Zebrafish Neuronal Nicotinic Acetylcholine Receptors: Cloning, Expression, and Functional Analysis

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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*****

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ABSTRACT

Cigarette smoking is a major public health concern, in that, over one billion people or one in three adults in the world smoke (WHO, 2006). Even more alarming is that approximately 20% of pregnant women in the western world continue to smoke during pregnancy despite a number of fetal complications linked with cigarette smoking (CDC, 2004, 2006 and U.S. Surgeon General’s Report (Chapter 5; pages 180–194). Smoking during pregnancy can cause serious health problems for both mother and child, such as pregnancy complications, premature birth, low-birth-weight infants, stillbirth and infant death (U.S. Department of Health and Human Services, 2004). Nicotine exposure in utero can also result in long-term anatomical, behavioral and cognitive deficits (Sexton et al., 1990; Olds et al., 1994; Slotkin 1992, 2004, 2008). Nicotine, the main addictive chemical component in cigarette tobacco, is a likely contributor to the adverse outcomes, as fetal brain and spinal cord are primary targets. Nicotine mediates its actions through nicotinic acetylcholine receptors, nAChRs, which are widely distributed throughout the body. This class of receptors belongs to the ligand gated ion channel superfamily, which includes GABA A, glycine, 5-HT3, and NMDA-type glutamate receptors. The nAChR is widely distributed in a vast majority of organisms and can be classified into two groups, muscle or neuronal. The muscle type is found at the neuromuscular junction (NMJ), while the neuronal type is found in nervous tissue. Each nAChR, despite its localization, is composed of five subunits that assemble around a central channel.

To date many disease states have been associated with the nAChRs in adult including Alzheimer’s disease, Parkinson’s disease, schizophrenia, Tourette’s syndrome,
epilepsy, and nicotine addiction. By conducting studies in a developing animal we are addressing the one million pregnant women in the United States and 20% of pregnant women in the western world that continue to smoke regardless of the harm they may be causing the fetus. The primary function of nAChRs is to modulate synaptic transmission. However, findings over the last 20 years indicate that neuronal nAChRs may play a potential role in the development of the nervous system. To date, it remains unclear as to how specific nAChRs subtypes function during normal developmental processes and how exposing the fetus to chronic nicotine affects fetal development.

The zebrafish (*Danio rerio*) has shown incredible potential for the examination of events that occur during development. The results from our laboratory presented in this manuscript represent a continuation in the study of neuronal nAChR in the vertebrate zebrafish model. Our research efforts have resulted in the cloning of zebrafish *chrna4* and *chrna6* neuronal nAChR genes. Full-length cDNAs for these zebrafish neuronal nAChR subunit genes were determined and sequence analysis showed that these cDNAs demonstrated a high degree of homology to nAChR genes found in other species. To date, it does not appear that the *chrna4* and *chrna6* genes were duplicated in the zebrafish genome. Additionally, our laboratory has determined RNA expression patterns and examined the functional roles that nAChRs may play in nervous system development. Time course studies using RT-PCR and subunit-specific primers to each of the α2, α4, and α6 subunits determined when each gene was expressed. All of these subunits were expressed very early in development, with *chrna2* RNA being expressed maternally in unfertilized embryos, *chrna4* RNA expressed by 3 hours post fertilization (hpf), and *chrna6* RNA present by 10 hpf.
*In situ* hybridization studies using sense and antisense digoxigenin-labeled RNA probes were also used to localize the temporal and spatial expression of nAChR RNA in developing embryos. Each of the subunits displayed unique expression patterns that were transiently regulated. The zebrafish *chrna4* RNA was expressed in a subset of hindbrain neurons in rhombomeres 4-7 and in cells consistent with *dlx2* expressing neural crest cells migrating along the pharyngeal arch at 24 hpf. Additionally, limited expression was observed in forebrain and midbrain structures. At 48 hpf significant bilateral expression was seen in both midbrain and hindbrain consistent with the nucleus of the medial longitudinal fascicle and reticulospinal neurons, with no expression detected in the spinal cord. At 72 and 96 hpf, *chrna4* continued to be highly expressed in specific midbrain and hindbrain areas.

At 24 hpf, zebrafish *chrna6* RNA was expressed in a subset of Rohon Beard sensory neurons, ventral neurons in the spinal cord, diencephalon, trigeminal ganglion, pineal, and in the first hindbrain rhombomere ventral to the cerebellum. At 48 hpf *chrna6* was no longer observed in spinal neurons, but was still expressed in trigeminal ganglion, pineal, and was now co-localized to the TH + locus coeruleus and the midbrain diencephalic catecholaminergic cluster. *chrna6* was highly expressed in retina at 48 hpf and minimally expressed in tectum which was not detected 24 hpf. At 72 and 96 hpf, zebrafish *chrna6* continued to be expressed in trigeminal ganglion, retina, and pineal. *chrna6* RNA was highly expressed in tectum in 72 and 96 hpf zebrafish, at robust levels with more widespread distribution than at 48 hpf. Additionally, at 96 hpf *chrna6* expression was detected for the first time with a pattern consistent with cranial sensory neurons in the hindbrain. At 72 hpf and 96 hpf *chrna6* RNA continued to be expressed in
the diencephalic catecholaminergic cluster, but was also present in non-catecholaminergic cells in both midbrain and hindbrain.

At 10 hpf, around the time of somitogenesis, *chrna2* expression was heavy in the anterior head region with some labeling in the dorsal spine. By 18 hpf, there was diffuse labeling in the brain and a well defined pattern in the anterior spinal cord. At 24 hpf *chrna2* was expressed in the forebrain consistent with the olfactory bulb, interneurons in the first 1-7 hemisegments of the spinal cord, interneurons localized down the entire length of the spinal cord, and cells along the midbrain/hindbrain boundary. At 48 hpf *chrna2* forebrain expression had disappeared and midbrain expression was prominent with very limited expression in hindbrain. The distinct pattern in the spinal cord remained intact. By 72 hpf all the spinal cord labeling had disappeared, but robust expression remained in the midbrain with limited expression in the hindbrain. By 96 hpf *chrna2* expression was still evident in the midbrain.

An antisense oligonucleotide morpholino gene knock down approach was used to determine the function of *chrna2* during development. Knock down of the *chrna2* resulted in swimming deficits, paralysis, and a disruption in the number, morphology, and extension of dorsal projecting motor neurons to the dorsal myotome. The loss of *chrna2* did not affect ventral projecting motor neurons.

Expression of the zebrafish nAChR α2 subunit RNA in *Xenopus* oocytes provided preliminary evidence of functional nAChRs in the zebrafish model. Whole-cell recordings demonstrated that the zebrafish nAChR α2 subunit was able to assemble as a heteromeric receptor and, upon activation, produced currents characteristic of α2 nAChRs in other species.
Treatment of zebrafish with nAChR agonists and antagonists was done to further examine the effects activating or blocking nAChRs on development. Treatment of zebrafish embryos with 50-200 µM concentrations of the nAChR agonist nicotine during specific periods of development resulted in motor behavior deficits, paralysis, and the induction of apoptosis. This nicotine-induced cell death was blocked when the nAChR antagonist, DHβE, was co-applied with nicotine, confirming this mechanism involved nAChRs. Embryos treated with nicotine displayed a nicotine-mediated upregulation of *chrna2, chrna 4, chrna 6, chrna7, cfos, p53, islet 1* and *shh* transcripts.

In addition to the *in vivo* work using zebrafish embryos, preliminary screening the zebrafish cell line, ZEM2S, indicated that α2, α4, α6, α7, and β2 nAChR subunit RNAs, along with *shh, islet 1, p53* and *cfos* were present. α3 and α8 RNAs were not detected. β2 and β4 nAChR RNAs were not screened. The expression of these genes in cell culture perfectly modeled the nAChR expression in 10 hpf zebrafish embryos where α2, α4, α6, α7, β2, and β3 nAChRs, along with Shh, Islet 1, p53 and cfos were also expressed. Preliminary studies indicate that nicotine induced upregulation of cfos transcript is evident in the cell line after 12 or 24 hours of drug exposure.

