frequency declined (Figure 2.1).
It has been shown in vertebrates that the rate of spontaneous motor activity can influence development (Hanson et al., 2008; Hanson and Landmesser, 2006; Borodinsky et al., 2004; Spitzer et al., 2004; Hanson and Landmesser, 2004). In acute exposure paradigms, nicotine can dramatically increase the bend rates of the spinal musculature in zebrafish embryos (Figure 2.2). A dose response was apparent with higher waterborne concentrations of nicotine producing a more robust motor output. The increase in the musculature bend rate of nicotine had a significant increase at 22 hpf (Figure 2.3). We hypothesized that this increased activity alone could alter aspects of cell biology in the developing embryo.

**Desensitization of the Nicotine Induced Response**

When the dose response experiments were performed, we noticed that if the embryos were exposed to high concentrations of nicotine first, then exposed to lower nicotine concentrations following a wash-out period, they typically did not show an increase in the musculature bend rates. Therefore, this suggested a potential desensitization phenomenon was occurring in the acute exposure paradigms.

Desensitization experiments were performed in 22-27 hpf embryos. Exposure to 30 µM nicotine caused approximately a 4-fold increase in the musculature bend rate of 25 hpf embryos in the chorion (Figure 2.4). However, after a two hour wash period, 30 µM nicotine failed to elicit the same magnitude response in 27 hpf embryos. This attenuated response to nicotine was not associated with the developmental stage; since stage matched 27 hpf control embryos had a robust response to nicotine (Figure 2.4, bottom; open circles).

**Nicotine Influx and Efflux in Zebrafish Embryos**

Radioisotopic flux experiments were performed to assess the amount of nicotine incorporation in embryos during exposures, and to determine the clearance rates during the washout phases of the experiments. Similar types of experiments have been performed analyzing
dioxin uptake in embryonic zebrafish (Henry et al., 1997). Zebrafish embryos were bathed in 
$[^{3}\text{H}]$-nicotine and the accumulated amount of nicotine was determined (Figure 2.5). At 30 µM, 
$[^{3}\text{H}]$-nicotine was rapidly incorporated in embryos reaching a steady state burden with a 10 minute exposure (Figure 2.5, A). These data validate results obtained from behavioral assays, 
where the nicotine induced behavioral response peaked within 5 minutes of exposure onset. Subsequent influx exposures showed that $[^{3}\text{H}]$-nicotine uptake increased as the external nicotine concentrations increased up to 30 µM. However, in each case, the amount of incorporated $[^{3}\text{H}]$-nicotine was less than the waterborne concentrations in the external medium (Figure 2.5, B).

The radioactive nicotine incorporation assays were then combined with the behavioral assays. After embryos were fluxed in the $[^{3}\text{H}]$-nicotine and the behavior recorded, $[^{3}\text{H}]$-nicotine uptake was quantified (Figure 2.5, C). In this example, only a fraction of the waterborne concentration of nicotine was incorporated in the embryo. However, this was a sufficient amount of nicotine to induce a robust motor output.

In this chapter, we observed a “desensitization” of the nicotine induced behavior. One explanation for this would be if nicotine was incorporated into the embryo and then was not cleared in a timely manner. If residual nicotine was left in the embryo that nicotine would potentially be able to bind to and activate nAChRs, causing the observed dampening of the behavioral response. To test this hypothesis, nicotine efflux experiments were performed to determine the clearance rates of nicotine from embryos during washout phase. Following the $[^{3}\text{H}]$-nicotine influx period, embryos were found to clear nicotine quickly over the first 180 minutes of wash, after which internal levels plateaued. Even up to 5 hours following the exposures, an equivalent of 1 µM nicotine was still left in the embryo (Figure 2.6). In previous experiments not shown here, we exposed embryos to 30 µM of $[^{3}\text{H}]$-nicotine for 5 min while simultaneously monitoring the behavior. After a 1-2 h wash period, the efflux rate for half of
those embryos was determined. The other half of the embryos were exposed to $[^3]$H-nicotine for a second time. Again, the nicotine-evoked response was dampened when compared to stage matched controls now exposed with $[^3]$H-nicotine for the first time. Moreover, an equivalent of 1 µM nicotine was still left in the embryo 2 h following the exposure.

**Residual Nicotine and Desensitization**

We wondered if the residual 1 µM nicotine was enough to inhibit the ability of an additional application of nicotine to elicit a motor output. Using the flux assays mentioned earlier, we determined that when embryos were exposed to a waterborne concentration of 1 µM nicotine, the nicotine would be completely incorporated and equilibrated into the embryo within the first two hours of exposure onset (Figure 2.7, A). So, in a behavioral paradigm we desired to mimic the level of residual nicotine that was not cleared from embryos during the wash periods in the acute exposures (refer to figure 2.6). Embryos were pre-incubated in 1 µM nicotine for 4 hours. When challenged with 30 µM nicotine, they had a significantly reduced motor output (Figure 2.7, B). In this experiment, the embryos pre-incubated in nicotine had a robust response when placed in embryo medium containing high KCl. These results suggest that the residual nicotine was likely responsible for the desensitization effect observed earlier in this study and is likely antagonizing nAChRs on neurons that play a role in activating the embryonic motor behavior.

**Spinal Neurons Express nAChRs**

Based on previous studies, we found that nicotine exposure in developing zebrafish embryos can produce two behavioral phenotypes: an increase in the early embryonic motor behavior and paralysis in older embryos. Embryos exposed chronically to 33 µM nicotine exhibit almost complete paralysis by 72 hpf (Svoboda et al., 2002). Also, during the early minutes of the exposure, the embryos exhibited an increase in their embryonic motor output. At 18 hpf, 30 µM
nicotine (embryos exposed while in the chorion), caused a doubling in the embryonic tail bend rate (Figure 2.8, A).

According to the data presented in this chapter, nicotine is most likely acting through nAChRs. We then wanted to know which nAChR combinations were likely mediating the nicotine response. Using behavior as a diagnostic tool, we monitored the behavioral output of embryos exposed to specific nAChR agonists, namely epibatidine and ABT 418. Similarly, when embryos (20-21 hpf) were exposed to epibatidine, a potent α4/β2 nAChR agonist, we observed an increase in the tail bend rate that resembled the nicotine-induced behavioral response (Figure 2.8, A-B). A dose response was apparent with higher concentrations of epibatidine producing a more robust motor output at 22 and 24 hpf (Figure 2.8, C-D). The ability of nicotine or epibatidine to induce an abnormal increase in behavior suggests that nAChRs are functional early on, but are not immensely activated by endogenous ACh. Likewise, ABT 418, a potent α4/β2 nAChR agonist in mammals, dramatically increased the embryonic motor output at 22 and 24 hpf (Figure 2.9). The epibatidine and ABT 418 induced increase in the musculature bend rate resembles the nicotine induced effect. Overall, these results imply that nicotine is likely functioning through subtype specific nAChRs, possibly those containing an α4 and/or β2 composition.

Candidate cell types that could express nAChRs that mediate the nicotine induced response include spinal interneurons and RB cells. This is based on previous work demonstrating that nicotine can evoke a motor output in the absence of brainstem mechanisms (Thomas et al., 2009). β2 containing nAChRs are considered high-affinity receptors for nicotine binding (Picciotto et al., 1998). To determine if β2 containing nAChRs were present early in zebrafish development, isl3 embryos which express GFP in RB neurons were labeled with either (mAb
a monoclonal antibody which recognizes the $\beta_2$ nAChR subunit in mammals or a polyclonal antibody directed against the $\beta_2$ nAChR in zebrafish (Figure 2.10, A-B). We found that RB neurons did indeed label with the $\beta_2$ nAChR antibody.

![Graph showing embryonic motor output in zebrafish](image)

Figure 2.1 The embryonic motor output in zebrafish. A: The motor output is quantified as the number of bends per minute that occurred in 5 minute epochs between 18 hpf and 25 hpf whole the embryos were in their protective chorions. The experiment was performed for embryos raised continuously at 28°C (n=16 embryos; behavior analyzed at 28°C) and embryos raised at 25°C after 13 hpf (n=15 embryos; behavior analyzed at 25°C). The black dashed circle highlights the 22 hpf time point in each experiment for purposes of comparison. Asterisks denote significance with $p$ value < 0.05, Student $T$-test. Comparisons of bend rates were made between the two groups of embryos at the individual developmental time points.

2.4 Discussion

In this chapter, we outlined the nicotine-induced modulation of early embryonic motor behaviors in zebrafish. Also, this work provides insight into the kinetics of nicotine accumulation in zebrafish embryos during early development. We provide evidence that nicotine, at low ($\mu$M) concentrations, leads to altered behavior associated with nAChRs expressed on spinal neurons, namely RB neurons.
Figure 2.2 Variability in the potency of nicotine stock. 23-24 hpf embryos were exposed to 30 \(\mu M\) nicotine (black bar) made from either the “clear” nicotine stock (n=10; clear circles) or the “tinted/yellowish” nicotine stock (n=10; orange circles). Nicotine “clear” induced an increase in the tail bend rate compared to controls (n=20; black circles) and the nicotine “tinted” induced bend rates.

Figure 2.3 Dose-response of nicotine induced motor behavior. ~22 hpf embryos (n=18, dechorionated) were videotaped for 5 minutes. After this initial recording, the embryos were separated into 3 groups. They were then exposed to 5, 15, or 30 \(\mu M\) nicotine at minute 5 (n=6 for each concentration). Video stills from one of the embryos exposed to 15 \(\mu M\) nicotine are shown to the right. Asterisks denote significant difference from control bend rates. \(\dagger\) denotes significance between all exposure conditions (p value < 0.001, Anova).
Figure 2.4 Desensitization of the nicotine induced motor output. A: (Top) behavior quantification from 25-27 hpf embryos (n=6) in the chorion. Application of 30 μM nicotine occurred at the first black bar resulting in an increased bend rate. After 120 min, the bend rate almost returned to baseline. The second application of 30 μM nicotine did not produce an increase in the bend rate. Stage matched controls not previously exposed to nicotine (open circles) respond to the nicotine exposure with an increased bend rate. (Middle) minutes 1 through 10 are shown as expanded view. The nicotine exposure occurred between minute 5 and minute 10. (Bottom) minutes 131 through 140 are shown as expanded views. The nicotine exposure occurred between minute 135 and minute 140. Asterisks denote significant difference from bend rates obtained prior to minute 135. ‡ denotes significant difference in bend rates when comparing the experimental (exposed to nicotine at minute 5) and control embryos (not previously exposed to nicotine) at the second application of nicotine (p value < 0.001, Anova).
**Embryonic Motor Behavior in Zebrafish**

Within the past 10-15 years, the zebrafish system has emerged as a prominent vertebrate model system to study the development of motor circuits, as well as the cellular and molecular factors that influence locomotor production (Chen et al., 2008; Fetcho et al., 2008; Fetcho, 2007; McDearmid et al., 2006; Bhatt et al., 2004; Granato et al., 1996). In recent studies using written computer software, the behavior of larval zebrafish swimming simultaneously in a Petri dish was easily tracked (Burgess and Granato, 2007). The behavior in these “older” fish is readily quantifiable as it is easy to keep track of the heads and tails of swimming larva with high speed cinematography.

To date, very few research groups have quantified the bend rates in young embryos with computer algorithms because the embryos tend to “tumble” within their chorions making their heads and tails sometimes difficult to track. The work of Sipple and colleagues (1998) quantified the early embryonic motor behaviors of zebrafish embryos in their chorions using computer software in a manner similar to the work of Burgess and Granato. In this particular study, bend rates of individual embryos were quantified by eye in various experimental paradigms. This analysis and quantification was somewhat tedious, but our results are in line with the results obtained by Sipple as we also observed a peak in bend rates occurring at about 19 hpf.

Also, we noticed that raising embryos at slightly cooler temperatures affected the rate of embryonic motor activity. However, the peak of activity still occurred at ~19-20 hpf. Therefore, when looking at the general biology of zebrafish embryos, the act of temperature fluctuations (also reported by Saint-Amant and Drapeau, 1998) can easily influence early embryonic motor behaviors.
nAChRs and Embryonic Motor Output

The results reported here add to a list of vertebrates, either as adult or embryos that have nAChRs distributed within spinal circuits that modulate or produce locomotor behaviors. In adult lamprey and Xenopus embryos, exogenous ACh can modulate the motor output via an excitatory mechanism. The fact that ACh can modulate the motor output implies that nAChRs are located in the circuit itself that produces the output, or on neuronal substrates that activate the circuits. In Xenopus, it is now known that spinal motoneurons feed back to interneurons within the central pattern generator, release ACh onto interneurons and this aids in providing a sustained motor output (Roberts and Perrins, 1995; Perrins and Roberts, 1994). The direct application of exogenous ACh to the spinal cords of Xenopus embryos actually activates a motor output (Panchin et al., 1991). In embryonic chick spinal cord, administration of DHβE, an α4/β2 nAChR antagonist slows down the embryonic motor output, but does not abolish it (Milner and Landmesser, 1999). This indicates that the α4/β2 nAChR is most likely distributed within the spinal circuit that produces the embryonic motor output in chick. Thus, in a variety of vertebrates including zebrafish, nAChRs are present early in embryonic spinal cord development. The nicotine induced behavioral output reported here, was reduced with repeated nicotine exposure, suggesting a possible receptor desensitization of neuronal nAChRs. This phenotype was evident between 22 and 29 hpf. In a variety of systems, nicotine is very effective at desensitizing the same neuronal nAChRs it activates, particularly the nAChR composed of all α7 subunits and the one composed of α4/β2 subunits (for review, see Mudo et al., 2007). Pharmacological evidence from our previous work suggested that neurons in embryonic zebrafish spinal cord could express α4/β2 nAChRs (Svoboda et al., 2002). Importantly, the muscle nicotinic acetylcholine α1 subunit, CHRN1A, does not appear to desensitize as quickly as the neuronal nAChRs because the 28 hpf...
embryo still responded to tactile stimulation even when that embryo failed to respond to repeated nicotine exposure (Thomas et al., 2009).

Also in this study, we show that embryos exposed to epibatidine as early as 18 hpf exhibited increased motor output which strongly suggests the presence of functional $\beta_2$ containing nAChR in embryonic zebrafish. We don’t have conclusive physiological data indicating that these subunits form functional receptors with their alpha subunit counterparts. However, the fact that epibatidine and ABT 418 can increase embryonic motor output suggests that functional $\beta_2$ containing nAChR are located in the embryonic CNS to produce the behavior. Thus, it may be the case that desensitization of an $\alpha_4/\beta_2$ like nAChR or other neuronal nAChRs is underlying the desensitized behavior in zebrafish reported here.

**Embryonic Motor Output: A Diagnostic Tool for Studying Vertebrate Nicotine Toxicity and nAChR Distribution**

Using a combination of behavior and pharmacological techniques, we have demonstrated that nicotine exposure at a very low concentration (1 µM) can disrupt zebrafish embryonic physiology. When embryos were pre-incubated in one µM nicotine for 4 hours and then challenged with 30 µM nicotine, they did not exhibit the robust increase in bend rates observed in embryos exposed to higher concentrations of nicotine. The ability of 1 µM nicotine exposure to have an impact on embryonic physiology is a significant finding because this low concentration was clearly interacting with nAChRS in the zebrafish embryo without producing overt toxic effects. In mammalian studies utilizing embryonic explants, concentrations between 0.6 µM and 6 µM nicotine can result in abnormal development. Embryonic lethality occurred in those explants when they were exposed to 6 µM nicotine (Zhao and Reece, 2005). We are interested in using the zebrafish as both a model system to study the consequences of nicotine exposure.
toxicity and also to understand the normal role of nAChRs in vertebrate spinal neuron development.

Our results obtained with 1 µM coupled with our observations that 5 µM nicotine can disrupt motoneuron axonal pathfinding (Menelaou and Svoboda, 2009) suggest that the nAChRs in vertebrates may be behaving in a similar manner when exposed to nicotine. It has been shown with epibatidine binding studies that there are two specific types of binding sites which recognize epibatidine in the 48 hpf developing zebrafish CNS. These binding sites may possibly be α4/β2 nAChRs or other related nAChRs (Zirger et al., 2003). Similarly, two epibatidine binding sites also exist in 9-11week prenatal human brain with binding constants that are similar to those detected in the zebrafish. Therefore, nAChR during zebrafish embryogenesis appear to be very similar to mammalian (human) nAChRs expressed during embryonic development. Our behavioral data are consistent with those observations.

**RB Neurons Likely Express Functional nAChR Early in Development**

We used the nicotine mediated behavioral phenotype reported here as a diagnostic endpoint to elucidate specific nAChR subtypes that may underlie this behavior. Nicotine applied to zebrafish embryos activated an embryonic motor output that could also be activated in the absence of brainstem inputs indicating an activation of nAChRs located on spinal neurons (Thomas et al., 2009). Candidate cell types to be activated by nicotine include interneurons within the spinal rhythm generator itself and sensory neurons known as RB neurons which are known to make synapses with spinal interneurons. Thus, we hypothesized that nicotine is activating nAChRs located on RB neurons. This is consistent with other work showing that the β2 nAChR mRNA is located in dorsal spinal cord and that the antibodies that recognize β2 nAChR protein labeled RB neurons. Using behavior as a guide, we were able to elucidate what particular nAChRs where likely to be involved in mediating the nicotine induced response. We
then analyzed the embryonic motor output in response to epibatidine and ABT 418, \( \alpha_4/\beta_2 \) nAChR agonists. These specific nAChR agonists increased the musculature bend rate in a manner similar to nicotine.