Although our preliminary work has generated a substantial amount of data in several areas, much work remains to be done in the zebrafish model. Future plans using the zebrafish to study nAChRs include the determination of expression patterns of the remaining nAChR subunits, the use of morpholino oligonucleotides to detect the functions of specific nAChRs subtypes, and the examination of the effect of other cholinergic agents on nervous system development. Our results indicate that zebrafish
will provide an excellent model for the continued study of the role of cholinergic receptors in neural development.
DEDICATION

I would like to dedicate this work to my family for all of their love and support.
ACKNOWLEDGMENTS

Foremost, I wish to acknowledge my advisor, Dr. R. Thomas Boyd for all of his patience, guidance, and support. He has provided me with invaluable neuromolecular biology training and I am forever grateful.

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FIELDS OF STUDY

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Area of Emphasis: Molecular Neurobiology
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<tr>
<td>α</td>
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</tr>
<tr>
<td>AP</td>
<td>alkaline phosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chlor-3-indolyl phosphate, touluidine salt</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>g</td>
<td>gram(s)</td>
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<tr>
<td>hpf</td>
<td>hours post fertilization</td>
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<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>INT</td>
<td>2-[4 Iodophenyl]-3-[4-nitrophenyl-tetrazolium chloride)</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium Phosphate</td>
</tr>
<tr>
<td>L</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>μl</td>
<td>microliter</td>
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<tr>
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<td>millimolar</td>
</tr>
<tr>
<td>M</td>
<td>moles per liter</td>
</tr>
<tr>
<td>MAB</td>
<td>maleic acid buffer</td>
</tr>
<tr>
<td>MABT</td>
<td>maleic acid buffer with .01% tween</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MetOH</td>
<td>methanol</td>
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<td>MgSO₄</td>
<td>magnesium sulphate</td>
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<tr>
<td>MOPS</td>
<td>morpholinopropanesulfonic acid</td>
</tr>
<tr>
<td>NH₄OAc</td>
<td>ammonium acetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
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<td>PBT</td>
<td>phosphate buffer solution with 2% tween</td>
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<tr>
<td>PTU</td>
<td>N-Phenylthiourea</td>
</tr>
<tr>
<td>PVA</td>
<td>poly vinyl alcohol</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TTBST</td>
<td>TBS with 2% Triton and 5% Tween</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>times</td>
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CHAPTER 1

INTRODUCTION

1.1 Significance

Cigarette smoking is a major public health concern, in that, over one billion people or one in three adults in the world smoke (WHO, 2006). Even more alarming is that approximately 20% of pregnant women in the western world continue to smoke during pregnancy despite a number of fetal complications linked with cigarette smoking (CDC, 2004, 2006 and U.S. Surgeon General’s Report (Chapter 5; pages 180–194). Smoking during pregnancy can cause serious health problems for both mother and child, such as pregnancy complications, premature birth, low-birth-weight infants, stillbirth and infant death (U.S. Department of Health and Human Services, 2004). Nicotine exposure in utero can also result in long-term anatomical, behavioral and cognitive deficits (Sexton et al., 1990; Olds et al., 1994; Slotkin 1992, 2004, 2008). Nicotine, the main addictive chemical component in cigarette tobacco, is a likely contributor to the adverse outcomes as fetal brain and spinal cord are primary targets. Nicotine mediates its actions through nicotinic acetylcholine receptors, nAChRs, which are widely distributed throughout the body. To date many disease states have been associated with nAChRs in the adult including Alzheimer’s disease, Parkinson’s disease, schizophrenia, Tourette’s syndrome, epilepsy, and nicotine addiction. By understanding the relationship of nAChR subtype selectivity in animal models for different disease states, it should be possible to develop innovative and highly efficacious therapeutics for diseases in which there are currently
large unmet medical needs. By conducting studies in a developing animal we are addressing the one million pregnant women in the United States and 20% of pregnant women in the western world that continue to smoke regardless of the harm they may be causing the fetus. To date, it remains unclear as to how specific nAChRs subtypes function during normal developmental processes and how exposing the fetus to chronic nicotine affects fetal development. Additionally, concerted efforts are presently ongoing to discover highly subtype-selective drugs to help smokers quit smoking. Understandably with drug discovery, special consideration must be taken for pregnant women because the health and development of the fetus must also be considered.

1.2 Brain Development and Cholinergic Signaling

The chemical compound acetylcholine, abbreviated ACh, was the first neurotransmitter to be discovered (Loewi, 1921) and is highly studied. It functions in both the peripheral nervous system (PNS) and central nervous system (CNS) as an excitatory transmitter in many organisms including humans. Additionally, ACh is the neurotransmitter in autonomic ganglia. There are two main classes of cholinergic receptors including nicotinic acetylcholine receptors (nAChR) which are ionotropic channels and muscarinic acetylcholine receptors (mAChR) that are G-protein coupled receptors. Classically, ACh mediates fast synaptic action through the nicotinic acetylcholine receptor (nAChR) and slower actions via muscarinic receptors. Nicotine, a major drug of abuse, is a selective agonist at the nAChR.

In the developing brain, neurotransmitters orchestrate a cascade of events necessary for normal structure and function. A growing body of evidence over the last 30
years using a variety of model systems, now suggests that ACh is not just involved in synaptic transmission, but a number of developmental processes such as cell division, differentiation, cell movement, synaptogenesis, neurite growth, apoptosis, etc (Slotkin, 2004) in virtually all model systems. Proper cholinergic signaling is vital for normal brain development. In the sea urchin, ACh seems to regulate cell movements during gastrulation and post-gastrulation stages (Gustafson, Toneby, 1970) and ACh receptors are detectible before neurulation in mouse (Atluri et al, 2001), indicating that ACh may be an important modulator in induction and patternization of the early nervous system. It is also thought that acetylcholine plays a role in the modulation of neuronal differentiation (Biagioni, 2000) and Slotkin, et al (1998,1999) found that ACh, acting as a trophic factor, can either promote mitosis in the developing neuron, or at later stages in development, can promote differentiation. Additionally, ACh has been found in migrating crest cells (Smith et al., 1979). At later stages of development, ACh may play a role in the maintenance of synaptic structures (Lipton and Kater, 1989) and neurite outgrowth. In Drosophila photoreceptor neurons are shown to have ACh on their axons during pathfinding events to target sites, well before synaptic connections are made (Yang and Kunes, 2004). Addition of the nicotinic agonist, nicotine, to PC12 cells and ciliary ganglion cultures produced a decreased rate of growth and retraction of neurites (Chan and Quik 1993, Pugh and Berg 1994). As multiple models support the role of ACh in development, the zebrafish is no exception. One of the best markers for identifying primary neurons at early stages of differentiation is the enzyme acetylcholinesterase (Hanneman & Westerfield1984, 1988; Wilson et al 1990). The first set of neurons to
express ACh in zebrafish are present at 14 hours of development suggesting that primary neurons in the embryonic zebrafish contain AChE before they sprout axons.