If RB neurons do express functional \( \beta_2 \) nAChR containing receptors, their development would likely be altered by exposure to nicotine or specific molecules targeting \( \beta_2 \) nAChR subunits. We will ultimately determine the effect embryonic nicotine exposure on Rohon-Beard neuron development. But first, a thorough characterization of Rohon-Beard development in zebrafish must be done in order to fully uncover abnormal developmental phenotypes caused by early nicotine exposure. Once this characterization has been established (Chapter 3 of this thesis), we will then be in a position to systematically analyze the effect of nicotine exposure on RB neuronal function and development which will be presented under chapter 4.
Figure 2.5 Quantification of nicotine uptake in zebrafish embryos. A: 23 hpf embryos (n=9, for each concentration) were exposed to 30 µM of [³H]-nicotine for 10, 30, 60 and 120 min, respectively. Nicotine incorporation was quantified via scintillation counting. In this example after a 2-hour exposure, nicotine equivalent to 10 µM was incorporated into the embryos just as it was for the ten minute time point. B: 23 hpf embryos were exposed to varying concentrations of [³H]-nicotine for 10 minutes. Nicotine incorporation into the embryos was less than the waterborne concentration of nicotine at all waterborne concentrations analyzed. The dashed lines in A and B corresponds to a nicotine concentration of 30 µM. C: 24 hpf embryos (n=6) were exposed to 30 µM [³H]-nicotine (black bar) and an increase in the bend rate occurred. At minute 9, the embryos were then processed for scintillation counting. The inset corresponds to the amount of nicotine into the embryos; roughly 4 µM. The amount of nicotine incorporation is much less than the waterborne concentration. Moreover, in this example 4 µM nicotine is producing the behavioral phenotype. The dashed line in C corresponds to a nicotine concentration of 6 µM. Asterisk denotes significance from control bend rate with p value < 0.05, Student T-test.
Figure 2.6 Nicotine efflux rates in zebrafish embryos. Efflux was quantified for embryos exposed to 30 μM [3H]-nicotine. 22-23 hpf embryos exposed to nicotine for 10 min and the incorporation was quantified (point highlighted by circle). The efflux rate was measured for a subset of the embryos hour by hour. There was 1 μM nicotine present in the embryos 5 h after the exposure.
Figure 2.7 One μM nicotine, equilibration and effect on embryonic physiology. A: Embryonic equilibration of $[^3]$H-nicotine was quantified for 1, 5, and 15 μM nicotine. (Left) thirty six embryos were separated into three groups, transferred to $[^3]$H-nicotine, and videotaped for 5 min to verify that nicotine was exerting an effect. (Right) influx was then quantified at minutes 5, 10, 60, and 120. After 2 h, equilibration was reached for the 1 mM $[^3]$H-nicotine. B: 25 hpf (n=8) were pre-incubated in 1 μM for 4 h (filled circles). Stage matched control embryos (n=7) are denoted by the open circles. At minute 5, both groups of embryos were exposed to 30 μM nicotine. The embryos pre-incubated in 1 μM nicotine do not respond to 30 μM nicotine compared to the controls. However, those embryos still respond with a robust motor output when exposed to embryo medium containing high KCL. Asterisks denote significant difference from control bend rates. In B, ‡ denotes significant difference in bend rates between the pre-incubated and control embryos produced by exposure to nicotine ($p$ value < 0.001, Anova).
Figure 2.8 Cholinergic modulation of the embryonic motor output. A: Peak bend rates are plotted for 18 hpf embryos exposed to 30 μM nicotine and 500 nM epibatidine. Both nicotine and epibatidine evoked a significant increase in tail bend rates. B: Tail bend rates are plotted for 20-21 hpf embryos exposed to varying concentrations of epibatidine (125 nM, 250 nM, and 500 nM respectively). Control bend rates are shown from minutes 1-5 (first black bar). At minute five, embryos were transferred to embryo medium containing epibatidine. Epibatidine exposure evoked an increase in the tail bend rate in a dose-dependent manner (second black bar). C: 22 hpf embryos exposed to epibatidine (50, 150, and 250 nM). D: 24 hpf embryos exposed to 125, 250, 500, 1000, and 2000 nM epibatidine. Epibatidine evoked a behavioral response similar to that of nicotine in a dose-dependent manner.
Figure 2.9 ABT 418, a human $\alpha_d/\beta_2$ nAChR agonist increases the musculature bend rate in young fish. Zebrafish embryos exposed to ABT 418 (100 $\mu$M) at 22 and 24 hpf. Within two hours of development embryos did not exhibit a significant change in bend frequency when exposed to ABT 418. Asterisks denote significant differences between control group and exposure group with $p$ value < 0.05 (Student’s $T$-test).

Figure 2.10 RB neurons express $\beta_2$ nAChR subunit early in development. A: At 22 hpf, GFP-expressing RB neurons of the $isl3$ zebrafish line expresses the $\beta_2$ nAChR subunit. (Middle) The monoclonal antibody 290 (mAb 290), which recognizes the $\beta_2$ in mammals, also detects $\beta_2$ on RB cells (white arrows) in zebrafish. (Right) merged views of RB neurons in $isl3$ and the $\beta_2$ antibody. B: At 30 hpf, the zebrafish antibody that specifically recognizes the $\beta_2$ subunit of nAChRs is expressed by GFP expressing RB neurons. Scale bars = 10 $\mu$m.
3.1 Introduction

Previously, we showed that early nicotine exposure could increase the embryonic motor behavior by possibly activating subtype specific nAChRs expressed on Rohon-Beard neurons (refer to chapter 2). We hypothesized that nicotine could modify Rohon-Beard neuron development in the context of its functionality and survival. However, before we tested this, a systematic approach to understanding RB neuron development had to be performed. In this chapter, we characterized the RB neurons across many stages of development in zebrafish. We then used this information to further investigate aspects of RB programmed cell death and removal during zebrafish embryogenesis; in the context of nicotine exposure presented under Chapter 4.

Programmed cell death (PCD) plays a necessary role in the development of the vertebrate central nervous system. During embryonic development, neurons can be completely removed or greatly reduced via a cell death process. This early removal of neurons has been attributed to the precise tuning of the primary neuronal network involving the elimination of unwanted, nonviable, or destructive cells as well as the reconstruction of the developing structures (Jacobson et al., 1997; Oppenheim, 1991). In addition, neuronal cell death is a rapid process characterized by an initial cell shrinkage and condensation stage (Jacobson et al., 1997).

Zebrafish may serve as powerful vertebrate model system to study PCD (for review, Pyati et al., 2007). In the context of neuronal PCD, there are few transient populations of neurons in the developing vertebrates which lend themselves to detailed analysis of PCD. In mammals, Cajal –Retzius cells are a transient population of neurons located in the hippocampus and neocortex (Derer and Derer, 1990). They function by producing reelin (Frotsher, 1997) which
likely acts as an inhibitory molecule in guiding migrating neurons and axons to their final destinations. In zebrafish, there are two distinct transient populations of spinal neurons; the variable primary motoneuron (VaP, Eisen and Melancon, 2001) and the spinal mechanosensory Rohon-Beard (RB) neurons. RB neurons serve a behavioral function mediating an identifiable touch response at ~27 hpf (Ribera & Nusslein-Volhard, 1998). Ultimately, the function of RB neurons is replaced by dorsal root ganglion neurons.

Zebrafish RB neurons are primary spinal mechanosensory neurons derived from the same neural plate region that produces neural crest cell derivatives (Epperlein et al., 2007; Rossi et al., 2008). The onset of RB neuron development is first apparent at 11 hours post fertilization (hpf) (Rossi et al., 2009) and the entrance of RB neurons into a cell death pathway is evident by 19 hpf (Williams et al., 2000). RB neurons are easily recognized by their large nuclei, giant cell bodies, and granular proplasms. These are common characteristics among lamprey, Xenopus, axolotl, and zebrafish RB neurons. RB neurons are located in the most dorsal lateral region of spinal cord early in development. Their axons project rostrally to the hindbrain as well as caudally within the dorsal longitudinal fasciculus (DLF) (Reyes et al., 2004; Bernhardt et al., 1990). RB neurons also have peripheral processes extending over the segmental myotomes where their free nerve endings innervate the skin and respond to light tactile stimuli (Paulus et al., 2009; Roberts, 2000; Clarke et al., 1984). In this context, they are analogous to dendrites.

Zebrafish RB neurons are a tractable cell population to study programmed cell death in an in vivo context as already demonstrated by previous researchers. However, there are several issues that make studying RB development complex as the zebrafish ages. The first problem is that most studies have used immunohistochemical techniques to label RB neurons with a variety of colorimetric detection assays. All rely on the ability of the antibodies to reliably penetrate the fish. The second is the transient nature of the markers. The best markers reported in the literature
include zn12, aat, anti-hu and an antibody against PKC (Patten et al., 2007 and Slatter et al., 2005). Although the antibody to PKC labels RB cells out to 13 days of age, it is reported only to label a subset of RB neurons.

We systematically characterized RB neuron development in a transgenic line of zebrafish (isl3) where GFP is expressed in RB neurons in order to identify a stable marker of all RB neurons across development. We show that the development of RB neurons in isl3 zebrafish mirrors the development of RB neurons in wildtype zebrafish where antibody markers were used to label RB neurons. Importantly, we were able to detect RB neurons at times when the conventional markers of RB neurons failed to detect RB neurons in non transgenic fish. We detected GFP positive RB neurons as late as 33 days post fertilization, but we don’t know if those late surviving RB neurons are functional.

3.2 Materials and Methods

Animal Care and Maintenance

Fertilized embryos were obtained from natural spawnings of breeding zebrafish and staged according to the Zebrafish Book (Westerfield, 1995). Adult fish were housed at 28.2°C on a 14 h light/10 h dark cycle. All embryos collected from group or paired spawns were rinsed in system water, transferred to 100 mm Petri dishes containing embryo medium, and placed in an incubator at 28°C until further analysis. The embryos utilized in this study were obtained from either Tg (isl3: GFP) (referred to as isl3 from here onward) or wildtype zebrafish (EkkWill Waterlife Resources, Ruskin FL). Isl3 is a zebrafish transgenic line where embryos of that line express green fluorescent protein (GFP) in retinal ganglion cells as well as their axons which project through the optic nerve, lateral line ganglia, trigeminal ganglia, RB neurons, and dorsal root ganglia. For the purpose of this study, isl3 zebrafish were used to study RB neuron
development. The *isl3* line was kindly provided by Chi-Bin Chien and Andrew Pittman at the University of Utah, School of Medicine.

**Immunohistochemistry**

Whole-mount immunohistochemistry was performed using a modified version of a previously published protocol (Svoboda et al., 2001, 2002; Pineda et al., 2006, Menelaou and Svoboda, 2009). All antibodies were prepared in NCS/PBST or PBST. The primary antibodies anti-acetylated tubulin (1:500 dilution, Sigma Aldrich), zn12 (1:500 dilution, Developmental Hybridoma Bank, University of Iowa), anti-Hu (1:250 dilution, referred to as Hu in the figures; Molecular Probes, Eugene, OR) were used to reveal RB neurons in wildtype lines. Anti-Hu recognizes an RNA binding protein that is abundantly expressed in RB somata (Henion et al., 1996), developing dorsal root ganglia neurons (Marusich et al., 1994) as well as spinal neurons. Anti-acetylated tubulin labels RB neurons, posterior lateral line ganglia, dorsal root ganglia, and neurons that have already begun axonogenesis. The zn12 antibody recognizes the L2/HNK-1 epitope and labels the surface of RB somata, as well as axons, growth cones, and a cytoplasmic region positioned near the nucleus (Metcalf et al., 1990).

The monoclonal antibody, F59 (1:50 Dilution, Developmental Hybridoma Bank, University of Iowa), was used to stain slow muscle fibers. In some experiments, the polyclonal antibody anti-GFP (1:500 dilution, Molecular Probes) was used to amplify endogenous GFP signals.

The generation of a zebrafish specific polyclonal antibody directed against CHRN2B (nAChR β2 subunit) has been previously described (Welsh et al., 2009). It was used at a 1:250 dilution. We have found it to be a reliable marker of RB somata and RB processes.

The next day, embryos were incubated in PBST and washed for 60-120 minutes at room temperature and then incubated for an additional 60-120 minutes in goat anti-mouse or anti-
rabbit secondary antibodies conjugated to Alexa 488 or Alexa 546 (1:1000 dilutions, Molecular Probes, Eugene, OR). Following fluorescent antibody incubation, embryos were washed in PBST for an additional 60-120 minutes at room temperature. Embryos were either immediately analyzed or stored in PBST at 4°C until examination.

**Agarose Embedding of Zebrafish**

At 18-72 hours post fertilization (hpf), transgenic (*isl3*) or wildtype zebrafish were mounted in 1.25% agarose (Fisher Scientific, Fair Lawn, N.J.) made in PBS. All embryos were mounted dorsal side up. In some experiments, living embryos were anesthetized in 0.01% tricaine methanesulfonate (MS222), individually mounted dorsally in agarose and transferred to 12-well plate dishes containing PBS or PBST. At the time of imaging, the agarose cubes were trimmed and individually placed on glass bottom Petri dishes for viewing. Afterwards, the live zebrafish were immediately viewed using fluorescent microscopy. All images of dorsal spinal cord are presented with the rostral region towards the top. Following image acquisition, the samples were removed from the agarose, anaesthetized in MS222, and fixed in individual labeled glass vials (one embryo per vial) and stored at 4°C until further anatomical analysis. At that time, the previously imaged *isl3* embryos were removed from fixative and washed in PBST for 30 minutes. Afterwards, embryos were re-mounted individually in 1.25% agarose and viewed using fluorescent microscopy.

**Lateral Mounts**

Fixed wildtype zebrafish (18-72 hpf) or *isl3* (18-96 hpf) processed for immunoreactivity were mounted laterally in PBS on microscope slides, coverslipped, and sealed with nail polish for viewing. All images are shown with the head orientated to the left and dorsal to the top.

**Image Acquisition**
Images were captured on a Zeiss Axiovert 200 M inverted microscope (Thornwood, NY) equipped with epifluorescence, a Zeiss ApoTome and an ORCA-ER digital camera (Hammamatsu, Japan). Images were obtained using a 40 x oil immersion objective (N.A. 1.30). Serial z-stacks were acquired with thicknesses of individual sections (step size) ranging between 0.3-0.7 μm. Images were then transferred to and reconstructed using Imaris 5.7.2 (Bitplane, St. Paul, MN). The Imaris imaging platform has provided our lab with the optical capability to perform detailed analyses of neuronal structures during development. Further adjustments for brightness and contrast were performed using Photoshop 7.0 (Adobe, San Jose, CA). Figures were assembled using CorelDraw Graphics Suite 12 (Ottawa, Ontario, CA).

**Analysis of RB Development**

RB somata were identified by their dorsal position in spinal cord, cell size, and antibody labeling in wildtype lines as well as GFP expression in the isl3 line. RB neuronal migration and soma size were quantified in embryos ranging from 18-96 hpf. The region of interest where most of the analyses were performed spanned 4-6 segments above the yolk sac extension (Figure 3.1, top). RB migration distances and cell size measurements were obtained using AxioVision 4.7.1 (Carl Zeiss Imaging Solutions). Specifically, the midline of spinal cord was established by measuring the distance between the most lateral left and right boundaries of spinal cord then dividing by half. RB cell movement to the midline was then quantified by calculating the distance from the center of RB nuclei to the midline. RB cell size was then calculated by multiplying the lengths of the horizontal and vertical axes of individual cells (Figure 1, A-C).

**Statistics**

All values are presented as means ± standard error of the means (SEM). A Student’s T-test was performed in order to assess levels of statistical significance. P-values < 0.05 are indicative of
statistically significant data. All experiments were replicated three or more times. All results shown are representative data selected from each group of replicated experiments.

Figure 3.1 Imaging methodology. At the top is a cartoon depicting the generalized region of interest in zebrafish spinal cord where RB analysis was performed. A-C: Methodology for quantifying RB neuronal development. A: A cropped image of a cell located in the dorsal region of spinal cord in *isl3* embryos showing intense nuclear GFP expression. B: RB cell sizes were individually measured in square micron units by calculating micron distances from the outermost visible horizontal and vertical boundaries of the cell. C: RB neuronal migration was quantified by measuring the distance from the center of the nucleus to the midline of spinal cord.
3.3 Results

Characterization of the *Isl3* GFP Reporter Zebrafish

Transgenic zebrafish have become popular for studying nervous system development (Kucenas et al., 2006; Goldman et al., 2001; Lele et al., 1996; Houdemine and Chourrout, 1991). Studies have used transgenic lines of zebrafish including the *isl3* transgenic to study vascular development (Lawson et al., 2002) and motoneuron development (Menelaou and Svoboda, 2008; Higashijima et al., 2000) in young fish. Recently, researchers have used the *isl3* transgenic zebrafish line in their experiments. GFP is expressed in RB neurons and other sensory neurons in *isl3* zebrafish including the retina (Yonkers and Ribera, 2009, Pittman et al., 2008). We chose this line to study RB development hypothesizing that it may allow for the most stable and reliable identification of RB neurons across development.

We first determined the identity of GFP expressing cells in *isl3* embryos using various antibodies. Interestingly, GFP expression appeared to be found specifically in sensory structures, with the exception of slow muscle fibers and nuclei which were only evident at earlier stages of development (presented later in this chapter). At 48 hpf, retinal ganglion cells and their axons traveling in the optic nerve (opn), lateral line ganglia (llg), trigeminal ganglia (trg), and dorsal root ganglia (drg) all robustly expressed GFP (Figure 3.2). These structures were also detected with appropriate antibodies typically used to label them in wildtype zebrafish. The monoclonal antibody zn5 labeled the optic nerve and retinal ganglion neurons. The antibody zn12 labeled lateral line, trigeminal ganglia, and RB neurons. Lastly, the antibody anti-Hu labeled RB neurons and dorsal root ganglia neurons (Figure 3.2). At 30 hpf, RB neurons in *isl3* zebrafish were reliably labeled with RB specific cellular markers, zn12, aat, and anti-Hu (Figure 3.3, A-C). Thus, at the anatomical level, all of these sensory neurons were behaving in a manner similar to
their corresponding structures in wildtype fish. Thus, we felt confident in using the *isl3* zebrafish to characterize RB neuron development.

**Fixation Alters RB Morphology**

Many groups have used GFP expressing transgenic fish lines to facilitate anatomical studies where experiments can be performed in living or fixed tissue. Fixation often reduces the GFP signal but does not completely quench the signal. However, it is known that fixation can cause shrinkage in tissue. To determine if fixation altered the stability of GFP expression in *isl3* embryos or caused other anatomical artifacts, RB neuronal morphology in young fish was compared in living and fixed tissues. At 30 hpf, living *isl3* embryos were anesthetized, mounted in agarose, and quickly imaged with fluorescent microscopy. Afterwards, embryos were incubated in 4% paraformaldehyde, stored and viewed again the following day at 48 hpf. RB cell bodies in fixed tissue were approximately 17% smaller in cell size when compared to living embryos (Figure 3.4, C). In addition, the GFP expression in RB processes in the fixed embryos appeared to have a beaded distribution compared to intact GFP expression in living embryos. It is possible that the beaded appearance could be the result of RB cell death, dim GFP expression, or simply a fixation artifact. To address this issue, we labeled *isl3* embryos with the anti-GFP antibody to enhance the GFP signal in *isl3*. RB peripheral processes still appeared to have a beaded pattern in spite of enhanced GFP labeling (Figure 3.4 D). We were able to demonstrate that RB peripheral processes in fixed 48 hpf *isl3* and wildtype zebrafish are intact, even when GFP expression or aat distribution was still fragmented. (Figure 3.4, E-F). These experiments were similar to those performed in 60 hpf zebrafish (Svoboda et al., 2001) where zn12 was used to detect intact RB peripheral processes. In the present study, an antibody directed against zebrafish CHRNB2 (Welsh et al., 2009) was used to detect intact RB peripheral processes.
It is important to note that RB peripheral processes in living *isl3* embryos remained intact out to 48 hpf, in spite of an apparent entrance into a PCD pathway earlier in development. This is consistent with results from studies done in living zebrafish embryos demonstrating that RB neurons and their processes appear healthy out to 48 hpf. However, RB neuron peripheral processes begin degenerating by 3 dpf with only traces of these processes remaining detectable out to 6 dpf (Reyes et al., 2004). Consequently, the morphological sustainability of GFP expression in RB peripheral projections in fixed *isl3* embryos should not be compared to wildtype zebrafish embryos because the GFP fragmentation occurred at 48 hpf. This change in GFP expression in RB processes, an apparent fixation artifact, does not influence the overall analysis of RB neuron migration (Figure 3.4 A) and cell size in those fish when compared to wildtype zebrafish processed for anti-Hu immunohistochemistry presented below.

**RB Neuron Migration Patterns in Wildtype and Isl3 Zebrafish**

Early in development, RB cells appear as two rows of cells in the dorsal lateral aspect of spinal cord (refer to figure 3.3). From 18-30 hpf, RB neurons are positioned bilaterally at the most dorsa-lateral region in spinal cord. They enter a migratory phase around 36 hpf and continue to transition to the midline forming an almost linear row of cells by 48 hpf (Figure 3.5 and 3.6, A; Tables 1 and 2). From 18-72 hpf, RB cells in *isl3* and wildtype fish migrate towards the midline in 2-4 micron increments every 6 hours of development, and completely reach the midline by 72 hpf (Figure 3.6, C). Like many aspects of vertebrate nervous system development which proceeds from rostral to caudal, RB migration occurred in the rostral part of cord first followed by migration in the caudal region. At 48 hpf, RB neurons were observed to have already migrated and formed one row (Figure 3.6, B; rostral), whereas in more caudal regions, RB neurons still remained localized in the lateral periphery appearing as two distinct rows of cells (Figure 3.6 B; caudal).
For most of our image acquisition, optical stacks through spinal cord were acquired spanning many microns of tissue. When these optical stacks were rotated, RB neurons appeared to migrate dorsal to ventral in conjunction with their lateral to medial migration. To perform this analysis, the acquired z-stacks of spinal cord in *isl3* embryos were rotated to present an orthogonal plane equivalent to a coronal (cross) section through spinal cord (Figure 3.7). In these experiments, the antibody aat was also used to label the RB peripheral processes innervating the skin. We utilized these rotated cross sectional views to reveal RB neuronal migration/displacement as a result of embryonic maturation. At 22 hpf, RB neurons are located at the most dorsal aspect of spinal cord. They remain at this dorsal position until 36 hpf. Once they reach the midline at 48 hpf, they appear to be displaced ventrally. The cells are even further displaced by 72 hpf.

**Changes in Somatic Morphology During RB Development**

Previous studies have shown that RB neurons undergo morphological changes during development. These changes involve soma shrinkage and degeneration of peripheral processes which are suggested to coincide with RB programmed cell death (Reyes et al., 2004; Svoboda et al., 2001). In the present study, a more thorough analysis of RB cell morphology from 18-72 hpf was performed in *isl3* zebrafish. During development, RB somata transition from a large oval shape into a smaller, angular-like shape (Figure 3.8, C). This is followed by changes in RB GFP nuclear expression. Early on in development (18-36 hpf), GFP is intensely and uniformly expressed in the nucleus. Later in development (48 hpf and older), nuclear GFP expression is either greatly reduced or absent, but remains intensely expressed in the cytoplasm. A degeneration in nuclear structure was partially evident in 48 hpf embryos; however in older fish (72 hpf and older), a well-defined nuclear structure was absent (Figure 3.8, B). These observations are consistent with those observations from previous research which showed that
the smaller, square-like shape of RB cell bodies suggested that they were dying cells (Patten et al., 2007) and that a decrease in nuclear density is a prominent feature of cell death (Lamborghini, 1987). These changes in cell biology as it pertains to nuclear structure may be a distinguishable feature indicative of RB PCD.

The morphology of RB neurons (cell size) along with their migration patterns was then quantified in isl3 zebrafish and wildtype zebrafish (Tables 1 and 2). In young embryos (18-24 hpf), as the number of small RB cells (less than 100 μm²) increased, the overall size of RB somata decreased. In contrast, by 30 hpf the number of small RB cells significantly decreased and RB somata become larger. Interestingly, from 36-96 hpf, the percentage of small RB cells markedly increased. This change in cell size occurred in a linear fashion. Thus, as the RB neurons aged, they became progressively smaller (~55% reduction in size for RB neurons in isl3 embryos) likely indicating entry into the PCD pathway.

**Antibody Labeling of RB Neurons: Characterization of Markers**

RB PCD has been extensively studied using a variety of approaches and immunohistochemical markers. The monoclonal antibodies aat, anti-Hu and zn12 seem to be the preferred markers used to label RB neurons. The latter was first produced and characterized in young zebrafish (Metcalfe et al., 1990) and has been used to study RB neuron development as well as RB PCD (Paulus et al. 2009; Reyes et al., 2004; Svoboda et al., 2001; Cornell et al., 2000; Williams et al., 2000; Metcalfe et al., 1990). Likewise, the monoclonal antibody aat labels RB somata in addition to RB peripheral arbors during embryogenesis (Piperno and Fuller, 1985; Svoboda et al, 2001). Although, these markers provide consistent visualization of RB neurons, the stability of these markers across development remained questionable. In fact, studies have shown that most markers used to identify RB neurons are not stable and therefore set limitations for studying RB neurons at later stages of development (Patten et al., 2007 and Slatter et al.,
In order to accurately study RB neuronal development on the basis of function or survival, a stable marker for RB neurons must be first established. We initially investigated the stability of preferred RB neuronal markers, zn12 and aat, from 24-72 hpf by characterizing their labeling patterns during development (Figure 3.9, A-B).

At 24 hpf, the zn12 signal was detected on the surface of RB somata, central axons, and peripheral processes. Also, zn12 labeling appeared in a unique pattern in the form of “hot spots” which were localized in the ventral region of RB neurons (Figure 3.9, A). In contrast, at 24 hpf, aat labeled RB axons projecting into the dlf and peripheral processes, but barely detected the cell bodies (Figure 3.9, B). By 36 hpf, aat labeling of RB somata became more apparent. In contrast to zn12, aat labeling is confined to the cytoplasmic regions of the cell providing a silhouette of the large nucleated domain.

Zn12 expression at 36 hpf was similar to the zn12 expression pattern observed at 24 hpf. At later times, RB cell bodies and peripheral processes were still detected by zn12 (Figure 3.9, C; 48 hpf), the labeling of RB neuron somata by zn12 and aat is reduced as the boundaries of cells appear less defined. At 72 hpf, zn12 and aat both failed to label RB somata, but still detected the peripheral processes. To determine whether or not the absence of zn12 and aat of labeling in RB somata at 72 hpf was due to the cells not being there, we performed labeling experiments in isl3 zebrafish (Figure 3.9, D). GFP expressing RB neuron somata were still present at 72 hpf but were not detected by the antibodies, zn12 and aat. Thus, we conclude that despite the early reliable labeling capabilities of zn12 and aat to detect RB somata, these antibodies are not the best markers to study RB somata at later stages of development. On the other hand, anti-Hu was able to detect RB neurons as late as 6 dpf. This finding will be further elaborated upon later in the chapter (see figure 3.12).
Figure 3.2 Structures expressing GFP in Tg(isl3:GFP) zebrafish. A-E: All representative photomicrographs show isl3 labeled structures in the left panels, cell specific antibody staining in the middle panels, and merged views of isl3 with antibody in the right panels. All images are of compressed z-stacks acquired at 48 hpf. A: Left, in isl3 zebrafish, GFP expression can be detected in the optic nerve and retinal ganglia; inset reveals the entire retina using a 20 x magnified view. Middle, zn5 which is an antibody that labels retinal ganglion cells in wildtype zebrafish labels the optic nerve; inset also shows the full view of the retina using a 20 x magnified view. Right, merged image shows GFP positive optic nerve and retinal ganglia cells labeling with zn5. B: Left, GFP expression in the lateral line ganglia (llg). Middle, zn12, an antibody that labels llg neurons as well as trigeminal ganglion (trg) and RB neurons (rbn) in wildtype zebrafish, labels llg neurons in isl3 zebrafish. Right, merged image shows GFP positive llg neurons labeled with zn12. C: Left, image is of the GFP positive trigeminal ganglion neurons in isl3 zebrafish. Middle, zn12 labels trigeminal ganglion neurons in isl3 zebrafish. Right, merged image shows GFP positive trigeminal ganglion neurons labeled with zn12. D: Left, image is a lateral view of GFP positive RB neurons in isl3 zebrafish. Middle, anti-Hu, an antibody that labels spinal neurons including RB neurons as well as dorsal root ganglion neurons in wildtype zebrafish, labels spinal neurons in isl3 zebrafish. Right, merged image shows GFP positive RB neurons labeled with anti-Hu. E: Same conventions as shown under D now focusing on dorsal root ganglion neurons. Left, image is a lateral view of GFP positive drg neurons in isl3 zebrafish. Middle, anti-Hu labels spinal neurons and drg neurons in isl3 zebrafish. Right, merged image shows GFP positive drg neurons labeled with anti-Hu. Solid arrowheads show dorsal and ventral projections arising from developing dorsal root ganglia. Scale bars: A=50 μm, B=20 μm, C=10 μm; opn-optic nerve, llg-lateral line ganglia, trg-trigeminal ganglia, rbn-rohon-beard neuron, drg- dorsal root ganglion
Figure 3.3 *Isl3* RB neurons are detected with cellular markers that label RB neurons in wildtype zebrafish. All images are dorsal views of the zebrafish. A: Left, GFP expression in RB neurons of a 30 hpf *isl3* embryo. Middle, anti-hu labeling of RB neurons in dorsal spinal cord. Right, merged image reveals all GFP positive RB neurons label with anti-Hu. B: Left, GFP expression in RB neurons of a 30 hpf *isl3* embryo. Middle, aat labeling of spinal neurons including RB neurons. Right, merged image reveals that GFP positive RB neurons label with aat. Open arrows depict cells that don’t express GFP signifying that GFP is specifically expressed in RB neurons. C: Left, GFP expression in RB neurons of a 30 hpf *isl3* embryo. Middle, zn12 labeling of spinal neurons in dorsal spinal cord of that same zebrafish. Right, merged image reveals GFP positive RB neurons labeled with zn12. Closed arrows depict zn12 labeling on the surface of RB neurons. Scale bar = 10 μm.
Figure 3.4 Morphology of RB neurons in *isl3* zebrafish: alterations caused by fixation. All images represent dorsal side up, 3-D flatten z-stacks captured using a 40 x oil objective. A: Left, An image of rbn GFP expression in a living 30 hpf embryo. Middle, the same fish imaged live at 48 hpf. Right, after fixation, the same fish was observed at 48 hpf. Open arrowheads show RB neuron processes projecting dorsally out into the periphery. B: Left, A magnified view depicting changes in GFP expression due to fixation. Open arrowheads show that at 48 hpf, RB axonal projections are continuous in living fish compared to fixed tissue (right). C: Bar graph showing average RB cell size in living tissue compared to fixed tissue. D: Comparison of RB peripheral processes in 48 hpf *isl3* (GFP) and *isl3* zebrafish labeled with anti-GFP at 48 hpf. E-F: 48 hpf *isl3* (E) and wildtype (F) zebrafish labeled with a polyclonal antibody made against the zebrafish CHRN2β subunit. This antibody recognizes the main peripheral processes of RB neurons. Continuous labeling demonstrates that RB processes are not degenerated at this time. Just the GFP signal appears fragmented. F: Distribution in RB peripheral process of wildtype zebrafish. Asterisk in C denotes a statistically significant difference in RB cell size, p< 0.05. Scale bars: A-B= 10 μm, D-E = 20 μm.
Figure 3.5 RB neuronal migration patterns during zebrafish development. Top: Dorsal views of RB neurons from 24-72 hpf *isl3* zebrafish. Bottom: Dorsal views of RB neurons from 24-72 hpf wildtype zebrafish. The RB neurons were labeled with the anti-Hu antibody. RB neurons form a double row of cells at the most dorsal lateral region of spinal cord. As development occurs, they then begin to migrate to form an almost linear row of cells by 48 hpf. During this migration, RB cell size appears to get smaller. Scale bar = 10 μm.
Figure 3.6 RB neuronal migration in the developing embryo. A: Cartoon depicts RB neurons originating as two rows in dorsal lateral spinal cord and later migrating towards the midline to eventually form an almost linear row (by 48 hpf). B: At 48 hpf, it was detected that RB neuronal migration occurs in a rostral-caudal fashion. At the most rostral portion of spinal cord in 48 hpf embryos, RB neurons have migrated to the midline forming one row. However, at the most caudal region of spinal cord, a double row of RB neurons can still be seen. Dotted arrows outline the most lateral position of spinal cord. C: graph compares RB neuronal migration patterns in 18 - 72 hpf isl3 and wildtype zebrafish. The dotted line labeled (m) represents the midline. Scale bar = 10 μm.
Table 3.1 GFP expressing RB cell sizes and migration was quantified. RB cell sizes were determined by measuring the horizontal and vertical boundaries of the cell body. RB cell migration was determined by calculating the distance from center nucleus (intense GFP expression) to the midline. At later stages of development, the nuclear region of RB neurons may have degenerated. In those cases, migration measurements were taken from the center of the entire cell body to the midline. Abbreviations are as follows: n, number; AVG, average.