Previous literature would also suggest that interfering with cholinergic signaling, either by activating or blocking signal, would result in disruptions in structure and behavior. Neonatal lesions of mouse cholinergic neurons (or depletion of cholinergic signaling) produce abnormal cortical development (Bachman et al., 1994; Hohmann et al., 1988, 1991), including permanent alterations in cortical morphology, alterations in dendritic branching patterns, changes in spine morphology in sensory motor cortex and cognitive deficits as the animals grew to adulthood. In addition literature citing rat models indicate that nicotine exposure increases apoptosis, changes neuron pathfinding events, alters brain size, and influences mitosis (Slotkin, 1999, 2004). Chronic cholinergic stimulation/activation by nicotine causes cell damage, reduced cell number, impaired synaptic activity, premature differentiation, and apoptosis. Finally, overstimulation of the cholinergic system in cell culture at the wrong time during development causes abnormalities such as apoptosis. The model used undifferentiated primary and immortalized hippocampal progenitors that were introduced to an exogenous cholinergic agonist; the cells experienced increased apoptosis when undifferentiated, but no change was observed in the same cells once differentiation was induced (Berger, et al 1998). As stated above, this growing body of evidence would indicate that deviation from normal cholinergic signaling during development causes adverse effects. Although cholinergic receptors have been linked to a variety of developmental processes, the specific receptor subtypes involved in these processes have yet to be determined.
As there have been little studies to examine the functional properties and/or role of nAChR in the developing brain, we will examine cholinergic signaling through the nicotinic acetylcholine receptor using zebrafish as a model system. To date, little work has been done to examine the effects of nicotine on zebrafish. Svoboda et al, in 2002, found that zebrafish exposed to 15 μM displayed abnormal swim behaviors and embryos treated with 33 μM nicotine for 66 hr experienced paralysis and abnormal motor neuron growth and extension. When a nicotinic antagonist was an added condition, the paralysis and motor neuron pathfinding errors were not observed. The Svoboda study demonstrates the ability to analyze the effects of nicotine and other drugs in the developing zebrafish; in addition, the phenotypes mentioned were nAChR dependent as evident by the nicotinic receptor blockade.

1.3 Acetylcholine Receptors

The cholinergic system, which utilizes acetylcholine as its main chemical messenger, includes two types of receptors – muscarinic and nicotinic so named because of selective stimulation by muscarine and nicotine, respectively. Nicotine is a selective agonist at nicotinic acetylcholine receptors of the autonomic ganglia, adrenal medulla, neuromuscular junction, and in the brain (Benowitz, 1998). The neuronal nAChR receptors are found in the central nervous system and the peripheral nervous system; meanwhile, the muscle nAChRs are found at neuromuscular junctions of somatic muscles and activate muscle contraction. The nAChRs, including both neuronal and muscle subtypes, are members of the neurotransmitter gated superfamily of ion channels (LGIC) including 5-HT\textsubscript{3}, GABA\textsubscript{A} and GABA\textsubscript{C}, NMDA-type glutamate, and glycine receptors (Figure 1 A). Members of this family are cation specific ion channels that open upon
binding of two molecules of ligand, allowing ions to pass through the channel. The channels are pentameric (Figure 1 B) or formed by combinations of five subunits organized around a central ion pore.

Figure 1: Molecular structure of the Ligand Gated Ion Channel (LGIC) Superfamily

(A) The superfamily comprises acetylcholine, serotonin, glycine and GABA<sub>A</sub> receptors. The receptors possess a large extracellular N-terminal domain containing the Cys-loop signature (*), followed by four transmembrane domains (shown by boxes). Intracellular (In) and extracellular (Ext.) domains are identified. (B) Receptors are constructed as pentameric ion channels from the assembly of five subunits creating an ion pore. Taken From “The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function.” Connolly and Wafford 2004.
Each of the nAChR subunit genes encode a protein (Figure 2) with four principal domains: (1) a large amino-terminal extracellular domain which includes agonist and competitive antagonist binding sites, (2) four transmembrane spanning domains (TM1-TM4) which share very high homology and are responsible for the opened or closed channel conformation, (3) a cytoplasmic loop domain between TM3-TM4 that varies greatly between subunits even in the same species and (4) an extracellular carboxy terminal domain (Changeux, et al, 1998).

**Figure 2: The structure of a single nAChR subunit**
Individual nAChR subunits include 4 transmembrane domains responsible for ligand binding and the confirmation state of the channel, an N-terminal domain involved with the binding of ligand, and a C-terminal domain. Taken from “Expert Reviews in Molecular Medicine: [http://www-ermm.cbcu.cam.ac.uk](http://www-ermm.cbcu.cam.ac.uk). 1999.

Binding of agonist at the junction of the α/β subunits causes rapid opening of the channel caused by conformation changes in the transmembrane segments, especially TM
2. Early electron microscopy and image reconstruction determined the structure of the *Torpedo marmorata* nAChR (Unwin, 1993) and provided the shape of the complex, the arrangement of the subunits, and some secondary structural predictions. This model predicted that within the aqueous channel that a bundle of five TM2 α-helices proteins formed the wall of the pore. Each helices was contributed by one subunit of the pentamer, whereas the rest of the segments were relatively structureless (Unwin, 1995). In the closed channel state, the TM2 α-helices were kinked by the Leu residues (Unwin, 1993, 1995). This ring of five aligned Leu residues was suggested to constitute the gate of the channel and a $15^\circ$ rotation or twist of the TM2 segments would destabilize the gate and stabilize an open structure of the TM2 bundle (Miyazawa et al., 2003). Another important structural feature of the nAChR is three rings of negatively charged amino acids that are involved in charge selectivity (Changeux, 1993; Corringer et al., 2000; Karlin, 2002). These elements constitute the structural and functional features of the ion-channel.

Functional properties of nAChR subunit combinations are determined largely by (1) their permeability to Ca++ and (2) their desensitization properties (Cachelin and Jaggi 1991; Gross et al., 1991; Vernino et al., 1992). Generally brain nAChRs have a higher relative permeability to Ca++ than those at the neuromuscular junction, with the most extreme example being the homomeric $\alpha 7$ receptor which has a relative permeability to Ca++ even higher than that of the NMDA type of glutamate receptor. The $\alpha 7$ subtype of nAChRs also displays the most rapid desensitization kinetics. Another property of nAChRs is that all types expressed in neurons exhibit inward rectification, that is, they conduct inward currents at negative potentials but at positive membrane
potentials current does not flow through them (Haghighi and Cooper 1998; 2000). Both α and β subunits confer distinctive functional properties to the receptors they form (Luetje and Patrick 1991; Gerzanich et al., 1998; Parker et al., 1998). As demonstrated in Figure 3, the changing of a subunit alters the associated properties of the channel including conductance and channel open time.

**Figure 3: Properties of different nAChR subtype combinations expressed in Xenopus oocytes**

A comparison of the diverse properties of several nicotinic acetylcholine receptor subtypes. Channel open time, PCa/PNa, and affinity of subtypes to several nAChR agonists and antagonists are compared. The relative Ca++/Na+ permeability of α7 nAChRs is higher than that of NMDA-receptors and they provide a major route of Ca++entry at rest or at hyperpolarized membrane potentials when NMDA-receptors or voltage-gated Ca++ channels are silent (Rogers and Dani 1995). Taken from “Neuronal Nicotinic Receptors: Pharmacology and Therapeutics Opportunities”. Edited by Stephen P. Arneric and Jorge D. Brioni.

After agonist is removed from the synapse, the gate of the channel returns to the closed position. If an excess concentration of agonist persists and remains bound, the receptor may also display another conformation, known as the desensitized state (Figure 4).
Receptors in the desensitized state have a channel that is closed, even though ligand remains bound. Historically, the phenomenon of nAChR desensitization was first described in muscle by Katz and Thesleff in 1957. Desensitization is an intrinsic property of the protein complex. When subjected to prolonged exposure to agonists, nAChRs expressed in neurons also undergo a conformational change resulting in an inactive receptor that does not allow for the passage of ions. Desensitization is a readily reversible phenomenon and can occur both at activating and subactivating agonist concentrations (Grady et al., 1994; Fenster et al., 1997; Lu et al., 1999). A distinct phenomenon from desensitization is permanent inactivation of nAChRs with a time course of hours rather than seconds to minutes as has been described for desensitization (Aoshima 1984; Rowell and Duggan 1998; Fenster et al., 1999a). A relatively short exposure of 5 min to relatively small concentrations of nicotine can produce long-lasting nAChR inactivation in rat striatal synaptosomes, with the subunit composition of nAChRs possibly determining the degree to which the receptors are desensitized (Fenster et al., 1997; Rowell and Duggan 1998). Desensitization and persistent inactivation of nAChRs are the processes that most probably contribute to the behavioral changes in humans who are exposed to nicotine through the use of tobacco products. Chronic exposure to a level of nicotine found in smokers (low nanomolar) resulted in inactivation of α4β2- and α7-containing nAChRs, but inhibited α3 containing receptors to a lesser extent (Olale et al., 1997; Alkondon et al., 2000). However, at present there is no consensus to what degree nicotine’s effects are due to inhibition rather than activation of nAChR signaling. Continuous administration of nicotine results in upregulation of nAChRs in both laboratory animals and chronic smokers (Marks et al.,
1983; Rowell and Li 1997; Court et al., 1998). Currently, this phenomenon is not well understood. The mechanism behind upregulation has been hypothesized to be due to decreased degradation of nAChRs (Peng et al., 1994). Receptor upregulation has been proposed to reflect receptor desensitization, which may underlie functional tolerance to nicotine’s effects. Accordingly, the half-maximal nicotine concentration necessary to produce desensitization was the same as that needed to induce up-regulation (Fenster et al., 1999b). Furthermore, up-regulation has also been found to be initiated by the AChR antagonist mecamylamine (Peng et al., 1994). However, upregulation does not always reflect desensitization, since some up-regulated nAChRs are still functional (Wang et al. 1998.)
Upon agonist binding, nAChRs undergo an allosteric transition from the closed, resting conformation to an open state which conducts the cations. In the active (open) conformation, the nAChR binds agonists with low affinity. The continued presence of agonist leads to ion channel closure and receptor desensitization. In this condition, the nAChR is refractory to activation although it displays higher affinity for agonist binding. A multiplicity of desensitized states is proposed to exist.