<table>
<thead>
<tr>
<th>Age (hours post fertilization)</th>
<th>Fish (n)</th>
<th>RB cell (n)</th>
<th>Average RB cell size (µm²)</th>
<th>Percentage of RB cells less than 100 µm² AVG</th>
<th>Fish (n)</th>
<th>RB cell (n)</th>
<th>Average RB cell distance from midline (µm)</th>
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<tr>
<td>18-20</td>
<td>6</td>
<td>91</td>
<td>123±2.5</td>
<td>15%</td>
<td>9</td>
<td>86</td>
<td>15.7±0.5</td>
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<tr>
<td>22-24</td>
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<td>118.4±1.7</td>
<td>19%</td>
<td>10</td>
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<td>30</td>
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</tr>
<tr>
<td>36</td>
<td>9</td>
<td>81</td>
<td>133.8±3.5</td>
<td>19%</td>
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<td>87</td>
<td>6.3±0.3</td>
</tr>
<tr>
<td>48</td>
<td>12</td>
<td>98</td>
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<td>138</td>
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<td>173</td>
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<td>138</td>
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<tr>
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<td>94%</td>
<td>13</td>
<td>70</td>
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Table 3.2 Anti-Hu labeled RB cell sizes and migration was quantified. RB cell sizes were determined by measuring the horizontal and vertical boundaries of the cell body. RB cell migration was determined by calculating the distance from center nucleus (negative Hu expression) to the midline. At later stages of development, the nuclear region of RB neurons may have degenerated. In those cases, migration measurements were taken from the center of the entire cell body to the midline. Abbreviations are as follows: n, number; AVG, average.

<table>
<thead>
<tr>
<th>Age (hours post fertilization)</th>
<th>Fish (n)</th>
<th>RB cell (n)</th>
<th>Average RB cell size (µm²)</th>
<th>Percentage of RB cells less than 100 µm² AVG</th>
<th>Fish (n)</th>
<th>RB cell (n)</th>
<th>Average RB cell distance from midline (µm)</th>
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<td>81</td>
<td>128.5±3.4</td>
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<td>11</td>
<td>117</td>
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<td>108.1±1.4</td>
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<td>6</td>
<td>68</td>
<td>10.0±0.3</td>
</tr>
<tr>
<td>30</td>
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<td>87</td>
<td>133.3±3.3</td>
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<td>6</td>
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<td>30</td>
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<td>93%</td>
<td>8</td>
<td>29</td>
<td>0.7±0.2</td>
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Figure 3.7 Dorsal-ventral repositioning of RB neurons coincides with fish maturation. 3-D rotational views of zebrafish spinal cord are shown with the posterior end facing toward the reader. In 22 hpf *isl3* zebrafish, GFP positive RB neurons (arrows) are positioned at the most dorsal portion of the spinal cord. From 36-72 hpf, RB neurons move in a ventral direction while migrating towards the midline (dotted line) and forming a linear row. In each image, the red signal corresponds to aat labeling of the RB peripheral processes. Scale bar = 20 μm.
Figure 3.8 Morphological and anatomical characteristics of RB neurons during development. A: Representative drawing of GFP expression in RB somata. B: Intense nuclear expression of GFP is observed in RB neurons of isl3 zebrafish. A well defined nucleus is observed (arrows) in early developing embryos (24 hpf). At 48 hpf, the nuclear region becomes less defined, but continues to intensely express GFP. By 72 hpf, RB nuclear GFP labeling in isl3 larvae is reduced or completely removed. C: From 24-72 hpf, RB cells undergo morphological changes that may correspond to their diminished functionality and viability. Scale bar = 5 μm.
Figure 3.9 aat and zn12 are transient markers of RB somata. A-B: Dorsal views of spinal cord in 24-72 hpf wildtype zebrafish. A: zn12 labeling of RB somata (open arrowheads) and processes is seen at 24-48 hpf. At 72 hpf, RB neurons are not labeled with the zn12 antibody, but labeling is still apparent in the peripheral processes (arrows). B: At 24 hpf, aat labels RB neuronal processes (arrows), but barely detects RB somata. At 36 hpf, aat intensely labels RB somata and processes. By 48 hpf, aat staining of RB somata (open arrowheads) appears to be weak. By 72 hpf, the aat labeling of RB somata is greatly reduced or not evident, but RB peripheral processes are still labeled with aat. C: Lateral views showing the presence of aat and zn12 positive RB neurons (open arrowheads) at 48 hpf. D-E: Image of a 72 hpf isl3 larva shows complete loss of aat (D) and zn12 (E) staining of RB somata. Scale bar = 10 μm.
Comparison of aat and zn12 Distribution in Peripheral Processes of RB Neurons

Few studies exist that actually have studied the development of RB peripheral processes in zebrafish in detail. It has recently been shown that the simple bending of the musculature influences the directionality of migrating peripheral processes (Paulus et al., 2009). In another study, fragmented tubulin distribution within RB peripheral processes of 48 hpf embryos was found to coincide with RB neuronal cell death (Svoboda et al., 2001). Likewise, microinjected DiI filled RB neurons revealed morphological changes in RB peripheral processes to be indicative of a programmed cell death mechanism (Reyes et al., 2004). We performed a relative anatomical comparison of RB peripheral arbors using the aat and zn12 antibodies in parallel. At 24 hpf, zn12 labeling reveals extensive labeling in RB growth cones and peripheral processes; however in aat labeled embryos, RB peripheral processes are barely seen (Figure 3.10, A-B). At 30 hpf, aat immunoreactivity has a more elaborate and continuous distribution pattern in peripheral processes, whereas peripheral processes labeled with zn12 reveals both a continuous and fragmented expression pattern. By 48 hpf, zn12 and aat distribution along RB peripheral processes was fragmented. These results are consistent with a previous study analyzing aat distribution in zebrafish RB neuron peripheral processes at different stages of development (Svoboda et al., 2001).

RB neurons extend their peripheral processes over the underlying slow musculature in zebrafish. However, in Xenopus and lamprey, the peripheral processes of RB neurons extend within the muscle (Nakao and Ishizawa, 1987; Clarke et al., 1984). To further determine if RB peripheral processes in zebrafish project through the muscle or over the top of the muscle, isl3 embryos were labeled with zn12 and F59. At 24 hpf, GFP was sometimes detected in the lateral slow muscle fibers, thus F59 was not needed to label the slow muscle. When zn12 was used to label the RB processes and then revealed with the fluorophore Alexa 546, the processes (shown
as red in Figure 3.11, A) were easily detected just lateral to the slow muscle fibers (green signal). At 36 hpf, the GFP expression in the slow muscle of isl3 embryos is down regulated, but the GFP can still be detected in RB processes. When the slow muscle was labeled with F59 and then revealed with the fluorophore Alexa 546, the RB processes (green) were seen lateral to the slow muscle (Figure 3.11, B). Thus, the RB neurons in isl3 zebrafish do not appear to traverse the muscle to get to the skin. Instead, they project laterally and then ventrally to innervate the skin. These results are consistent with data showing RB peripheral processes in 4 dpf wildtype zebrafish extending over the musculature (Reyes et al., 2004).

3.4 Discussion

The development of RB neurons has been studied at the morphological, physiological, and molecular levels. Although there have been many advances in molecular biological techniques in recent years, there still seems to be limited reliable methods available to accurately describe the development of RB neurons. In fact, most of the research has been restricted to retrograde dye labeling, or antibody labeling, coupled with fluorescent microscopy offering a somewhat limited anatomical description of RB neuron development. We took advantage of the stability of GFP expression in the isl3 reporter zebrafish line to systematically analyze RB neurons at various stages of development. In doing so, we were able to visualize aspects of RB neuronal development not previously described. This characterization revealed that the RB neurons in the isl3 transgenic fish have a developmental profile virtually identical to those RB neurons in wildtype zebrafish with the major caveat being that they may live much longer than previously shown using conventional anatomical techniques.

RB Neuron Markers

isl3 zebrafish exhibits GFP expression in a variety of sensory structures including RB neurons. Anatomical analyses utilizing the monoclonal antibodies anti-acetylated tubulin, anti-
Hu, and zn12 in the *isl3* reporter line confirmed that these transgenic zebrafish were not significantly different than wildtype zebrafish. In the *isl3* line, the different classes of GFP positive sensory structures were detected with appropriate antibodies including: the retinal ganglion cells and optic nerve were detected with zn5 (Sakai and Halloran, 2006; Zolessi et al., 2006), dorsal root ganglia and RB neurons were detected with anti-Hu, and the lateral line ganglia and trigeminal ganglia were identified with zn12. The aat and zn12 markers are further discussed in the context of RB development.

Anti-Hu recognizes an RNA binding protein that abundantly expressed in RB somata (Henion et al., 1996) as well as later developing dorsal root ganglia (Marusich et al., 1994). We found it to be the most stable marker for detecting RB neurons as it labeled them reliably out to 6 days of age. By 7 days, anti-Hu failed to detect RB neurons in *isl3* embryos (see figure 3.12A).

Anti-acetylated tubulin is a good marker to label RB neuron processes. We noticed a peculiar phenomenon in that it seemed to detect the main branch of RB peripheral processes before it labeled RB somata. It may be the case that the tubulin is targeted to the process first providing structural integrity for the quickly elongating process. What is interesting is that the somatic expression of tubulin as revealed by aat labeling is upregulated at around 27-30 hpf and then down regulated and almost not detectable by 72 hpf on RB somata. However, even though the tubulin appears to fragment by 48 hpf, it is still easily detected in RB processes at 72-96 hpf (96 hpf data not shown). To us, this seems somewhat peculiar as it appears that tubulin, despite fragmentation is being maintained in the peripheral processes.

Lastly, zn12 reliably labeled RB somata early in development at times when aat failed to do so. As was the case for aat, zn12 failed to detect RB neuron somata at 72 hpf. However, in the *isl3* zebrafish line gfp expressing RB neurons are still there. Similar to aat, zn12 was able to detect RB processes at 72 hpf.
In summary, of the 3 antibody markers described, anti-HU was the most reliable marker of RB somata across development (19 hpf-6 dpf). Anti-acetylated tubulin and zn12 both are reliable markers for detecting RB processes in the skin and even growth cones which can be detected by zn12. Zn12 is typically the antibody of choice to label RB neurons and RB peripheral processes in embryos less than 36 hpf of age.

**RB Migration and Morphology Changes: Potential Indicators or Triggers of PCD**

In wildtype and *isl3* zebrafish, RB neurons migrate from a dorsal-lateral position in spinal cord towards the midline to form an almost linear row by 48 hpf. During the migration, a corresponding decrease in cell size also occurred.

We found that RB neurons migrate in anterior to posterior, extend their processes over the underlying musculature, and are displaced in a ventral direction during embryonic maturation. RB neurons also appeared to undergo two waves of cell size reduction. The first wave occurred at 18-24 hpf, where the ratio of small cells to larger cells increased over this time span. However, by 30 hpf the number of small RB cells greatly declined. The second wave in size reduction was observed from 36-96 hpf, where the ratio of small cells to large cells increased substantially. We infer that the first wave is most likely associated with RB neurons reaching an apex in development and the second wave may imply that RB neurons completely entered into programmed cell death and are undergoing cell shrinkage and degeneration.

During development RB neurons undergo many morphological changes which may be indicative of their demise. Previous studies have shown that the smaller, square-like shape of RB cell bodies suggests that they are dying (Patten et al., 2007) and that a decrease in nuclear density is a prominent feature of cell death (Lamborghini, 1987). In this study, we have reported that RB neurons undergo morphological changes throughout the course of development. Early on, RB neurons have a large, round-like shape and a prominent nuclear region is observed. However, at
later stages, RB neurons take on a smaller, hexagon-like shape and the nuclear region appears degenerated. These observations are consistent with the cell undergoing apoptosis.

**How Long do RB Cells Actually Survive?**

Differentiated RB neurons are first detected as early as 11 hpf (Rossi et al., 2009). Beginning at approximately 19 hpf, they undergo a cell death process which involves several proposed mechanisms. Previous studies have claimed that RB neurons are completely removed early on during development. A study utilizing the HNK-1 antibody to identify RB neuronal somata reported that RB neurons are entirely lost by 5 dpf (Williams et al., 2000). In contrast, RB neurons retrogradely labeled using Dil were observed as late as 7 dpf (Reyes et al., 2004). In our experiments, the most stable marker of RB neurons was anti-Hu which detected RB neurons as late as 6 dpf. At 6 dpf, the RB neuron somata were reduced in size, but when labeling was performed in *isl3* fish, the identity of these small cells was confirmed by their GFP expression (Figure 3.12A); they were indeed small RB neurons. Studies investigating the expression patterns of PKC in zebrafish have reported the presence of RB neurons out to 13 dpf (Patten et al., 2007). Again, the cell size of the PKC positive RB neurons was reduced. Thus, there are many inconsistencies concerning the exact survival age of RB neurons. This likely has to do with the available markers used to detect RB neurons in zebrafish.

We have also observed RB neurons at later stages of development than previously reported using the *isl3* reporter fish. Interestingly, we show that RB neurons are seen as late as 33 days post fertilization (Figure 3.12B); however evidence of whether or not these cells are functional at 33 dpf has not been provided. At 33 dpf, the GFP positive cells were small, about 5-6 microns in diameter. We conclude that due to the specified limitations of stable, reliable RB markers used in non-transgenic fish, an accurate assessment of the presence of RB neurons at late stages of development was not obtained in previous studies.
Figure 3.10 Comparison of aat and zn12 labeling in wildtype zebrafish during development. A: At 24 hpf, zn12 labels RB neuron peripheral processes and recognizable growth cones (open arrows), axons, and somata. From 30-72 hpf, zn12 labeling becomes more elaborate in RB peripheral processes. From 48-72 hpf, the zn12 label appears fragmented. B: At 24 hpf, aat detects the dorsal portion of RB processes. By 30 hpf, RB processes exhibit a more elaborate and continuous distribution of aat labeling. At 48 hpf, aat distribution in RB peripheral processes is fragmented. Bottom: Cartoons of aat and zn12 labeling in RB neurons from 24-48 hpf. Anti-acetylated tubulin only detects RB processes at 24 hpf. Scale bar = 20 μm. dlf- dorsal longitudinal fasciculus, lt- lateral line
Figure 3.11 RB neurons project their processes out in the skin. A: Left: A 24 hpf *isl3* embryo was labeled with zn12 to reveal RB somata and processes. B: At 36 hpf, F59 labeling in *isl3* reveals slow muscle fibers as well as GFP expression in peripheral RB processes. At the right, the rotated views emphasize RB processes extending over musculature (open arrows). Scale bars: A-B (left) = 10 μm, rotated views = 20 μm.
Figure 3.12 RB neurons in “aging” isl3 zebrafish. A: At the top are lateral views of 2 day old and 6 day old isl3 embryos showing GFP expressing RB neurons. In each case, anti-Hu was used to label RB neurons (middle panels). In the merged images (bottom panels), GFP positive RB neurons were detected with anti-Hu (open arrowheads). Note the reduction in size of the RB neurons in the 6 day old zebrafish. They range in size from 4-7 microns in diameter. B: RB neurons and the dorsal longitudinal fasciculus (open arrows) are still detected in isl3 zebrafish at 17 dpf, and 33 dpf respectively. The RB neurons are less than 6 microns in diameter. Scale bars = 10 \( \mu \text{m} \).

### Studying RB PCD in Zebrafish

The transient nature of markers used to study RB development as well as the rapidly changing morphology of RB neurons can potentially be used as diagnostic tools to further study various aspects RB PCD. Transient markers such as zn12 and aat detect RB somata early in development, but not at later stages. For example, if the PCD pathway for RB neurons was potentially modified by genetic or pharmacological manipulations, observations could be taken to monitor the presence of these markers at later stages of development and see if there are any differences in their ability to detect RB neurons when the markers should not detect the RB
neurons. The onset of tubulin fragmentation in RB peripheral processes can also be used as a diagnostic tool. Svoboda et al. 2001, used alterations in aat labeling as an indicator of RB neuron survival in a mutant zebrafish known as macho. Likewise, changes in RB cell size, cell number and migration are consistent processes occurring at specific times during development.

With our characterization of RB development in zebrafish complete, a variety of anatomical phenotypes can be utilized to investigate PCD and manipulations that alter PCD. Using a PCD model, preferably a zebrafish line that expresses GFP in RB neurons (isl3), researchers could observe differences in the size and morphology of RB cells that would typically not be seen at the stage of development analyzed. For example, one could perform a manipulation resulting in RB neurons failing to migrate to the midline by 48 hpf and then determine whether or not tubulin fragmentation is evident in the RB epidermal processes of those 48 hpf embryos. If tubulin distribution remained continuous, this would potentially indicate that either the RB neurons have failed to enter into a PCD pathway or the biology of the cell has been altered. We would predict that cells would have not have migrated to the midline by 48 hpf. On the other hand, a genetic or chemical manipulation resulting in a continuous distribution of tubulin in the processes coinciding with RB cells completely migrated to the midline by 48 hpf would indicate that the manipulation was directly influencing RB peripheral process development.
CHAPTER 4
THE EFFECT OF EMBRYONIC NICOTINE EXPOSURE ON ROHON-BEARD NEURON DEVELOPMENT

4.1 Introduction

Results presented in Chapter 2 indicated that early embryonic nicotine exposure produces an increase in the embryonic motor behavior by activating nAChRs on RB neurons. We predicted that nicotine could disrupt normal RB development in zebrafish. To date, there are limited amounts of data describing RB neuron development in detail. Therefore, we decided to perform a baseline characterization of RB development in which we described aspects of RB development never reported before. In this chapter, the effect of nicotine on RB development and cell death is analyzed by using a variety of anatomical phenotypes described in Chapter 3.

Nicotine exposure can alter vertebrate nervous system development. For instance, abnormalities in axonal targeting occur in zebrafish as a result of embryonic nicotine exposure (Menelaou and Svoboda, 2009; Svoboda et al., 2002). Nicotine exposure in larval zebrafish causes growth and behavioral defects as well as increased morbidity (Parker and Connaughton, 2007). In mice, prenatal nicotine exposure can induce irreversible changes in behavior (Nordberg et al., 1989), impair cholinergic angiogenesis (Cooke et al., 2009), and alter hippocampal morphology (Huang et al., 2007; Slawecki et al., 2000). In rats, early exposure to nicotine affects normal patterning within the cholinergic system (Falk et al., 2005; Navarro et al., 1989), impairs muscarinic receptors (Slotkin et al., 1999) and induces profound changes on the dopaminergic and serotonergic systems (Muneoka et al., 2001). These studies suggest that nicotine can be neurotoxic early in development.

In contrast, administering low doses of nicotine during critical times of development has been shown to produce positive effects on vertebrate nervous system development (Ryan et al., 2001). Recent studies have shown that nicotine can act as an anti-inflammatory agent, an anti-
depressant, or as a neuroprotectant in specific cases (Ferrea and Winterer, 2009; Tunez et al., 2009; Picciotto and Zoli, 2008). In rats, nicotine rescued cortical neurons and motoneurons from apoptosis induced by glutamate toxicity (Akaike et al., 2009; Nakamizo et al., 2005; Hildebrandt et al., 2003) and reduced mitochondrial damage in the brain (Cormier et al., 2003). The neuroprotective qualities of nicotine are likely mediated by specific nAChRs, preferably those comprised in part of \( \alpha_6, \alpha_4, \alpha_7, \) and \( \beta_2 \) subunits (Huang et al., 2009; Hejmadi et al., 2003; Stevens et al., 2003).

Here we examine the effects of nicotine on a unique population of transient neuron, the mechanosensory RB neuron. Importantly, RB neurons are not innervated by other neurons. Thus, perturbations altering their development are likely not the indirect consequence of altering the function of upstream neurons.

Similar to amphibian RB neurons, zebrafish RB neurons are born early and are quickly removed via a programmed cell death (PCD) pathway. Anatomical characteristics of RB PCD include (1) a fragmentation of tubulin distribution in RB peripheral processes (Reyes et al., 2004; Svoboda et al., 2001), (2) changes in the morphology of the cell (Patten et al., 2007), and (3) the presence of fragmented DNA. Alterations in RB cell migration patterns may also be linked to cell death. We examined these four characteristics of RB development in embryos exposed to agents that modulate nAChR activity including nicotine and provide evidence that embryonic exposure to these agents can rescue RB cells from dying, thus acting as a neuro-protective agent.

4.2 Materials and Methods

**Zebrafish Care and Maintenance**

Fertilized embryos obtained from natural spawnings of transgenic [Tg(isl3:GFP), referred to as *isl3* from here onward] and wildtype zebrafish (EkkWill Waterlife Resources, Rustin, Florida) were staged according to the *Zebrafish Book* (Westerfield, 1995). Adult fish were kept
at 28.0°C on a 14 h light/10 h dark cycle. All embryos collected from group or paired spawns were cleaned in system water, transferred to 100 mm Petri dishes containing embryo medium, and placed in an incubator at 28.0°C until further processing. Embryos of the isl3 zebrafish transgenic line express green fluorescent protein (GFP) in retinal ganglion cells and their axons which project through the optic nerve, lateral line ganglia, trigeminal ganglia, RB neurons, and dorsal root ganglia. The isl3 line was kindly provided by Chi-Bin Chien and Andrew Pittman at the University of Utah, School of Medicine.

**Agarose Embedding**

Zebrafish embryos were mounted dorsal side up in 1.25% agarose (Fisher Scientific, Fair Lawn, N.J.) made in PBS. In some cases, living isl3 embryos (~24 hpf) were anesthetized in 0.01% tricaine methanesulfonate (MS222) for 5-10 minutes, individually mounted in agarose blocks, and quickly placed on glass bottom petri dishes for viewing. Following image acquisition, individual fish were carefully removed from the agarose and transferred one-by-one to 12-well plate dishes containing embryo medium. Embryos remained individually housed in an incubator at 28.5°C and maintained daily until further processing. At later stages of development, living embryos previously viewed were anesthetize and re-mounted in agarose and imaged using fluorescent microscopy.

**Drug Administration**

Embryos were developmentally staged in accordance to previously described methods (Kimmel et al. 1995). 12-18 hpf embryos were exposed to varying concentrations of nicotine (1 μM-60 μM; Sigma, St. Louis, MO, Cat. No. N3876-5 ml), DHβE (10-40 μM; Sigma, St. Louis, MO) or epibatidine (125 nM-1 μM; Tocris, Saint Louis, Missouri) made in embryo medium (Westerfield, 1995). Epibatidine and DHβE stocks were stored at 4°C and -20°C respectively while nicotine stocks (31.2 mM) were made fresh each day. All stocks were made in distilled
water and then diluted in embryo medium to the desired working concentration. In most exposure settings, embryos were continuously exposed until 48 hpf. At 48 hpf, embryos were either fixed in 4% paraformaldehyde or rescued in embryo medium and fixed at 72 hpf. All embryos were manually dechorinated at 27-30 hpf and kept in an incubator at ~28°C until the desired stage of development was reached.

**Immunohistochemistry**

Embryos were fixed in 4% formaldehyde and stored overnight at 4°C. The following day, fixed embryos were washed at room temperature for 40-60 minutes in Phosphate Buffer Saline (PBS) containing 0.1% Tween-20 (PBST). Head and tail clippings were performed to identify control groups from exposed groups. This procedure allowed embryos from multiple groups to be processed in the same tube/vial to eliminate inconsistency with antibody staining. Details of the whole-mount immunohistochemistry protocol performed in wildtype and *isl3* zebrafish have been published previously (Svoboda et al., 2001, Pineda et al., 2006, Menelaou and Svoboda, 2009). Antibodies were prepared in NCS/PBST or PBST. The primary antibodies anti-acetylated tubulin (1:250 dilution, Sigma Aldrich) and anti-Hu (1:250 dilution, referred to as Hu in the figures; Molecular Probes, Eugene, OR) were used to reveal Rohon-Beard (RB) neuron somata and peripheral processes.

Antibodies which recognize the β2 nAChR subunit in mammals (mAb 290, 1:500 dilution, Sigma Aldrich) or zebrafish CHRN2B (Welsh et al., 2009, 1:250 dilution, Antibodies Incorporated, Davis CA) were also used in this study. A fluorescent secondary antibody (Alexa 488 and 546; Molecular Probes, Eugene, OR) was used at a 1:1000 dilution to reveal primary antibody labeling.
**TUNEL Labeling**

For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling of GFP positive RB neurons in *isl3* embryos, embryos were pre-washed in PBST for 30-40 minutes. This pre-wash period was followed by a 15 min (24-36 hpf), 20 min (48-60 hpf), or 30 min (72 hpf and older) digestion in protease (1mg/ml) at 37ºC which was followed by brief washes (3 x 1 min) in PBST. The samples were then fixed in 4% paraformaldehyde for 10 minutes to stop the protease reaction. Embryos were then rinsed in PBST (6 x 1 min) and allowed to stand in PBST for 20 minutes. Afterwards, embryos were preincubated for 15 minutes in a 1x TTase buffer solution (5x:125mM Tris buffer, 1 M Na cacodylate, 1.25 mg/ml bovine serum albumin (BSA), 1% Tween 20, pH 6.6 room temperature) with CoCl$_2$ added to the final mixture for a one millimolar concentration. Following this preincubation, embryos were re-incubated (20-25 embryos per 1.5 microcentrifuge tube) in a reaction solution (20 μl of TTase buffer, 0.5 μl of TdT, 0.50 μl of rhodamine dUTP, 71 ml of milliQ water, and 8 μl of CoCl$_2$) on ice and in the dark for 60 minutes. They were then washed in the reaction solution at 37ºC in the dark for an additional 60 minutes. After this, the embryos were washed in PBST (3x 1 min) and either immediately viewed or stored in PBST at 4ºC. TUNEL positive cells expressing GFP were counted within the head, trunk, and tail regions of spinal cord. Quantifications were done by calculating the average percentage of TUNEL positive RB neurons in comparable regions of interest spanning the anterior posterior axis of the fish.

**Morphological Analysis**

**RB Migration and Cell Size**

RB neurons were identified by their large somata, dorsal position in spinal cord and by anti-hu immunoreactivity in wildtype lines as well as GFP expression in the *isl3* line. Anti-Hu recognizes an RNA binding protein that is abundantly expressed in RB somata (Henion et al.,
1996), developing dorsal root ganglia neurons (Marusich et al., 1994) and spinal neurons. RB neuronal migration in all experimental groups was quantified at 48 hpf and soma size was measured in embryos ranging from 72-120 hpf. The region that was analyzed extended 4-6 segments above the yolk sac extension.

RB migration and cell size measurements were made using AxioVision 4.7.1 (Carl Zeiss Imaging Solutions). Specifically, the midline of spinal cord was determined by measuring the distance between the most lateral left and right boundaries of cord then dividing by half. RB cell movement to the midline was then quantified by calculating the distance from the center of RB nuclei to the midline. RB cell size was measured by multiplying the lengths of the horizontal and vertical axes of individual cells.

**Distribution of aat in RB Peripheral Processes**

At 48 hpf, wildtype zebrafish were labeled with the aat antibody to reveal acetylated tubulin in RB peripheral processes. For each exposure paradigm, RB processes were examined for an obvious fragmented or continuous aat immunoreactivity distribution. The following qualitative measurements were applied: Completely fragmented, partially fragmented, and continuous (no fragmentation is apparent). All observations were made in the region above the yolk sac extension.

**Visualization of Antibody Labeling**

Images were captured using a Zeiss Axiovert 200 M inverted microscope (Thornwood, NY) equipped with epifluorescence, a Zeiss ApoTome and an ORCA-ER digital camera (Hammamatsu, Japan). Images were obtained using either a 20 x dry objective (N.A. 0.6) or a 40x oil immersion objective (N.A. 1.30). Serial z-stacks were acquired with thicknesses of individual sections ranging between 0.3-0.7 μm.
For each experiment, the embryos were either mounted in agarose (see above) or placed in a drop of PBST on 1-mm-thick microscope slides, cover slipped, and sealed with nail polish for viewing. In all cases, the same exposure time (ms) was used to acquire images for control embryos and drug exposed embryos of the same trial. All samples were first viewed at a 20x magnification and captured as 2-D single focal plane images. This was done to document the overall results of each experimental group. Then, samples were randomly chosen and imaged at a 40 x magnification and captured in serial z-stacks for a more detailed analysis. All lateral views are shown with the rostral region orientated to the left and dorsal to the top. All dorsal views are shown with the rostral region to the top.

Images were then transferred to and reconstructed using Imaris 5.7.2 (Bitplane, St. Paul, MN). Further adjustments for brightness and contrast were performed using Photoshop 7.0 (Adobe, San Jose, CA). Figures were assembled using CorelDraw Graphics Suite 12 (Ottawa, Ontario, CA).

**Statistics**

All values are presented as means ± standard error of the means (SEM). Kruskal-Wallis One Way Analysis of Variance (Anova) was performed in order to assess levels of statistical significance. P-values < 0.05 are indicative of statistical significance. All experiments were replicated three or more times. All results shown are representative data selected from each group of replicated experiments.

**4.3 Results**

**Acetylated Tubulin Distribution in RB Peripheral Processes is Altered by Nicotine Exposure**

In zebrafish, mechanosensory RB neurons are located at the most dorsal region of spinal cord. These neurons are identified by their large soma and most dorsal position in cord. They are
the first neurons to project growth cones and extend their axons into the dlf as well as project peripheral processes out in the skin. In young embryos, 24--27 hpf, tactile stimulation to the skin activates RB neurons which in turn activate excitatory CoPA to produce swimming.

In previous work, aat distribution in RB peripheral processes has been characterized during RB development based upon the appearance of fragmentation and continuity (Svoboda et al, 2001; Chapter 3). In that work, continuous aat distribution in RB processes was evident from 27 hpf to 36 hpf. Beginning approximately at 40 hpf, a more fragmented aat distribution became apparent. Uniform aat distribution within RB processes was indicative of viable RB neurons, whereas altered aat distribution suggested that the cells had entered into PCD.

Chronic embryonic exposure to nicotine or epibatidine altered the aat immunoreactivity pattern in RB processes out in the skin. Embryos in their chorions were chronically exposed to either 30 µM nicotine or 250 nM epibatidine from 12-48 hpf. At 48 hpf, control embryos displayed a fragmented aat distribution, whereas exposed embryos appeared to have continuous aat distribution (Figure 4.1, A-C). The percentage of fragmented aat distribution was greatly reduced in exposed embryos (11-44%) when compared to controls (87%) (see table 4.1). Interestingly, at 48 hpf the aat distribution within RB neurons of exposed embryos resembled aat distribution in younger fish (27-36 hpf) suggesting that RB PCD has been either delayed or rescued. Similar results were obtained in a previous study where RB PCD was shown to be regulated by activity dependent mechanisms (Svoboda et al, 2001). Therefore, it seems reasonable to conclude that RB development may be regulated via a mechanism involving specific nAChRs, namely α4/β2 expressed on RB somata.

**Alterations in RB Somata Morphology in Nicotine Exposed Embryos**

RB somata undergo characteristic morphological changes as they are removed by a programmed cell death process and replaced by dorsal root ganglion neurons. Observable
phenotypes include: changes in their somatic structure-going from an oval/round shape to more of a hexagonal-like shape (Patten et al., 2007), decreases in cell size and nuclear density (Lamborghini, 1987), and a reduction in RB cell number (Slatter et al., 2005). In *isl3* embryos, GFP-expressing RB neurons appear smaller over time with a less defined nuclear structure by 72 hpf. Embryos exposed to nicotine or epibatidine from 12-48 hpf exhibited larger RB cells at 72 hpf compared to RB cells from 72 hpf controls (Figure 4.2, A-C). Moreover, they appeared to retain some nuclear integrity. This type of cellular morphology is actually what is seen in younger fish possessing healthy, viable RB cells. By 96 hpf there was no observable difference in RB cell size between controls and exposed groups (see table 4.2). These results provide additional evidence that nicotine is behaving partially as a neuroprotective agent during RB development in zebrafish.

Figure 4.1 Programmed Cell Death (PCD) of RB neurons examined by anti-acetylated tubulin (aat). Distribution of aat in RB peripheral processes at 48 hpf. A, aat distribution appears fragmented by 48 hpf in control fish. Anti-acetylated tubulin intensely labels the lateral line (black arrow). B-C, Wildtype embryos were chronically exposed (12-48 hpf) with 30 μM nicotine (B) or 250 nM epibatidine (C). When compared to controls, embryos exposed to either nicotine or epibatidine remained continuous. Scale bar = 20 μm.
Table 4.1 The effect of nicotine and epibatidine on the peripheral processes of RB neurons. A qualitative analysis was used to determine acetyl distribution in RB peripheral processes. All observations were taken specifically above the yolk sac extension. Embryos were individually scored based on complete fragmentation (no continuity; an overall beaded appearance), partially fragmented (some continuity; mostly fragmented), and continuous (no fragmentation; complete continuity).

<table>
<thead>
<tr>
<th>Exposure window (hpf)</th>
<th>Treatment group</th>
<th>Fish (n)</th>
<th>Anti-acetylated Tubulin Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Completely fragmented (%)</td>
</tr>
<tr>
<td>12-48</td>
<td>Control</td>
<td>63</td>
<td>87% (n=55)</td>
</tr>
<tr>
<td></td>
<td>Epibatidine 125 nM</td>
<td>9</td>
<td>44% (n=4)</td>
</tr>
<tr>
<td></td>
<td>Epibatidine 250 nM</td>
<td>42</td>
<td>16% (n=7)</td>
</tr>
<tr>
<td></td>
<td>Epibatidine 500 nM</td>
<td>34</td>
<td>23% (n=8)</td>
</tr>
<tr>
<td></td>
<td>Nicotine 1 μM</td>
<td>9</td>
<td>11% (n=1)</td>
</tr>
<tr>
<td></td>
<td>Nicotine 5 μM</td>
<td>4</td>
<td>0% (n=0)</td>
</tr>
<tr>
<td></td>
<td>Nicotine 15 μM</td>
<td>12</td>
<td>25% (n=3)</td>
</tr>
<tr>
<td></td>
<td>Nicotine 30 μM</td>
<td>27</td>
<td>29% (n=8)</td>
</tr>
<tr>
<td></td>
<td>Nicotine 60 μM</td>
<td>8</td>
<td>25% (n=2)</td>
</tr>
</tbody>
</table>

Figure 4.2 Morphological changes of RB neurons observed in chronically exposed embryos. Isl3 embryos expressing GFP in Rohon-Beard neurons at 3 days post fertilization (dpf) A, RB neurons (arrow) appear smaller (less than 10 microns), whereas in embryos exposed with 250 nM epibatidine (B) and 30 μM nicotine (C) from 12-48 hpf appeared to be larger (10 microns and greater). This is indicative of RB neurons survival. Scale bar = 10 μm.
Table 4.2 The effect of nicotine and epibatidine on RB cell morphology. TUNEL positive RB neurons were counted in regions spanning the rostral, trunk, and caudal sections of the embryo at the specified stages. All results are presented as mean ± SE. Asterisks (*) denote significant differences between nicotine and epibatidine exposed groups and controls (p value < 0.05, Anova).