The nAChR presents a number of sites which may be targeted by ligands (Figure 5). Agonists and competitive antagonists compete for the agonist binding site in the extracellular domain of the α subunits. As this site exists at the interface between adjoining subunits, it offers the prospect of nAChR subtype-selective interactions. Non-competitive antagonists and allosteric modulators act at sites distinct from the agonist binding site.
Figure 5: Structural overview of a single nAChR subunit
View of a nAChR subtype with a single subunit removed to reveal the central cation channel. Binding sites for agonists/competitive antagonists, non-competitive antagonists, allosteric modulators, and channel blocking non-competitive antagonists are shown. Flow of ions through the channel is also indicated. Taken from: Sharples C.V.G and Wonnacott S. Neuronal Nicotinic Receptors. Tocris Reviews 19, 2001

Approximately 50 years ago the first clear distinction between the nicotinic receptors (nAChRs) at the neuromuscular junction (NMJ) of skeletal muscle and those on postganglionic autonomic neurons was described pharmacologically and remained essentially unchanged until molecular cloning revealed that the subunits are extremely diverse. The acetylcholine receptors at the NMJ were the first studied nAChR and are now the most characterized and well understood nAChR. For the original cloning studies the *Torpedo californica* electric organ was used because it contains a large number of nAChRs. From electron crystallography, these receptors were found to be pentameric membrane proteins consisting of four separate types of subunits assembled around the
channel (Unwin, 1993). Muscle nAChRs (Figure 6) are pentamers of four subunits 
\((\alpha_1, \beta_1, \gamma, \delta)\) for fetal and denervated muscle and \((\alpha_1, \beta_1, \delta, \varepsilon)\) for adult muscle arranged around a central pore in a stoichiometry \(\alpha_1\beta_1\gamma\delta\) of 2:1:1:1 (Changeux et al., 1987).

Figure 6: Muscle nAChR putative subunit arrangements around the central pore
The muscle nAChR subunits are depicted arranged as pentamers around a central cation ion channel in a general stoichiometry of \(2\alpha:1\beta:1\delta:1\gamma\) in the fetal form with a switch in the adult form of \(2\alpha:1\beta:1\delta:1\varepsilon\). ACh binding sites are indicated at the appropriate interfaces between subunits. 
Taken from: http://www.med.upenn.edu/nscience/neuro_lindstrom.html

At the arrival of a presynaptic action potential, the concentration of ACh in the synaptic cleft rises to millimolar levels in milliseconds. Two molecules of ACh bind to the receptor at the \(\alpha_1\) subunit and ligand binding induces a conformational change that leads to an open channel that is now permeable to cations. The flow of ions induces depolarization which triggers muscle contraction. The channel remains open only briefly before becoming desensitized. When desensitized it is essentially closed to ions and is refractory to agonist activation, but agonist can bind with enhanced affinity. Very low
concentrations of agonist can create a desensitized state without being in the open state
first.

Much research has been done characterizing the structure and function of
nAChRs, especially those found at the vertebrate neuromuscular junction. However, less
is known about the neuronal nAChRs. In the mammalian brain there are at least nine
α (α2, α3, α4, α5, α6, α7, α8, α9, α10) and three β (β2, β3, β4) subunit encoded by
distinct genes similar in sequence to the genes encoding muscle nAChR subunits (Boulter
et al., 1986; reviewed by McGehee and Role 1995). These proteins combine to make
functional channels (Sargent, 1993) with a putative stoichiometry in vitro of 2α:3β
(Anand et al. 1991, Cooper et al. 1991). These subunits may constitute heteromeric or
homomeric receptors (Figure 7). The α4β2 heteromeric subunit combination, formed of
both α and β, is the most common receptor subtype in the brain. The α7 (also α8, and
α9) homomeric combinations, formed from only α subunits, are located in the CNS or
PNS. In the PNS the α3β4α5* are the most prominent subtype.
The neuronal nAChR receptors are depicted as arranged pentamers around central cation channel. They may exits in heteromeric form, comprised of both $\alpha$ and $\beta$ genes or in homomeric form comprised of only $\alpha$ subunits. The most common heteromer in the CNS is the $\alpha4\beta2$ and in the PNS is $\alpha3\alpha5\beta4*$ with the most common homomer being the $\alpha7$ subtype.

The diversity resulting from the assembly of the multiple neuronal subunits in different combinations results in a wide spectrum of structurally and functionally distinct states (Lloyd and Williams, 2000 nAChRs (Figure 8), with different pharmacological specificities and ion channel properties (Role 1992). Both $\alpha$ and $\beta$ subunits seem to contribute to the functional diversity of nAChRs. Brain nAChRs have been implicated in neural development, neuroprotection (via Ca++ entry), mood, learning and memory and various disease states including Alzheimer’s disease, Parkinson’s disease, schizophrenia, Tourette’s syndrome, epilepsy, and nicotine addiction. (for reviews see Role and Berg 1996; Levin and Simon 1998; Adler et al., 1998; Belluardo et al., 2000, Lloyd and Williams, 2000).
Different subtypes of brain nAChRs have been implicated in neural development, neuroprotection (via Ca++ entry), mood, learning and memory and various disease states including Alzheimer’s, Parkinson’s, Schizophrenia, Tourette’s syndrome, Epilepsy, and Nicotine addiction. Taken from “Neuronal Nicotinic Acetylcholine Receptors as Novel Drug Targets”. Loyd and Williams. 2000.

The generation of knock-out mice lacking specific nAChR subunits has given new information for their physiological importance (reviewed by Cordero-Erausquin 2000). Thus far, only the α3 subunit appears to be necessary for survival (Xu et al., 1999a). This subunit forms a nAChR that mediates fast synaptic transmission in the autonomic nervous system. Mice lacking the α4, α7, α9, β2, or β3 subunits survive to adulthood without any severe behavioral deficits (Picciotto et al., 1995; Orr-Urtreger et al., 1997; Booker et al., 1999; Marubio et al., 1999; Ross et al., 1999; Vetter et al., 1999). In contrast to the knock out of a single subunit, simultaneous knock out of β2 and β4
subunits is lethal (Xu et al., 1999b). Therefore, it is possible that the absence of a single nAChR subunit can be compensated for during development, and future studies using inducible genetic modifications are needed for a more detailed characterization of nAChR function in the CNS.