<table>
<thead>
<tr>
<th>Age (dpf)</th>
<th>Fish (n)</th>
<th>Treatment group</th>
<th>RB cell size (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 hpf</td>
<td>14</td>
<td>Control</td>
<td>76.9±2.9</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Epibatidine 250 nM</td>
<td>101.3±3.6 †</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Nicotine 30 μM</td>
<td>92.8±3.5 †</td>
</tr>
<tr>
<td>96 hpf</td>
<td>17</td>
<td>Control</td>
<td>54.9±1.6</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Epibatidine 250 nM</td>
<td>53.2±2.4</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>Nicotine 30 μM</td>
<td>52.1±1.5</td>
</tr>
</tbody>
</table>

**Nicotine Exposure Delays RB Cell Migration to the Midline**

We previously characterized the migration of RB neurons in dorsal spinal cord (Chapter 3). To recap, RB neurons in young embryos (18-34 hpf) start out as two rows in the most dorsolateral position in spinal cord. Nearing 36 hpf, they begin to migrate independently towards the midline forming one row by 48hpf in the most rostral region of the fish (refer to Chapter 3). Because RB neurons likely express functional nAChRs, we hypothesized that nicotine would modulate RB migration to the midline. To test this, we chronically exposed wildtype embryos or *isl3* embryos (12-48) hpf in the chorion to nicotine and epibatidine and observed RB migration at 48 hpf. There were no observable gross anatomical changes at 48 hpf caused by the exposures (Figure 4.3, Top). However, RB neurons failed to completely migrate to the midline in exposed embryos (Figure 4.3, A). RB cells in control embryos have migrated to the midline and are linearly arranged by 48 hpf. In contrast, RB cells in nicotine and epibatidine exposed embryos remained at the periphery of dorsal cord arranged as two rows. The percentage of RB cells...
present at the midline was reduced approximately by 50% in exposed groups when compared to controls (Figure 4.3, B). These results also suggest that the activation of β2 nAChRs expressed on RB neurons plays a role in RB development in the context of cell migration.

**Ventrally-Directed RB Neuronal Migration is Delayed by Nicotine Exposure**

Rohon-Beard neurons have been characterized in a variety of ways. The basic anatomy of RB neurons was first described decades ago in *Xenopus* and later outlined in zebrafish by the usage of simple fluorescence and DIC microscopy (Roberts, 2000; Clarke et al., 1984; Jacobson, 1981). Although we have gained tremendous insight about RB neuron development and structure, fluorescent microscopy alone limits our ability to completely visualize the morphological profile of RB somata, axons, and peripheral processes. Therefore, we utilized fluorescent microscopy in conjunction with detailed image analysis to observe characteristics of RB neuron development not previously described. Early in development, RB neurons form two rows in the most dorsal-lateral region of spinal cord and then independently migrate to the midline to form one row. In parallel, RB neurons undergo a migration in a ventral direction within spinal cord (refer to Chapter 3). 3-D optical stacks revealed that RB neurons in 72 hpf zebrafish exposed to either nicotine or epibatidine from 12-48 hpf exhibited a delay in ventral migration when compared to RB neurons in 72 hpf control fish (Figure 4.4, A-C; table 4.3).

**Nicotine Delays RB Neuron Programmed Cell Death (PCD)**

The appearance of RB neurons occurs early in zebrafish development (~15hpf) and they are quickly removed via a programmed cell death pathway. RB neurons enter into a cell death process around 19 hpf with peak cell death occurring around 34 hpf (Williams et al., 2000). Up to this point, we have shown that nicotine and epibatidine can modulate RB neuron development making 48 hpf RB neurons look like 30-36 hpf RB neurons. This could be explained by nicotine directly influencing RB PCD.
Wildtype embryos were exposed to either nicotine or epibatidine from either 12-34 hpf and then processed for TUNEL labeling at 34 hpf (Figure 4.5, A) or 12-48 hpf and processed for TUNEL labeling at 72 hpf (Figure 4.5, B). We observed a significant reduction in the number and intensity of TUNEL positive GFP-expressing RB neurons in exposed embryos when compared to controls. Interestingly, we observed a similar reduction between controls and exposure groups for both 34 hpf and 72 hpf. The average percentage of TUNEL positive RB neurons was significantly lower in embryos exposed to either nicotine or epibatidine at both time points (see table 4.4). However, by 72 hpf, some variability was evident in TUNEL labeling of embryos exposed epibatidine (refer back to Figure 4.5, B). Overall, these data suggest that nicotine may play a neuro-protective role in RB neuronal development. Since epibatidine can produce similar if not the same effects as nicotine on RB development, it is likely that nicotine mediates its effects via β2 subunit containing nAChRs.

Alterations in RB Morphology in DHβE Exposed Embryos

Given that nicotine and epibatidine likely mediate their effects on RB neuron development via neuronal nicotinic acetylcholine receptors containing the β2 subunit, we wanted to test whether or not blocking this receptor subtype would inhibit the action of nicotine. We initially antagonized receptors containing the β2 nAChR subunit by pre-incubating embryos with DHβE, an α4/β2 nAChR antagonist. In control experiments, embryos were chronically exposed to DHβE from 12-48 hpf.

To our surprise, RB neurons in DHβE exposed embryos were delayed in migration to the midline (Figure 4.6, A) and also exhibited continuous aat distribution in RB peripheral processes at 48 hpf (Figure 4.6, B). The percentage of RB neurons with continuous aat distribution in peripheral processes and delayed migration was increased in DHβE exposed embryos compared
to non-exposed embryos (see table 4.5). These results were somewhat unexpected given that DHβE has an antagonizing effect on α4/β2 nAChRs.

4.4 Discussion

Nicotine can function as a neuro-protective compound as well as a neurotoxic compound (Ferrea and Winterer, 2009). In the context of neuro-protection, nicotine protects against neuronal cell death in PC12 cells (Yamashita and Nakamura, 1996). Other studies have also shown that nicotine protects cortical neurons from the neurotoxic effects of glutamate (Akaike et al., 2009).

In this chapter, we have shown that embryonic nicotine exposure can increase the survival rate of RB neurons in zebrafish. We provide evidence that this neuro-protective effect is mediated through specific nAChRs, likely those containing the β2 nAChR subunit. In support of this, experiments performed on primary cortical cultures from mice show that the neuro-protective effects of nicotine involve the activation of β2 containing nAChRs (Stevens et al., 2003).

**Variations in RB Development Describes a Neuro-Protective Role to Nicotine**

In zebrafish, RB neurons enter a PCD pathway early on during embryogenesis. Several studies have provided possible mechanisms describing how RB neurons die (Behra et al., 2002; Svoboda et al., 2001; Williams et al., 2000), but exactly why RB neurons are short-lived remains to be fully investigated. A growing body of work provides evidence of certain characteristics seen in RB neurons that are indicative of a cell death phenomenon. For instance, RB neurons undergo somatic and axonal morphology changes that signify that they are dying. Their somata become smaller in size, they form more of an edgy hexagon-like shape from an oval-rounded shape, there is a decrease of nuclear volume, and degeneration of their peripheral arbors becomes apparent (Patten et al., 2007; Reyes et al., 2004; Lamborghini, 1987).
These morphological features along with RB cell migration were analyzed in this study. Examination of anti-acetylated tubulin immunoreactivity in RB peripheral processes showed continuous distribution at a time when distribution should appear fragmented (at 48 hpf). This distribution in older embryos (48 hpf) resembled the type of distribution that is seen in younger fish (24-36 hpf). We obtained similar results looking at aat immunoreactivity in RB peripheral processes of embryos exposed to varying concentrations of nicotine. In addition, embryos previously exposed to nicotine showed delayed RB cell migration that could only be observed at later stages of RB development (48 and 72 hpf). At 48 hpf, RB cells remained in two rows at the lateral boundaries of spinal cord in nicotine exposed embryos compared to control embryos where RB neurons formed one row at the midline of spinal cord. Likewise, at 72 hpf RB neurons failed to migrate ventrally within spinal cord, thus resembling a younger fish (~36-48 hpf).

At 3 days post fertilization the size of RB cell bodies was relatively large in nicotine exposed embryos compared to controls, thus suggesting that these neurons were healthy. We also noticed that by 4-5 days post fertilization, no difference was observed in RB cell size between controls and nicotine exposed embryos. Based on these results, we assume that certain developmental traits characteristic of RB PCD are manifested at different times. In addition, the results from morphological analysis suggested that nicotine and epibatidine were rescuing cells from PCD or at minimum, delaying their entry into the PCD pathway. However, the nicotine-induced phenotypes could possibly occur independently of PCD. For instance, studies done in Drosophila have shown that the inactivation or overexpression of PTEN, a tumor suppressor gene affects cell size (Huang et al., 1999). The PTEN mutants tend to have larger cell bodies. Likewise, PTEN knockout mice show changes in cell size and growth (Kishimoto et al., 2003). PTEN has the ability to dephosphorylate phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) thus,
acting as a negative regulator of the PI3K/AKT pathway. The PI3K/AKT pathway plays an important role in cell survival, growth, and motility (Thompson and Thompson, 2004). Two PTEN genes have been found in zebrafish and that play a vital role in its development (Finkielztein et al., 2006; Croushure et al., 2005). Zebrafish mutants that lack functional PTEN showed an increase in cell survival (Faucherre et al., 2008). In addition, in order for cells to migrate they must be polarized via a mechanism that involves the Rho family of GTPases, phosphoinositide-3-kinases, integrins, microtubules, and vesicular transport. The Rho family of GTPases includes Rho and CdC42 which are vital regulators of cell morphology and movement (Leskow et al., 2005). It has been found that the inhibition and inactivation of CdC42 can disrupt the directionality of cell migration (Ridley et al., 2003). Therefore, the nicotine-induced effect could be the result of disrupting these downstream mechanisms, without influencing PCD pathway.

**Embryonic Nicotine Exposure Delays RB Neurons from PCD**

RB neurons are first detected at 15 hpf using the HNK-1 antibody. They soon enter a cell death process as early as 19 hpf with peak cell death occurring around 34 hpf and 72 hpf (Williams et al., 2000). Embryos chronically exposed to nicotine showed significantly reduced TUNEL positive RB neurons at 34 hpf. Likewise, at 72 hpf, nicotine exposed isl3 embryos had reduced TUNEL activity in RB neurons.

We noticed the same trend in the ratio of TUNEL positive RB cells in controls compared to exposed groups at both stages development. Thus, in normal RB neurons, the peak of PCD occurs prior to tubulin fragmentation in peripheral processes (48 hpf), changes in cell size, and their migration patterns. Since nicotine and epibatidine were altering all of these characteristics in parallel, we believe the exposure is delaying entry into PCD and rescuing the cells from PCD at a time when they should dye off. In this context, nicotine is protecting RB cells via a neuro-
protective fashion, but ultimately they will die. It is possible that the other mechanisms which regulate zebrafish RB PCD will ultimately override the nicotine exposure and trigger PCD in the embryo.

**The Role of nAChRs in PCD and Neuro-Protection**

When these studies were first being conceived, we predicted that nicotine exposure would activate nAChRs and prematurely kill the RB cells. We observed exactly the opposite where RB neurons actually maintained morphological phenotypes indicative of young embryos instead of those typical of older RB neurons. One possible mechanism that may explain this resides in the ability of DHβE to produce anatomical phenotypes that were similar to those caused by nicotine and epibatidine exposure. DHβE may bind nAChRs and then prevent endogenous ACh from being able to bind nAChRs. The implication of this is that endogenous ACh may somehow be activating RB PCD in the embryo. This is supported by another study (Behra et. al, 2002) where RB neurons died faster in embryos that lacked functional acetylcholinesterase.

Nicotine and epibatidine are potent cholinergic agonists. In a chronic exposure paradigm, they may exhibit a bimodal range of function where they first activate nAChRs and then actually desensitize them making them unable to bind endogenous ACh. Thus, rendering nAChRs unavailable to bind endogenous ACh either by applying an antagonist or through desensitization would yield the same result. In a previous study, we demonstrated that 30 μM nicotine was able to desensitize neuronal nAChRs expressed in young embryos. When embryos were exposed to for 5 minutes nicotine (30 μM) and then re-exposed 2 hours later, the second exposure did not produce an increase in musculature bending. In acute exposure behavior paradigms (5 minute exposure), one μM nicotine will not typically produce a robust motor output. However, we were
able to demonstrate that one µM nicotine, applied for 2.5 – 4 hours to the embryo was able to desensitize nAChRs making them unavailable to respond to a second application of 30 µM nicotine. Thus, desensitization of neuronal nAChRs was caused by the exposure and we suspect that those desensitized nAChRs are located on RB neurons. If this were true, both low and high concentrations of nicotine in a chronic exposure paradigm should produce similar morphological phenotypes. Indeed, when embryos were exposed to [1 µM, 5 µM, 15 µM, and 60 µM] the anatomy of RB neurons was similar in all the embryos (Figure 4.7, A-E). Therefore, we find it reasonable to conclude that nicotine desensitizes nAChRs located on RB neurons and by doing so, the receptor is made unavailable for ACh binding and RB neurons survive longer. Based on our data showing that the β2 nAChR subunit is located on RB neurons and that epibatidine and DHβE, two molecules that interact with α4/β2 nAChRs can modulate RB development, it is likely that β2 containing nAChRs play a role in RB development and PCD. Likewise, DHβE exposure would also block nAChRs and prevent ACh from binding (Figure 4.8). Thus, the resulting anatomical phenotypes caused by these manipulations could be similar.

In summary, many studies have established that early exposure to nicotine can cause deleterious outcomes on vertebrate development. This is the first morphological study to examine the potential neuro-protective role of nicotine on a distinctive population of spinal sensory neurons in zebrafish. The development and death of Rohon-Beard neurons have been studied for decades now in Xenopus, lamprey, and zebrafish models. The fact that they are born early but then are quickly removed by a PCD pathway makes this neuronal cell type an excellent cellular model to study neuro-protection in an in vivo context.
Figure 4.3 Embryonic nicotine and epibatidine exposure delays RB migration towards the midline. Wildtype zebrafish at 48 hpf. A: Hu positive RB cells (asterisks) have migrated and formed an almost linear row at the midline (dotted line). However, in nicotine and epibatidine exposed embryos, RB neurons remain at the periphery of spinal cord as two rows respectively. (Top): Photomicrographs of 48 hpf wildtype fish for each exposure group. Embryos chronically exposed with epibatidine and nicotine showed no gross morphological changes when compared to controls ($p$ value $< 0.05$, Anova). Scale bar = 10 μm. B: Bar graph quantifying the distance of RB neuron migration from the midline. Asterisks denote significance from the control group C: A bar graph quantifying the percentage of RB cells that have migrated to the midline. There is approximately a fifty percent reduction in the number of RB cells at the midline in exposed embryos compared to controls.
Figure 4.4 Embryonic nicotine and epibatidine exposure delays the ventral migration of RB neurons. Rotated views of RB neurons (green cellular signal at midline) and processes in isl3 labeled with aat at 72 hpf are shown. A, In control fish, RB neurons migrate ventrally down the midline (indicated by dotted line) in spinal cord. B-C, RB neurons in embryos chronically exposed (12-48 hpf) to 250 nM epibatidine and 30 μM nicotine showed a significant reduction in ventral migration when compared to controls. Scale bar = 10 μm.
Table 4.3 The effect of nicotine and epibatidine on RB cell migration. The migration of individual RB neurons was scored by measuring the distance from the most dorsal region of the embryo to the first RB cell. All results are presented as means ± SE. † denotes significant difference between nicotine and epibatidine exposed groups and controls (p value < 0.05, Anova).

<table>
<thead>
<tr>
<th>Age (hpf)</th>
<th>Fish (n)</th>
<th>Exposure group</th>
<th>Rohon-Beard cell migration (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 hpf</td>
<td>14</td>
<td>Control</td>
<td>17.24±0.86</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Nicotine 30 μM</td>
<td>12.09±0.87 †</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Epibatidine 250 nM</td>
<td>12.53±1.35 †</td>
</tr>
</tbody>
</table>

Figure 4.5 RB neuron survival revealed by TUNEL labeling. A-B (left), isl3 embryos at 34 hpf and 72 hpf; (middle), TUNEL staining revealed by a rhodamine tag; (right), Merge. A, At 34 hpf, isl3 embryos exposed to nicotine or epibatidine from 12-34 hpf were TUNEL labeled. The number of TUNEL positive RB cells significantly declined in exposed embryos when compared to controls. B, At 72 hpf, isl3 embryos exposed to nicotine and epibatidine from 12-48 hpf were TUNEL labeled. The number of TUNEL positive RB cells was greatly reduced in nicotine exposed embryos when compared to controls. In the epibatidine exposed larva, 4 of the 5 RB neurons shown in this field of view were TUNEL positive. Scale bars = 10 μm.
Table 4.4 RB PCD analysis in nicotine and epibatidine exposed embryos. The area of RB cell bodies was individually measured for the indicated stages. All results are presented as means ± SE.

<table>
<thead>
<tr>
<th>Age (hpf)</th>
<th>Fish (n)</th>
<th>Treatment group</th>
<th>RB cells (n)</th>
<th>TUNEL pos RB cells (n)</th>
<th>Avg % of TUNEL pos RB cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>34-36 hpf</td>
<td>6</td>
<td>Control</td>
<td>327</td>
<td>159</td>
<td>50.01±0.03%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Epibatidine 250 nM</td>
<td>335</td>
<td>68</td>
<td>19.72±0.04%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Nicotine 30 μM</td>
<td>310</td>
<td>76</td>
<td>12.20±0.03%</td>
</tr>
<tr>
<td>72-74 hpf</td>
<td>9</td>
<td>Control</td>
<td>364</td>
<td>225</td>
<td>62.55±0.05%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Epibatidine 250 nM</td>
<td>181</td>
<td>61</td>
<td>33.44±0.07%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Nicotine 30 μM</td>
<td>412</td>
<td>99</td>
<td>25.51±0.05%</td>
</tr>
</tbody>
</table>

Figure 4.6 DHβE delays RB neuron development in 48 hpf embryos. A, dorsal view of RB neurons) located at the midline (red dotted line) in an almost linear row in control fish. In DHβE exposed embryos RB neurons remain in two rows and fail to completely migrate. RB neurons at the midline are denoted by the asterisks. B, aat distribution in RB peripheral processes in control (top) and DHβE exposed embryos (bottom). Scale bars: A=10 μm, B= 20 μm.
Table 4.5 Chronic DHβE exposure modulates RB cell death at 48 hpf. Anti-acetylated tubulin distribution of RB peripheral processes were scored as completely fragmented if they exhibited an overall speckled appearance similar to controls, respectively. RB processes that were scored as partially fragmented exhibited some continuity. Distribution that was scored as continuous exhibited complete continuity and no evidence of fragmentation. All results are presented as mean ± SE. Asterisks (*) denote significance with a p-value < 0.05, Anova.

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>Fish (n)</th>
<th>Anti-acetylated Tubulin Distribution</th>
<th>Fish (n)</th>
<th>RB cell migration to the midline (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Completely fragmented (%)</td>
<td>Partially fragmented (%)</td>
<td>Completely Continuous (%)</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>64% (n=9)</td>
<td>35% (n=5)</td>
<td>0% (n=0)</td>
</tr>
<tr>
<td>DHβE</td>
<td>18</td>
<td>11% (n=2)</td>
<td>66% (n=12)</td>
<td>22% (n=4)</td>
</tr>
</tbody>
</table>
Figure 4.7 Nicotine dose-dependent effects on the peripheral processes of RB neurons. Wildtype embryos chronically exposed with varying concentrations of nicotine (1 μM, 5 μM, 15 μM, 30 μM, and 60 μM) from 12-48 hpf. A- E: aat distribution is altered in exposed embryos when compared to controls. At 48 hpf, wildtype controls display a fragmented aat distribution pattern in RB peripheral processes: In nicotine exposed embryos aat distribution appears continuous. This continuity is also observed at lower concentrations of nicotine (1 μM; arrow). Scale bar = 20 μm.
Figure 4.8 Proposed mechanisms for RB PCD. Top: nAChRs are expressed on RB neurons and are composed of various combinations of α and β subunits (likely β2). A: Acetylcholine (ACh) binds and opens the receptor allowing the flow of positively charged ions (likely calcium) to flow through the channel pore. This is followed by a calcium-dependent cell death pathway leading to RB PCD. B: Nicotine, a competitive nAChR agonist, binds and activates the receptor. Ultimately, nicotine desensitizes the receptor, thus making it unresponsive to the binding of ACh which leads to delayed RB PCD. C: DHβE, a competitive nAChR antagonist, binds to the receptor making it unavailable for ACh to bind, thus leading to delayed RB PCD.
CHAPTER 5
CHARACTERIZATION OF A NOVEL PRIMARY MOTONEURON MARKER IN ZEBRAFISH

5.1 Introduction

The ability to study and compare neuronal structures simultaneously using vertebrate models can be challenging. Although, recent advances in molecular biology and genomics have provided us the uncanny ability to study and visualize specific aspects of neuronal development \textit{in vivo}, the ability to study multiple structures in the same animal has still proven to be a daunting task. To date, researchers have used credible methods in order to study neuronal structures of interest. Particularly, in zebrafish methods such as immunohistochemistry (IHC)/Immunocytochemistry (ICC), fluorescent dye labeling, and transgenic models have been the foundation used to study aspects of motoneuron development \textit{in vivo}. However, these methods are somewhat limited in their ability to perform ideal experiments, such as those that may require dual-labeling of neuronal structures, namely motoneurons. Studies using IHC or ICC usually involve the use of antibodies that recognize either primary or secondary motoneurons. For instance, in order to perform dual-labeling a polyclonal (poly) antibody labeling the pmn in conjunction with a monoclonal (mono) antibody labeling the smn would be preferred. Unfortunately, the production of these particular antibodies is not always available in the preferred manner. Although this method can still be performed using the same type of antibodies (mono-mono; poly-poly), the ability to achieve optimal results can be laborious. Also, fluorescent dye injection experiments can be useful in some cases, but this too is a tedious and time consuming method. In addition, transgenic models have been proven to be very effective in achieving dual-labeling in structures, but in some transgenic models, the GFP expression pattern can be transient, over-powering, or unstable. This can potentially inhibit the study of these structures across a broad range of development as well as allow for accurate observations.
In chapter two, we described an antibody that detects $\beta_2$ nAChR subunits on RB neurons in zebrafish. Unexpectedly, we noticed that it not only labeled RB neurons (Figure 5.1), but also what seemed to be pmn. At that point, we realized that we may have accidently made a stable marker for primary motoneurons. To date, no one has a good, clean pmn marker. Antibodies such as zn-1 and znp-1 are currently being used to label pmn (Figure 5.2). But, even these antibodies do not provide the best stability or optimal resolution needed to analyze subtle yet significant changes during motoneuron development. In fact, recent studies using the znp-1 antibody have shown that znp-1 labels primary as well as smn in 48 hpf zebrafish (Tallafus and Eisen, 2008). On the other hand, markers are available to reliably label smn in fish. The antibody zn5, which recognizes the adhesion molecule DM-GRASP, labels smn somata and axons out to eight days of age and transgenic lines of zebrafish, where GFP is expressed in differing populations of smn, are also available to study smn anatomy. When we first started using the zebrafish $\beta_2$ nAChR antibody, we felt that the antibody was detecting smn axons. However that analysis was performed in 3 day old transgenic zebrafish (isl1, Welsh et al., 2009). And although labeling of GFP positive smn axons was observed in that study, in retrospect, we could have been misled as it is possible that GFP may also be expressed in pmn axons of those fish at later times of development.

So we set out to characterize the $\beta_2$ nAChR antibody to determine conclusively if it labeled pmn or smn axons. In this chapter, we will systematically characterize the $\beta_2$ nAChR antibody and demonstrate that it is a reliable marker of pmn axons in zebrafish. At the end of the chapter, double labeling studies will be presented where IHC was used to monitor the consequences of embryonic nicotine exposure on both pmn and smn axons in the same fish. Since we knew that smn axons were altered upon nicotine exposure, the simple question we
wanted to address was: Did changes in smn axonal pathfinding caused by the exposure coincide with any changes in pmn axonal pathfinding, or were they occurring in an independent fashion?

Figure 5.1 Rohon-Beard neurons express the $\beta_2$ nAChR antibody. At 36 hpf, RB somata and axons robustly express $\beta_2$ nAChRs. Scale bar = 10 µm.

Figure 5.2 Primary motoneurons revealed by znp-1. At 30 hpf (left panel), the znp-1 antibody labels primary motoneurons CaP and MiP (arrows) very well. However, by 72 hpf, the znp-1 labeling becomes highly elaborate and busy, thus making the identification of CaP and MiP motoneuron axons difficult. Scale bars = 20 µm.

5.2 Materials and Methods

Animal Maintenance

Zebrafish embryos were collected and handled according to the protocol outlined in the materials and methods section of chapters 3 and 4. The embryos utilized in this study were obtained from several wildtype zebrafish lines and the transgenic Tg(\textit{gata2}:GFP) zebrafish (Meng et al., 1997) which expresses GFP in a subset of secondary motoneurons that innervate the ventral myotome (Zeller et al., 2002).
**Immunohistochemistry**

Whole-mount immunohistochemistry was performed using a modified version of a previously published protocol (Svoboda et al., 2001, 2002; Pineda et al., 2006, Menelaou and Svoboda, 2009). All antibodies were prepared in NCS/PBST or PBST. The primary antibodies zn-1 (1:200 dilution, Developmental Hybridoma Bank, University of Iowa), znp-1 (1:250 dilution, Developmental Hybridoma Bank, University of Iowa), zn5 (1:500 dilution, Developmental Hybridoma Bank, University of Iowa), and the zebrafish specific β2 nAChR antibody (Welsh et al., 2009; 1:250 dilution) were used to reveal primary and secondary motoneurons in wildtype lines. A fluorescent secondary antibody (Alexa 488 or Alexa 546 (primarily used to reveal β2 labeling) Molecular Probes, Eugene, OR) was used at 1:500, 1:1000, and 1:5000 dilutions to reveal primary antibody labeling. The zn-1 antibody labels the neuropil region of primary motoneuron cell bodies. The znp-1 antibody labels primary motoneuron axonal projections. The zn5 antibody is a neuronal cell surface marker that labels secondary motoneurons as well as hindbrain neurons.

**Imaging Acquisition**

Images were captured on a Zeiss Axiovert 200 M inverted microscope (Thornwood, NY) equipped with epifluorescence, a Zeiss ApoTome and an ORCA-ER digital camera (Hammamatsu, Japan). Images were obtained using a 40 x oil immersion objective (N.A. 1.30). Serial z-stacks were acquired with thicknesses of individual sections ranging between 0.3-0.7 μm. In this study we obtained 368 serial z-stacks from 184 fish (wildtype and transgenic). Images were then reconstructed using Imaris 5.7.2 (Bitplane, St. Paul, MN). Further adjustments for brightness and contrast were performed using Photoshop 7.0 (Adobe, San Jose, CA). Additional processing and assembling of all figures was done using the CorelDraw Graphics Suite 12 (Ottawa, Ontario, CA).
Analysis of $\beta_2$ Labeled Primary Motoneurons

Primary motoneurons were identified by their position in spinal cord, axonal trajectories, and antibody labeling in wildtype lines. Secondary motoneurons were identified by antibody labeling. Primary motoneuron axonal projections and branching were analyzed per segment in a rostral to caudal direction in zebrafish ranging from 30 hpf to 12 dpf. Axonal phenotypes were characterized manually by eye and include: a hook-like projection, extra branching, and unraveling and twisting.

5.3 Results

Systematic Characterization of the Primary Motoneuron Axons, CaP and MiP using the $\beta_2$ nAChR Antibody

In zebrafish, pmn are mostly identified by their positions within spinal cord. The caudal primary (CaP) motoneuron is the first to extend its axon ventrally to innervate the ventromedial myotome. This is followed by the middle primary (MiP) motoneuron which extends its axonal projection dorsally to innervate the dorosmedial myotome. Lastly, the rostral primary (RoP) motoneuron extends its process laterally. Early on, a fourth type of primary motoneuron, the variable primary (VaP) can be seen in a portion of the spinal cord (Eisen and Pike, 1990). However, this unique type of pmn is typically removed via PCD by 36 hpf (Eisen et al., 2001). Using the $\beta_2$ nAChR antibody, we show the axonal morphology of the CaP and MiP motoneurons throughout various stages of development. At 30 hpf, CaP somata and axons were first detected with anti-$\beta_2$ (Figure 5.3).

The $\beta_2$ nAChR Antibody Specific for Primary Motoneuron, but not Secondary Motoneurons

We speculated whether or not the $\beta_2$ nAChR antibody was truly labeling pmn somata and axons. We felt that we were clearly labeling the CaP somata and axon early on, but labeling of the dorsal trajectory was not clearly defined. Was the antibody labeling the MiP axon or dorsal
smn axons? To test this, we performed double staining experiments. First, we used znnp-1 in conjunction with the anti- β2 nAChR to confirm that the ventral axons labeled were CaP axon. The znnp-1 positive motoneuron axons were also labeled with anti-β2 nAChR at 36 and 48 hpf (Figure 5.4, A-B). Interestingly, the β2 labeled CaP and MiP axons projected down the core of the znnp-1 labeled motoneuron which was very easy to see at 36 hpf. This suggested that the β2 nAChR antibody is only labeling the main axonal branch. This further suggested that the β2 nAChR antibody was specifically recognizing pmn axons at these developmental time points.

We then performed double labeling experiments using two markers for smn: the transgenic GATA2 zebrafish, which expresses GFP in ventral secondary motoneuron populations and zn5, an antibody that recognizes the adhesion molecule DM-GRASP on secondary motoneurons (Fashena and Westerfield, 1999). Both markers were used in conjunction with the β2 nAChR antibody and examined. The zn5 labeled CaP-like and the GATA positive smn axons did not colocalize with β2 labeled axonal structures (Figure 5.5, A-C). Also, zn5 positive MiP-like smn axons projected differently from β2 labeled axons in the dorsal region (Figure 5.6).

Once we established that we were labeling pmn axons, we then decided to model these pmn axonal trajectories from 36 hpf all the way out to 12 days post fertilization (Figure 5.7 and 5.8). We noticed that as the fish aged, the structures labeled became more elaborate. Also, the signal did not seem to be down regulated.

In addition, there were various phenotypes associated with β2 labeled CaP motoneuron axons. At 36 hpf, in a specific region of CaP ventral axons an outward “hump-like” projection can be observed (Figure 5.8, A). This CaP phenotype is commonly found in 30-48 hpf embryos. In addition, by 48 hpf, a “hook-like” projection extends from the CaP ventral axon just below the MiP dorsal projection (Figure 5.8, B). Likewise, this phenotype is unique to CaP motoneurons at
48 hpf. By 72 hpf, CaP ventral axons begin to form “extra branches” and an “unraveling or twisting” of the axons is apparent (Figure 5.8, C). We quantified these unique phenotypes from 30-96 hpf in a select number of fish. Interestingly, from 3 to 8 dpf, the extent of these phenotypes become more elaborate (see table 5.1), but by 12 dpf these elaborations become more conserved (refer back to Figure 5.7; 8 and 12 dpf).

**β₂ nAChR Positive Primary Motoneuron Axons: Schematic Models**

The β₂ nAChR antibody appeared to only label the main branch of the primary motoneurons. Upon characterizing the labeling of the β₂ nAChR antibody, we were able to model characteristics of the pmn axonal trajectories. At 30 hpf, β₂ labeled CaP somata and axons only with no obvious branching. By 36 hpf, MiP projection were evident. Between 30-36 hpf, a hump-like pattern was observed on the ventral CaP axon. At 48 hpf, a stereotypical hook-like projections extended from the CaP ventral axon to the myotome. Also, a few branches had begun to extend towards the distal end of the axon. This branching became more elaborate by 72 hpf. A representative cross sectional view showed that these axonal branches project into the myotome (Figure 5.9). These phenotypes were specific to β₂ labeled structures. They also allowed us to identify CaP and MiP motoneuron axons at each stage of development. The patterning of β₂ labeled of pmn changed as development progressed. As MiP processes extended further posterior, the CaP axons become highly branched. Early on (36-48 hpf), both MiP and CaP axons were also found only within the mid myotomal region while at later stages (4-12 dpf), these projections extended just adjacent to the segment boundaries (Figure 5.10).

**5.4 Discussion**

Motoneuron development in zebrafish has been studied extensively over the years. Currently, only a few methods have been used to identify populations of motoneurons during
development. However, even these methods have not been consistent. In this chapter, we described an antibody that was initially made to verify expression of nAChR on RB neurons, but accidently was found to also label primary motoneurons, specifically CaP and MiP.

**Primary Motoneuron Axon Markers: Benefits and Pitfalls**

To date, the ability to identify motoneurons *in vivo* is limited to intracellular dye injections using horseradish peroxides (HRP), antibody labeling, and transgenic models. Dye injection techniques provide the capability to visualize motoneuron morphology in its entirety. Specifically, the regional arborizations of primary motoneurons, CaP, MiP, and RoP were easily identified using dye injected HRP (Westerfield, 1986). Although dye injection techniques result in a better signal-to-noise ratio and specific labeling of the structure of interest, it is more time consuming and tedious. In addition, the use of antibody markers has been in some cases beneficial to study aspects of motoneuron development. Pattern formation and basic morphological analyses can be assessed easily *via* antibody labeling methods (Beattie, 2000). However, only a few motoneuron antibody markers have been established, namely zn-1, zn-p-1, and zn5. Although the zn-p-1 antibody is an excellent marker to label primary motoneurons, it is only useful at early stages of development. At later stages, zn-p-1 labeling becomes very elaborate which makes it difficult to clearly distinguish primary motoneuron axons. In addition, the work of Tallafus and Eisen 2008, showed that zn-p-1 labels both primary and secondary neurons at later times in development. Therefore, the ability to study primary motoneuron development over time using the zn-p-1 antibody can be challenging. Furthermore, the use of transgenic zebrafish can be useful. In the case of CaP axons, the neuropilin 1-a (nrp1a:GFP) transgenic zebrafish can be used to examine GFP expressing CaP motoneurons, but only at early developmental stages because the GFP expression is transient (Sato-Maeda et al., 2008).
Figure 5.3 The caudal primary motoneuron is identified early during zebrafish development. At 30 hpf, CaP motoneuron somata (asterisk) and axon (arrow) labeled with the $\beta_2$ antibody. Scale bar = 20 $\mu$m.