Nearly every region of the CNS receives cholinergic input from either of the two major clusters of cholinergic nuclei, the basal forebrain complex and the pontomesencephalic system (Descarries et al., 1997). The diffuse nature of these projections largely explains the wide distribution of nAChRs, serving as targets for the endogenous agonist of cholinergic nerves, acetylcholine (see Sargent 2000 for a detailed review). In the brain, nAChRs are located in different parts of neurons and have been subdivided into “somatodendritic”, “preterminal” and “presynaptic” receptors (Rapier et al., 1988; 1990; Grady et al., 1992; Marshall et al., 1996; Wonnacott 1997). There is a large body of evidence that nAChRs are present in many types of neurons, including the cholinergic ones, in the brain. It is also commonly observed that individual neurons express more than one nAChR channel type. Therefore, it is not possible to predict nAChR compositions based solely on the set of genes expressed by a single neuron. The β2 subunit mRNA and protein are most widely expressed and their distribution is generally similar to pattern of binding seen with $^{3}$H_nicotine (Clarke et al., 1985; Hill et al., 1993). The distribution of α4 mRNA is less widespread than that of β2 mRNA, but these two subunits comprise at least 90% of brain high affinity nicotine binding sites (Wada et al., 1989; Flores et al., 1992). The mRNAs encoding α4 and α7 are found in equal amounts. The distribution of α7 mRNA correlates with the binding of 125I-bungarotoxin ( _-BgT) with the distribution being distinct from that seen with
According to in situ hybridization studies, areas expressing the strongest signal for $\alpha_4$ and $\beta_2$ include layers of cerebral cortex, medial habenula, thalamus, mesencephalic dopaminergic nuclei and area postrema (Léna and Changeux 1998). The highest intensity signals for $\alpha_7$ are found in olfactory bulb, layers VVI of cerebral cortex, hippocampus, supraoptic nucleus of the hypothalamus and vestibular nuclei. In contrast, the distribution of $\alpha_3$ and $\alpha_5$ is much more limited, and $\alpha_2$, $\alpha_6$, $\alpha_3$ and $\beta_4$ are only present in a few brain structures. Interestingly, mRNA coding for the $\alpha_6$ subunit is selectively concentrated in dopaminergic nuclei of the midbrain suggesting a role for $\alpha_6$–containing nAChRs in mediating nicotine’s locomotor activating and rewarding effects (Le Novere et al., 1996).

1.4 Nicotine and Cholinergic Agents

Nicotine, a selective agonist at the nAChR, is a tertiary amine with a pyridine and pyrolidine ring. There are two stereoisomers of nicotine being the (R)-nicotine which is less active and the (S)-nicotine that is active in cigarette tobacco. Nicotine is a weak base and therefore is readily absorbed across cell membranes in the proper pH environment. Nicotine from cigarettes in the lungs is buffered to an acceptable pH and rapidly absorbed by the vasculature of the lung aveoli. It has been estimated the nicotine reaches the brain in 10-19 sec. Nicotine levels in the brain and blood decrease rapidly and are eliminated from the body. Smokers smoke multiple cigarettes in a day; therefore, the nicotine concentration in the body oscillates. The half-life of nicotine is 2 hr. and can accumulate over 6-8 hr reaching plasma levels of 20-40 mg/ml which then drops off a night.
Nicotine is metabolized mainly by the liver, but also the lungs and brain. 70-80% of nicotine is broken down into cotinine metabolite by C-oxidation. The half-life of cotinine is 14-20hr, much longer than nicotine. Secondly, P450 cytochrome enzymes and mono-oxygenases are thought to play a role in metabolism. The major metabolite in the urine is trans-3’-hydroxycotinine. It is still unclear as to whether the harmful affects of cigarette smoking during pregnancy is caused by nicotine or the many toxins in the smoke, particularly CO, carbon gases, lead, or cadmium. Early animal studies show that CO at the concentrations seen in smokers induces fetal hypoxia (Longo, 1977), reduces birth weight (Garvey and Longo, 1977, Lynch and Bruce, 1989), produces structural and functional abnormalities in the brain (Fechter and Annau, 1977, and Fechter, et al, 1986) and cognitive defects after birth (Mactutus and Fechter, 1984 and Mactutus and Fechter, 1985). Experimental work by Slotkin and others show in the developing animal that nicotine impairs the development of nicotinic receptors in the brain that ultimately display behavioral abnormalities. Therefore, it is likely that both nicotine, by affecting the development of the fetus directly, and the toxins, by restricting oxygen and inducing fetal hypoxia, cause detrimental affects to the fetus.

In addition to nicotine and ACh, numerous other substances have been discovered that either activate or inhibit nAChRs, and these compounds have provided valuable pharmacological tools for understanding the function and pharmacology of nAChRs. Detailed reviews on the various agonists and antagonists can be found elsewhere (Holladay et al., 1997; Lloyd and Williams 2000), and thus only agents used in the present experiments are described here. Dihydro-β-erythroidine (DHβE) is a plant alkaloid derived from the seeds of *Erythina americana*. DHβE is a tertiary amine that can
penetrate the blood-brain barrier (Decker et al., 1995). In addition to its effects on muscle end-plates, DHβE binds to heteromeric brain nAChR subtypes (Williams and Robinson 1984). Various nAChR subtypes expressed in oocytes differ greatly in their sensitivity to DHβE-induced blockade of ACh-evoked currents (Harvey et al., 1996). According to Harvey and co-workers (1996), the α3β4 subtype is resistant to DHβE, with 10 – 120 fold differences in sensitivity compared to other heteromeric subunit combinations (α2β2, α2β4, α3β2, α4β2, α4β4). Furthermore, DHβE displaces [125I]-BgT binding from the putative α7 nAChRs in rat brain only at very high (millimolar) concentrations (Decker et al., 1995). Tritiated DHβE also labels sites that co-localize with high affinity nicotine binding sites composed of α4β2 nAChRs (Williams and Robinson 1984; Flores et al., 1992).

1.5 Zebrafish as a Model System

The zebrafish has been established in the last 20 years as an important model organism for vertebrate development, most notably by researchers in Oregon, Boston and Tübingen (Germany). The zebrafish (Danio rerio) is a tropical fish originating from Pakistan and India with an adult size of 3-4 cm. The major advantage of the zebrafish as a model system is the simultaneous application of embryological and advanced genetic techniques. Unlike rodent, genetic methods are limited by the lack of a gene knock-out through homologous recombination. Nevertheless, a huge number of mutant lines have been generated by reverse genetics. Several large-scale mutagenesis screens using the chemical mutagen ethynitrosurea (ENU) and extensive screening for developmental
defects have been performed (Driever et al., 1996; Haffter et al., 1996). These genetic and molecular studies can be done efficiently, as zebrafish embryos can be obtained in large numbers, develop ex utero, are easy to maintain in relatively small facilities (Haffter and Nusslein-Volhard, 1996), and the embryos develop quickly.

Stages, as defined by hours post fertilization (hpf) of zebrafish development, are well characterized and easily identifiable (Kimmel et al, 1993, 1995). Embryonic development of the zebrafish is rapid (Figure 9) and occurs in a transparent, extra-embryonic membrane known as the chorion which is approximately 1 mm in diameter. Following fertilization of the egg, zebrafish development proceeds through a number of morphologically distinct stages including: zygote (0-0.75 hpf; cleavage (0.75-2.25 hpf); blastula (2.25-5.25 hpf); gastrula (5.25-10 hpf); and segmentation and somite formation (10-24 hpf) (Kimmel et al., 1995).
Moreover in zebrafish, gain of function and transient gene knock-down analysis can be efficiently performed by mRNA overexpression as well as antisense morpholino oligonucleotide gene knock-down (Nasevicius and Ekker, 2000) respectively. This is possible by a well-established microinjection technique and the access to a huge number of embryos at earliest developmental stages. Furthermore, due to the transparency of zebrafish embryos, expression analysis by *in situ* hybridization can be easily performed to investigate genetic interactions. Thus, in zebrafish novel genes controlling functionally important developmental processes can be rapidly identified and functionally analyzed. Altogether, this shows that the zebrafish provides many important advantages for the analysis of vertebrate development and complements other established model organisms and experimental systems. As studies in diverse species have demonstrated the role of
acetylcholine in multiple developmental processes such as cell division, differentiation, cell movement, synaptogenesis, neurite growth, and apoptosis (Slotkin, 2004). I propose to use zebrafish (Danio rerio) as a model system to study cholinergic signaling via the nAChR during development of the brain and spinal cord. As cholinergic agents can easily penetrate zebrafish embryonic/larval skin, I can administer nicotine directly into the water, providing an effective non-invasive drug delivery.