Figure 5.4 Zn-1/znp-1 labeling with $\beta_2$ labeled pmn. A: At 36 hpf, znp-1 (left; green) and $\beta_2$ (middle; red) labeled pmn colocalized (right). B: At 48 hpf, znp-1 and $\beta_2$ labeled pmn colocalized. A representative cartoon illustration showing $\beta_2$ labeled pmn axons projecting down the core of znp-1 labeled motoneurons is shown at the far right side of A and B. Scale bars = 20 $\mu$m.
Figure 5.5 $\beta_2$ labeling is specific for primary motoneurons. A: At 48 hpf, zn5 labeled motoneurons (top; green) and $\beta_2$ labeled motoneurons (middle; red) does not project side by side (emphasized by arrows). B: Also at 72 hpf, zn5 labeled axons to not colocalize with $\beta_2$ labeled motoneurons. C: At 48 hpf, GFP positive ventral axons in GATA2 fish do not colocalize with $\beta_2$ labeled with axons (emphasized by arrows). The bottom insets show the regions highlighted by the white boxes at a higher magnification. Scale bars: B-C = 20 $\mu$m, insets = 10 $\mu$m.
Figure 5.6 $\beta_2$ labels the MiP motoneuron axon. At 72 hpf, $\beta_2$ and zn5 labeled dorsal axons project differently (indicated by white arrows). Right: At the right is a representative drawing taken from a lateral, flattened view of zn5 labeled secondary motoneuron axonal projection.

Figure 5.7 The $\beta_2$ antibody labels pmn during zebrafish development. From 36 hpf to 12 dpf, $\beta_2$ labeling in CaP (Asterisks shows CaP somata) and MiP (arrows). At 8 dpf, excessive axonal branching is observed. However, by 12 dpf less branching is observed. Also, at 12 dpf, dorsal root ganglion is apparent (arrow head). Notice the disappearance of CaP somata from 36 hpf to later stages.
Figure 5.8 Primary motoneurons in wildtype zebrafish. A: At 36hpf, a “hump-like” projection is found on the CaP axon (arrow). Right, rotated view of A. B: At 48 hpf, hook-like projections (arrows) and a hump-like pattern (open arrow heads on rotated view) are evident in CaP projections. C: At 72 hpf, CaP axons become more elaborate as MiP axons extend their projections in the dorsal musculature. Right, extra branching (arrows) on CaP axons. Scale bars: A = 15 μm, B-C = 20 μm.
Table 5.1: Phenotypes associated with $\beta_2$ labeled CaP motoneuron axons. A qualitative assessment was used to describe various CaP motoneuron phenotypes (finger-like projection, extra branching, and unraveled/twisted axons).

<table>
<thead>
<tr>
<th>Age (hpf)</th>
<th>Fish (n)</th>
<th>(%) of segments with finger-like projection on CaP ventral axons</th>
<th>(%) of segments with extra branching on CaP ventral axons</th>
<th>(%) of segments with unraveled/twisted CaP ventral axons</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>18</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>36</td>
<td>22</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
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<td>48</td>
<td>17</td>
<td>55%</td>
<td>53%</td>
<td>57%</td>
</tr>
<tr>
<td>72</td>
<td>32</td>
<td>83%</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>96</td>
<td>10</td>
<td>80%</td>
<td>100%</td>
<td>83%</td>
</tr>
</tbody>
</table>

Figure 5.9 Cartoon illustration of CaP and MiP axonal projections labeled by the $\beta_2$ nAChR antibody. A cross-sectional view of CaP and MiP projections at 36, 48, and 72 hpf using the $\beta_2$ antibody. At 48 and 72 hpf, notice the extra branching extending out from CaP axons towards the muscle. Also the labeling of the $\beta_2$ antibody in CaP somata is down regulated by 48 hpf. sc - spinal cord
Figure 5.10 Representative drawing of $\beta_2$ labeling in pmn at various developmental stages. CaP (blue) and MiP (green) motoneurons and axons were traced in fish at 36 hpf, 2, 3, 4, 8, and 12 dpf. At 8 and 12 dpf, dorsal root ganglion (black) is present. sc-spinal cord

**Potential Uses for a Stable Primary Motoneuron Marker**

In this chapter, we have described the $\beta_2$ nAChR antibody as a potential marker for primary motoneurons. It is not uncommon for a receptor subunit to label cellular (axonal) components. In fact, this is not the first study to show structural or cell-specific labeling using a receptor antibody. Recent studies show that a dopamine receptor antibody labels the somata as well as the processes of dopaminergic neurons in the rat retina (Veruki, 1997). In addition, the glutamate receptor subunit 2 (Glur2) was found to label bipolar cell somata and the outer
plexiform layer of the rat retina (Johansson et al., 2000). In zebrafish, a subunit of the \(N\)-methyl-D aspartate receptor (NMDAR) known as NR2A was found to colocalize with zn5p-1 labeled primary motoneuron axons at 6 dpf (Todd et al., 2004).

In order to thoroughly study motoneuron development in zebrafish, a reliable motoneuron marker must be used. Zn5 is a marker for smn axons and for pmn axons. We found that anti-\(\beta_2\) nAChR appears to be an unambiguous pmn marker. Having a stable pmn marker can make it easy for researchers to study primary and secondary motoneuron populations simultaneously. With this capability, in vivo comparative studies in the same fish can be performed. For our purposes, we will use the \(\beta_2\) nAChR antibody in conjunction with other markers to study the effect of nicotine on motoneuron development. Based on our previous work, we have shown that nicotine affects axonal pathfinding and development of secondary motoneurons (Menelaou and Svoboda, 2009; Welsh et al., 2009; Svoboda et al., 2002). Therefore, the \(\beta_2\) nAChR antibody would allow us to understand whether or not the effect of nicotine on secondary motoneurons is indirectly or directly linked to an effect on primary motoneurons. In the following figures, we briefly show that this indeed is feasible.

Nicotine exposure can cause several smn axonal phenotypes in zebrafish. First, the exposure can cause axons to stall before entering the periphery. Second, axons may fork upon entering the periphery and third, axons may simply take incorrect paths to the periphery. These observations are for the dorsal projecting smn axons. So a question asked is, are these phenotypes linked to alterations in pmn axon trajectories? Secondary motoneuron axonal stalls do not seem to be linked to alterations in pmn axons because pmn axons extended into the periphery in the nicotine exposed embryos (Figure 5.11). In this example, the embryos were exposed to nicotine from 22 to 72 hpf and analyzed at 72 hpf. However, if embryos were exposed from 12-30 hpf and analyzed at 72 hpf, smn axon errors appear linked to pmn axon
errors (Figure 5.12A). Recall that dorsal projecting smn axons are not present at 30 hpf. In the experiments shown under Figure 5.12, smn axons “slid” in a rightward direction below the spinal cord before making the appropriate turn to the dorsal periphery above spinal cord. In these same segments pmn axons also “slid” suggesting that the anomaly observed with the smn axons is linked to altered pmn axons. In addition, there are some smn axonal pathfinding errors that are not associated with pmn axons. For instance, smn axons projecting dorsally appears to “loop” back instead of extending down a straight path out into the periphery while pmn axons continue to extend out into the periphery (Figure 5.12, B).

In conclusion, the focus of this chapter was aimed at the characterization of a new pmn marker for zebrafish. We are confident that the anti-β2 nAChR antibody labels CaP and MiP motoneuron axons consistently throughout development. We then were able to show that nicotine exposure can alter pmn axon trajectories which then in turn can influence smn axonal trajectories.
Figure 5.11 Nicotine-induced “stalls” in smn axons are not linked to alterations in pmn axons. A: At 72 hpf, zn5 labeled dorsal, MiP-like smn axons stalled in all three segments (axons are numbered 1-3). B: $\beta_2$ labeled dorsal MiP axons (white arrows) extended “normally” into the periphery. C: Merged view of zn5 and $\beta_2$ labeled axons.
Figure 5.12 At 72 hpf, nicotine-induced pathfinding errors in smn axons maybe linked to alterations in pmn axons. Ai-Aii: (Top) Zn5 labeled axons; (Middle) \( \beta_2 \) labeled axons; (Bottom) Merge. Ai: zn5 labeled axons “slid” (number 3 white arrow) to the right before extending dorsally out into the periphery. Aii: zn5 labeled axons “dropped” or “slid” (number 2 white arrow) to far ventrally before extending dorsally out into the periphery. In both cases, alterations in pmn axons were linked to the pathfinding errors in smn axons. B: Smn axons (zn5 labeled; green axons shown by white arrow) “looped” instead of extending out into the periphery while pmn axons (\( \beta_2 \) labeled; red) continued to extend “normally” out into the periphery.
CHAPTER 6
SUMMARY AND CONCLUSIONS

Nicotine, a common drug of abuse has been associated with many adverse birth outcomes among women who smoke during pregnancy. Exactly how nicotine exerts its effects on the developing central nervous system is still not fully understood. In this work, we used the zebrafish model to study the neuro-toxic effects of nicotine during embryogenesis. Over the years, zebrafish behavior has been well characterized and studied across many developmental windows. Therefore, this behavior was used as a diagnostic means for studying the effects of early embryonic nicotine exposure. Nicotine exposure evoked a doubling in the musculature bend rate as early as 18 hpf. This same effect was observed in embryos lacking brainstem or supraspinal input. In addition, acute exposure to nicotine caused what seemed to be a “desensitizing” effect in which embryos responded to the initial exposure to nicotine but was unable to respond when re-introduced to nicotine some hours later. These data led us to conclude that nicotine was most likely activating nAChRs located in spinal cord.

Also, we determined how much nicotine was actually getting into the embryo to produce such robust effects. Using radioisotopic flux assays, nicotine incorporation into the embryo was quantified. We found that only a fraction of the waterborne nicotine concentration was actually being accumulated in the embryo and this was enough to elicit a behavior response. Moreover, we discovered that a residual nicotine is most likely the underlying cause producing the “desensitization” effect when embryos were returned to embryo medium. Taken together, these results suggest that nAChRs may be present early in development and mediate the actions of nicotine during embryogenesis.

We then set out to determine if specific nAChRs were involved in the nicotine-induced behavioral response. We applied the α4/β2 nAChRs agonists, epibatidine and ABT 418 to
developing embryos. These specific nAChR agonists produced an increase in the musculature bend rate similar to that caused by nicotine exposure. This suggested that nicotine may activate β2-containing nAChRs that localize in spinal cord. We hypothesized that nAChRs would be expressed in RB neurons based on previous data showing the β2 mRNA and a transgene encoding the β3 subunit of nAChR where both found in RB neurons. We then performed IHC in wildtype and isl3 embryos using a monoclonal β2 nAChR antibody made in mammals and a β2 nAChR zebrafish specific antibody. RB neurons were labeled by the β2 nAChR antibodies as early as 18 hpf.

Since RB neurons expressed nAChR subunits, and since cholinergic agonists were able to elicit an increase in bend rates, functional nAChRs were likely present early in zebrafish embryogenesis. If functional receptors were present on the RB neurons, we hypothesized that RB neuron development would be affected by embryonic nicotine exposure. However, before we could study the effects of nicotine on RB development, we first systematically characterized RB neuron development. The analysis included a thorough assessment of RB neuron peripheral process and cell morphology, dorsal and ventral migration patterns within spinal cord, and the stability of specific RB cellular markers (see table 6.1). We observed that from ~ 36 to 48 hpf, RB neurons begin to migrate towards the midline forming an almost linear row by 72 hpf. Also, RB neurons migrate in a ventral direction within spinal cord. In conjunction with migration, RB somata became smaller as they changed from a more of a rounded, oval-like shape to an edgy, hexagonal-like shape. We also found changes in RB peripheral processes revealed by anti-acetylated tubulin immunoreactivity. Early in development, RB peripheral processes have a “continuous” aat distribution, but by 48 hpf, this distribution appeared fragmented. These anatomical phenotypes are indicative of RB PCD.
We used the anatomical endpoints to study how nicotine could affect RB development and cell death. Surprisingly, we found that embryonic exposure to nicotine caused a delay in RB migration and cell shrinkage. In addition, aat distribution in RB peripheral processes appeared continuous at times when it should be fragmented in non-exposed zebrafish. We later performed a TUNEL assay which revealed a significant reduction of RB cell death in nicotine exposed embryos compared to controls. These data suggest that nicotine is delaying RB neuron cell death and providing a temporal form of neuro-protection.

The neuro-protective effect of nicotine is clinically relevant. Individuals who suffer from neurodegenerative diseases, such as Parkinson’s disease (PD) are treated with nicotine. In this context, nicotine has the ability to increase dopamine levels in the midbrain as well as extend the life of dopamine producing cells in PD patients. Likewise, in this study we have shown how early exposure to nicotine can delay RB neurons from PCD or delay the onset of RB neuron PCD. Although we mainly focused on the morphological changes of RB neurons, we suspect that the functionality of these neurons is extended as well. In order to fully understand the level at which nicotine delays RB neurons from dying, the functionality of RB neurons would need to be tested at times when they should be dying. This could be done by using electrophysiological techniques to monitor their electrical activity or by calcium imaging techniques.

In chapter 5, we characterized a β2 nAChR antibody and showed it is a good stable marker for primary motoneuron axons. We found that it labeled CaP and MiP axons respectively, as well as Rohon-Beard neurons. This observation led us to characterize the labeling pattern of the antibody across development. We found that the β2 nAChR antibody reliably labeled CaP and MiP axons out to 12 dpf. At 30 hpf, CaP somata and axons were detected by the β2 nAChR antibody. At 36 hpf, the MiP axons were detected with the antibody. Interestingly, the β2 nAChR antibody seemed to only label the main axonal projection and not the elaborate branches
extending from the main axon. To date, few if any reliable primary motoneuron markers have been identified. The primary motoneuron marker, znpl has been proven to be useful in studying aspects of motoneuron development, but only early in development. Some studies utilized the filling of primary motoneurons with fluorescent dyes to look at axonal trajectories, but this technique can be cumbersome and time consuming. We am confident that we have identified a unique and stable zebrafish pmn marker. This marker can be used to study both populations of motoneuron axons, pmn and smn axons within the same embryo/larva. In saying this, indirect or direct effects of embryonic nicotine exposure on motoneuron development could be easily studied. For example, in an exposure paradigm, pmn axons may be altered, which in turn, could influence smn axons. On the other hand, manipulations including nicotine exposure may only affect smn axons and spare pmn axons. These types of issues can be readily addressed using the \( \beta_2 \) nAChR antibody as a marker for pmn axons.

We have also shown that a \( \beta_2 \) containing nAChR likely mediates the actions of nicotine in early developing embryos. It would be interesting to see how nicotine effects the expression of the \( \beta_2 \) nAChR subunit. Will the expression of \( \beta_2 \) be upregulated, downregulated, or unaltered in exposed fish? This could provide us information that pinpoints \( \beta_2 \)-containing nAChRs as having a non-classical or classical function during aspects of CNS development. Also, my work did not examine the distribution of other potential subtypes such as \( \alpha_4, \beta_6, \alpha_2, \beta_3 \), etc. However, based on preliminary data from our laboratory, we do know that RB neurons express other nAChR subtypes. With the antibodies analyzed in this study, further characterizations can be done to determine which compliment of nAChR subunits are expressed by various neurons within the developing zebrafish CNS.
Table 6.1: Description of various cellular markers used to study specific neuronal populations during zebrafish development.

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<th>Staining</th>
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<tr>
<td>zn-1</td>
<td>Cytoplasmic neuronal</td>
<td>Primary motoneuron axonal projections</td>
<td>Tallafuss et al., 2008 Hanneman et al., 1988</td>
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<tr>
<td>zn-1p</td>
<td>Neuropil region</td>
<td>Primary motoneuron cell bodies</td>
<td>Trevarrow et al., 1990</td>
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<td></td>
<td>Recognizes microtubules; labels neurons that have already begun axonogenesis</td>
<td>RB neurons, motoneurons, DRGs. Posterior lateral line ganglia (pllg) nerve</td>
<td>LeDizet and Piperno, 1991</td>
</tr>
<tr>
<td>anti-Hu</td>
<td>Recognizes proteins found in the nuclei and cytoplasm of most neurons</td>
<td>RB neurons, dorsal root ganglia (DRG)</td>
<td>Henion et al., 1996</td>
</tr>
<tr>
<td>zn12</td>
<td>Recognizes an antigen associated with cell adhesion molecules; glycoproteins; neuronal cell surface marker</td>
<td>RB neurons, Trigeminal ganglia (TG), lateral line ganglia, primary motoneurons</td>
<td>Metcalfe et al., 1990 Wilson et al., 1990 Ross et al., 1992</td>
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<tr>
<td>zn5</td>
<td>Neuronal cell surface marker (SC-1, DM-GRASP, BEN)</td>
<td>Hindbrain commissural neurons; secondary spinal motoneurons</td>
<td>Fashena and Westerfield, 1999</td>
</tr>
<tr>
<td>β2</td>
<td>Recognizes the β2 nAChR subunit</td>
<td>Primary motoneuron axons, specifically CaP and MiP</td>
<td>Welsh et al., 2009</td>
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