Neural development occurs in a well-characterized pattern and molecular markers are available to identify brain regions and specific cell types (Kimmel et al, 1995, Kimmel 1993, Luo, 2000). During segmentation, the central nervous system (CNS) transforms from a flat sheet of ectodermal cells, the neural plate, to a solid three dimensional structure morphologically subdivided into compartments. In contrast to other species, cell movements at 12 hpf in the neural plate of zebrafish lead to the formation of the neural keel, a solid rod-like structure. Shortly after, by “secondary neurulation”, this transient tissue is hollowed to form the lumen of the neural tube (Papan and Campos-Ortega, 1994). By 16 hpf, compartmentalization of the zebrafish brain is evident by ten neuromeres, or swellings, that are visible along the rostro-caudal axis of the neural tube (Kimmel et al., 1995). The seven most caudal neuromeres are the segmental rhombomere hindbrain compartments and the anterior three neuromeres are thought to correspond to the telencephalon, diencephalon and midbrain. By the end of segmentation significant changes in the neural tube has led to the formation of the major subdivisions of the brain. At 24hpf, a small forebrain ventricle and morphological evidence of the epiphysis/pineal (Ross et al., 1992; Wilson and Easter, 1991) is visible. The midbrain has a prominent ventricle which separates the dorsal tectum and the ventral
tegmentum (Wilson et al., 1990; Kimmel et al., 1995). In addition, the cerebellum is also
now clearly evident in the region of the midbrain/hindbrain boundary. Rhombomere 1 is
located directly ventral to the cerebellum (Kimmel et al., 1995). The hindbrain at this
stage consists of seven discrete and functionally distinct rhombomeres (Trevarrow et al.,
1990; Kimmel et al., 1995). At 24 hpf the spinal cord is essentially a continuous structure
at this age with no obvious morphological distinctions along its rostrocaudal axis
(Kimmel et al., 1995). Special sensory areas are also beginning to form at 24 hpf, hence
the lens of the eye and the olfactory and otic placodes are clear. After approximately 2
days the larvae hatches from the chorion and becomes free swimming.

Primary neurons in the zebrafish spinal cord have been identified and divided into
three main classes: motor neurons, interneurons and sensory neurons (Bernhardt et al.,
1990; Kuwada et al., 1990; Eisen, 1991). These neurons are repeated in the somites along
the anteroposterior axis and are characterized based on the location of their cell body, the
projection of the axon, and their position within the somite boundaries. By 18-20 hpf,
each spinal cord hemisegment contains approximately 11 differentiated neurons: three
primary motor neurons, five interneurons, and three primary sensory Rohon-Beard
neurons (Kuwada and Bernhardt, 1990). At 15 hpf the first neurons to develop in the
spinal cord are the sensory Rohon-Beard neurons located in the dorsal neural tube
(Kuwada et al., 1990) which extend two axons, one anterior and one posterior by 17 hpf.
Three primary interneurons develop slightly later and project axons at 17 hpf (Kuwada et
al., 1990). Finally, three primary pioneer motor neurons (Figure 10) of the zebrafish
spinal cord develop at 15 hpf and extend axons at approximately 17 hpf. These have
been individually identified as the caudal primary (CaP), middle primary (MiP) and rostral primary (RoP) neurons.

![Diagram of 3 Primary Motor Neurons in the Spine with predicted projection pathways](image)

**Figure 10: Diagram of 3 Primary Motor Neurons in the Spine with predicted projection pathways**

Transverse and (b) lateral views showing wild-type projections of the three primary motor neurons, RoP (R; green), MiP (M; red) and CaP (C; blue), in each hemisegment. Axons from RoP, MiP and CaP project ventrally from the spinal cord (sc) along the common pathway to the HMS, and then diverge. RoP invades the myotome at the level of the HMS, whereas MiP retracts its common pathway projection and instead projects to dorsal myotome. CaP continues on and projects to ventral myotome. Taken from “Wiring the zebrafish: axon guidance and synaptogenesis” Review. Hutson and Chien 2002.

In addition, a fourth motor neuron, the variable primary neuron (VaP), (Eisen et al., 1989) which dies later in development is only located in certain segments. The axon pathways of the primary motor neurons have been well characterized and shown to follow highly specific trajectories (Beattie, 2000). The nomenclature of the primary motor neurons is set by the stereotypical positions relative to the somite boundary: the Caudal Primary and Variable Primary (CaP/VaP) neurons are located centrally relative to the somite and extend into the ventral somite, Rostral Primary (RoP) neurons are located
at a level immediately anterior to the boundary and innervates muscle cells around the horizontal myoseptum, and the Middle Primary (MiP) neurons are located immediately posterior to the boundary and send a collateral branch into the dorsal somite. The LIM homeobox genes are thought to dictate a combinatorial code leading to primary motor neuron development (Appel et al., 1995; Tokumoto et al., 1995; Segawa et al., 2001). Initially, all primary motor neurons express Islet 1, isl-1, but later in development they express different combinations of LIM genes. The CaP and VaP neurons express isl-2 and lim3, while the MiP and RoP neurons express isl-1 and lim3. While this expression pattern may provide a molecular code for primary motor neuron differentiation there is no evidence that it is involved in axon guidance in zebrafish (Segawa et al., 2001).

Zebrafish motor behavior begins at 17 hpf, only two hours after the first motor neurons begin extending axons, with spontaneous coiling of the tail that continues for hours. This coiling activity can be altered with cholinergic receptor blockers such as α-bungarotoxin and d-tubocurarine (Grunwald, et al, 1988 and Saint-Amant L, 1998) indicating that the motor activity is of neural origin, not specifically spontaneous muscle activity. At 21 hpf the embryo can response to touch and by 23 hpf the coiling activity is limited; therefore the touch response is more evident. At this stage contraction or movement can be elicited by probing the head or tail. It has been show that structures anterior to the hindbrain are not crucial for touch responses up to 24 hpf. (Saint-Amant L, 1998). Blockade of neurotransmitter systems eliminates the touch response indicating that by 21 hpf that chemical neurotransmission is already established (Saint-Amant L, 2006). At 28 hpf, zebrafish can be induced to swim and by 36 hpf the embryos can swim at an increased frequency (Saint-Amant, 1998). By 48 hpf, the spontaneous motor
activity is abolished and tactile stimulation induces swimming. At 72 hpf the embryo displays intermittent swimming in which the embryo burst forward and then stops. At 96 hpf, the embryo displays multiple motor behaviors including: 1) spontaneous routine turns followed by a 2) slow swim and 3) escape turns after tactile stimulation as a part of the startle response, followed by 4) burst swims. The motor behavior continues to evolve and become more complicated as the embryo reaches 6 dpf (Saint-Amant, 2006).

1.6 Neuronal Nicotinic Acetylcholine Receptors in Zebrafish

Our laboratory is pioneering the work in neuronal nAChR in zebrafish and the first to clone full length cDNAs and examine the mRNA expression patterns. To date we have cloned 9 zebrafish orthologs ($\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_6$, $\alpha_7$, $\alpha_8$, $\beta_2$, $\beta_3$, $\beta_4$) of the 12 subunits known to date and completed temporal/spatial expression patterns for 6 of the genes (Zirger, et al 2003, Ackerman, et al, unpublished). Zirger et, al showed that the $\alpha_2$ and $\beta_3$ nAChR RNA was present at 2 hpf (zygotic translation) and that $\alpha_7$ expression began at 8 hpf. $\alpha_2$ expression was localized to the anterior segments of the spine up to 48 hpf and in the olfactory bulb at 24 hpf. $\beta_3$ expression was limited to the retinal ganglion layer and an area consistent with the optic nerve exiting the eye. Finally, $\alpha_7$ was expressed in hindbrain and the eye at 72 hpf and 96 hpf. In short, each nAChR displayed unique transient expression patterns and seem to be differentially regulated during development, as evident by the fluctuating levels of mRNA expression in the PCR and in situ hybridization studies. Additionally, binding studies with epibatidine (high affinity ligand for nAChR) indicated that at 48 hpf and 5 dpf there are at least two
populations of nAChR binding sites. Most importantly, with a response noted to epibatidine, we now know that there are assembled nAChR as early as 48 hours after fertilization (Zirger, et al, 2003). Furthermore, treatment of zebrafish with 50 or 100 µM nicotine until 96 hpf induces abberant apoptosis in tectum and retina along with paralysis; meanwhile, pre-treatment of the embryos with DHβE (nAChR antagonist) blocks the increase in apoptosis (Zirger, unpublished). My work presented here extends these studies using the developing zebrafish as a model system to study nAChR function during development.
2.1 Animal Experiments

2.1.1 Zebrafish Husbandry

*Danio rerio*, zebrafish, were used for these studies. Adult animals were housed in constant temperature (28.5 °C) and humidity with a 12 hr light: dark cycle in the Ohio State University Center for Molecular Neurobiology zebrafish facility. The fish were fed twice daily with flake food (Tetramin, Aquatic Eco-Systems) and brine shrimp (Biomarine, Aquafauna Biomarine). Zebrafish embryos were reared, collected, and allowed to develop in our laboratory in an IsoTemp Incubator (Fisher) at 28.5 °C in Petri dishes with fish water. The embryos were staged as described by Kimmel, et al (1995) and around 24 hpf placed into N-Phenylthiourea (PTU) to block pigment formation. All studies were performed in accordance with the Guide for Care and Use of Laboratory Animals as adopted by the National Institute of Health, USA, and after approval by the
Ohio State University Institutional Laboratory Animal Care and Use Committee. Animals were anesthetized by tricaine (m-3-amino benzoic acid ethyl ester methansulfonate).

2.1.2 Nicotine Treatment of Zebrafish

In these studies, embryonic zebrafish were chronically exposed to nicotine [(-)-nicotine bitartrate] (Sigma) concentrations of 50, 100, or 200 µM diluted directly into fish water from 1-3 hpf (hours post fertilization) to 96 hpf. Larval growth, morphology, mortality, behavior, and gene expression were examined at 24 hpf, 48 hpf, 72 hpf and 96 hpf.

2.1.3 Antisense Oligonucleotide Morpholino Gene Knock Down in Zebrafish

Zebrafish (Danio rerio) of the AB* strain, Islet 1-GFP and HB9-GFP transgenics were used throughout these studies. They were maintained at 28°C and embryos were staged according to Kimmel et al. (Kimmel et al., 1995). Morpholino antisense oligonucleotides (MO) were designed by and obtained from Gene-Tools Inc. The MO were diluted and injected as previously described (Nasevicius and Ekker, 2000; Phillips et al., 2001). At least 30 embryos were examined for each experiment in triplicate. To knock down nAChRs, splice-blocking morpholinos were generated (Table 1) as follows:
Morpholino oligos (MO) can block nuclear processing events, in particular pre-mRNA splicing. Sequences were chosen to target exon splice donor or acceptor site regions of the corresponding mRNA. This resulted in aberrant splicing of the mRNA, which resulted in a frame shift. The effect of the splice variant-morpholino was verified by RT-PCR on total embryo RNA. Antibodies for zebrafish nAChR genes have not yet been developed; therefore, translation start site morpholinos and Western analysis could not be used for these studies.

The morpholino oligomers were diluted in ddH₂O to a working stock concentration of 4 mM and stored at -20 °C. Filtered phenol red dye was added to a concentration of 30% to visualize fluid during injections. Approximately 1 nl of diluted MO/dye solution per pulse was injected at the boundary of the yolk of one- to two-cell stage embryos. Final concentrations injected into the embryo ranged from 1 ng to 8 ng with a volume of 1 nl to 3 nl. Embryos were injected and allowed to recover in embryo
rearing medium (ERM) with 50 units/ml penicillin and 50 ug/ml streptomycin for 24 hr and were then transferred to fish water with PTU (.03 g N-Phenylthiourea/ 1 L of fish water).

2.1.4 Touch Response Motor Behavior Assay

Zebrasfish embryos display three stereotyped behaviors by 36 hours post-fertilization (hpf) (Saint-Amant and Drapeau, 1998). The earliest behavior consists of repetitive spontaneous alternating coiling of the tail that is independent of stimulation and starts at 17 hpf and declines by 26 hpf. After 21 hpf, embryos start to respond to mechanosensory stimulation with coils that are stronger than spontaneous coils. Typically embryos respond with two alternating coils. By 26 hpf mechanosensory stimulation initiates swimming episodes. The production of any touch response can be divided into several steps from sensory perception to muscle activation. In zebrasfish two types of mechanosensory neurons sense touch stimuli: trigeminal neurons in the head and yolk and Rohon-Beard neursons in the spine (Drapeau et al., 2002). Once triggered by sensory input, interneuronal networks located in the hindbrain and spinal cord produce the appropriate motor rhythm. Motoneurons are activated by these central networks, release acetylcholine at neuromuscular junctions (NMJs) and depolarize the muscle membrane. After various treatments of either gene knock down or cholinergic drug exposure, mechanosensory stimuli were delivered to the yolk, head, and spine with thin forceps. For each experiment at least 10 embryos per experimental group were probed 10 times in succession. The number of times the embryos swam in response to the touch
stimuli was recorded. A distinction between a normal and abnormal response was not recorded, only the ability to evoke any movement was noted.

2.2 Molecular Biology

2.2.1 Total RNA Isolation

The samples for RNA isolation were either freshly anesthesized zebrafish embryos or embryos that were previously collected and frozen at -70 °C until needed. The embryos were homogenized in 1 ml of TRIZol Reagent (Invitrogen) using a glass Teflon homogenizer. The samples was transferred to a microcentrifuge tube and incubated for 5 min at 15-30°C to permit complete dissociation of nucleoprotein complexes. Next, 200 µl of chloroform per 1 ml of TRIZol was added to the sample and it was inverted vigorously by hand for 15 sec. The sample was incubated for 3 min at 15 to 30°C and then centrifuged for 15 min at 4°C (12,000 x g). The aqueous phase was transferred to a fresh microcentrifuge tube, 500 µl of isopropanol alcohol was added, and the sample was incubated for 10 min at 15 to 30°C. Following incubation, the isolate was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was washed with ice cold 75% ethanol. At this point, the samples were either stored at -70 for future use or the sample was briefly vortexed and then centrifuged at 7,500 x g for 5 min at 4°C. After removing the supernatant, the pellet was air-dried for 5-10 min at room temperature. Finally, the RNA was dissolved in 20 µl of DEPC H₂O (Invitrogen). The solution was then incubated on ice for 30 minutes with intermittent hand mixing and
the concentration was determined by spectrophotometry. The RNA was stored at -70 °C for months without degradation.

2.2.2 Determination of RNA and DNA concentrations

A 3 µl aliquot from the sample was taken to determine the O.D. and concentration by spectrophotometry (Spectronic 21D, Milton Roy). Each 3 µl aliquot was added to 600 µl of ultraPURE Distilled Water (Gibco) and mixed. Absorbance was measured at 260 nm, and the µg of RNA was determined by the following formula: 0.1 O.D. at 260 nm = 40 µg RNA. Likewise, the formula to determine µg of DNA is 0.1 O.D. at 260 nm = 50 µg DNA. From these equations, the µg/µl of sample could be determined.

2.2.3 RNA gel electrophoresis

Samples were prepared for electrophoresis by preparing 3 µg of RNA as determined by the spectrometer. Each sample was brought to a common volume (8 µl) with ultraPURE Distilled Water (Gibco). 24 µl of RNA loading buffer (per 100 µl: 70 µl of deionized formamide (Invitrogen), 20 µl of 37% formaldehyde (Fischer), 10 µl of 10X MOPS, and 2 µl of ethidium bromide) was added to each sample and mixed by brief vortex. The samples were incubated at 60°C for 10 min and then immediately loaded into the gel wells. A 1% agarose gel was prepared using the following procedure: 2.15 g UltraPure agarose (Invitrogen) was added to 200 ml of distilled H2O and 15 ml 10X MOPS DEPC treated buffer (Ameresco) and dissolved by heating to boil. After cooling
to below 55°C, 30 ml formaldehyde was added, the solution swirled, the gel poured in the caster under the fume hood, and allowed to solidify for at least 30 min. After polymerization, the gel was placed in the electrophoresis chamber containing 1X MOPS. Samples, including a .5-10 Kb RNA ladder, were loaded and current applied at 10 mA over night. The gel was checked under UV light to confirm RNA integrity by the migration of the 28s and 18s bands and a photograph taken with a FOTODYNE camera.

2.2.4 Synthesis of double-stranded cDNA from total RNA

After determining the concentration and integrity of the purified RNA, total RNA was used for the synthesis of first strand cDNA using Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). 3 ug of total RNA was combined in a microcentrifuge tube with 1µl of random hexamers (50ng/µl), 1 µl of 10 mM dNTP mix, and DEPC H2O to a final volume of 10 µl. The samples were incubated at 65°C for 5 min and then put directly on ice. In a separate microcentrifuge tube, 2 µl of 10X RT Buffer, 4 µl 25mM MgCl2, 2 µl of 0.1 M DTT, 1µl of RNase Out (40U/µl), and 1 µl of Superscript III RT (200U/ µl) was combined. Next the RNA containing mix was briefly centrifuged and then 10 µl of cDNA synthesis mix was added to the RNA sample and mixed well. The mixed reaction was heated for 10 min at 25°C, 50 min at 50 °C, and terminated for 5 min at 85 °C using a PTC-100 Programmable Thermal Controller. The first strand reactions were placed immediately on ice, then 1µl of RNaseH was added to
remove the RNA template, and the sample was incubated for 20 min at 37 °C. The cDNA was stored at -20 °C for months.

### 2.2.5 RT PCR

Polymerase Chain Reaction (PCR) is a process that allows synthesis of DNA in vitro (Saiki et al., 1988). It can be used for the sequence-specific amplification of DNA, for quantification of gene expression at the level of mRNA (Metzker and Caskey, 2001) as well as for site-directed mutagenesis of DNA (El-Gewely et al., 2001). The synthesized cDNA was amplified by PCR using Platinum Taq DNA Polymerase (Invitrogen) following the manufacturers protocol combining the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration in Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer (-MgCl2)</td>
<td>5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>1 µl</td>
<td>0.2 mM each</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>1.5 µl</td>
<td>1.5 mM each</td>
</tr>
<tr>
<td>Primer mix (10µM each)</td>
<td>1.0 µl</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.0</td>
<td>1 µl (as required)</td>
</tr>
<tr>
<td>Platinum Taq Polymerase</td>
<td>0.2 µl</td>
<td>1 unit</td>
</tr>
<tr>
<td>DEPC H₂O</td>
<td>50 µl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The only exception to the cocktail was that 3.0 ul of MgCl₂ was necessary to synthesize the α4 gene. The components were often scaled into larger reaction volumes and included samples with no Taq enzyme and a control with no DNA. PCR was carried out in an automated thermocycler (PTC-100 Programmable Thermal Contoller, MJ Research, Inc). After a hot start of 94°C for 2 min, the following steps were programmed:

1) denaturation for 30 sec at 94°C,
2) sequence-specific annealing of primers for 30 sec ranging from 53°C to 58°C
3) elongation of the PCR product at 72°C (2 min).

Finally, a final elongation step at 72°C for 10 min finished the PCR process. PCR conditions were optimized for each specific primer set used. Included in Table 2 are the names of the gene amplified, the primer sequences, the annealing temperature, the cycle count, and the predicted product size. PCR was used to generate time course analysis of gene expression and also to generate the cDNA necessary to synthesize riboprobes for *in situ* hybridization. The PCR products were stored at -20 ºC
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α2</td>
<td>GGAGATCCTCCGAGCATCCAT GAGCTCTTCTAGATGGCAGGAGG</td>
<td>56</td>
<td>40</td>
<td>600</td>
</tr>
<tr>
<td>α2</td>
<td>GGAGATCCTCCGAGCATCCAT TTTTGCAATATTGCGACGCCTG</td>
<td>58</td>
<td>40</td>
<td>2000</td>
</tr>
<tr>
<td>α3</td>
<td>ATTTCTGTCGCCCGACTGT GAATACGAGAAGGTCAGAGA</td>
<td>56</td>
<td>30</td>
<td>1200</td>
</tr>
<tr>
<td>α4</td>
<td>TATGATCAAAAACGAGGAAGAAGAATCAGTGAGACGAGA</td>
<td>55</td>
<td>35</td>
<td>1200</td>
</tr>
<tr>
<td>α6</td>
<td>TTTGTTTTGGAAAGGGAGACGGT GAATACGAGAAGGTCAGAGA</td>
<td>55</td>
<td>30</td>
<td>890</td>
</tr>
<tr>
<td>α7</td>
<td>AGGAAGATGGGAATTTTGGGAAT AGCAGAATGTCAATGTACGGAGGTTTGGCTT</td>
<td>56</td>
<td>40</td>
<td>500</td>
</tr>
<tr>
<td>α8</td>
<td>GTTTCCGTCGCGCTTGTTGTA TTTCAACATTCTGGATATCA</td>
<td>54</td>
<td>32</td>
<td>600</td>
</tr>
<tr>
<td>β2</td>
<td>CTTTGTGTAAGTGTGGGACGGA GGAGATCAGTACCAGAAGA</td>
<td>55</td>
<td>34</td>
<td>870</td>
</tr>
<tr>
<td>β3</td>
<td>AGACTTACAGGGATTACCGTAATC GATCATGGACATACGATGGCACTATGG</td>
<td>56</td>
<td>40</td>
<td>1200</td>
</tr>
<tr>
<td>β4</td>
<td>GACACATCAGGTACCTTTCCGATTA TGTGAGGCGCGAATGGGACT</td>
<td>53-58</td>
<td>28</td>
<td>400</td>
</tr>
<tr>
<td>Islet</td>
<td>TGCAAAAACCGCGTGTGCT GTGCAGAATGTCAAGACGAGA</td>
<td>56</td>
<td>28</td>
<td>700</td>
</tr>
<tr>
<td>shh</td>
<td>AGACATCCGAGAAGCTGACAC GAACTGATGAAAGTCGCTGAAAC</td>
<td>55</td>
<td>26</td>
<td>600</td>
</tr>
<tr>
<td>p53</td>
<td>AAGAACAGCCTCAGCCATCC CTCAGTTTTTCTGGTTCGAC</td>
<td>53</td>
<td>28</td>
<td>900</td>
</tr>
<tr>
<td>c-fos</td>
<td>AAAGTCAAGGCGACAGCCTT GCAGGACTAGTGTTGTCAGA</td>
<td>54</td>
<td>35</td>
<td>1000</td>
</tr>
<tr>
<td>β-actin</td>
<td>CCCCCTGGTTCCCAATAACCT TCTGTTGGCGTTGGGATTCA</td>
<td>53-58</td>
<td>28</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 2: Oligonucleotide primers used to generate zebrafish specific nAChR
2.2.6 DNA gel electrophoresis

The synthesized PCR products or DNA fragments were analyzed by agarose gel electrophoresis. Samples were prepared for electrophoresis by preparing a total volume of 31 µl to be loaded into the gel wells. 25 µl of DNA (PCR product) was combined with 3 µl of 10X Blue Juice Gel Loading Buffer (Invitrogen) and 3 µl of 10X REact 3 buffer (Invitrogen). A 0.9% agarose gel was prepared using the following procedure: 1.8 g UltraPure agarose (Invitrogen) was added to 200 ml of 1X TBE (National Diagnostics) and dissolved by heating to boiling. After cooling to below 55°C, the solution swirled and the gel poured in the caster at the bench top and allowed to solidify for at least 30 min. After polymerization, the gel was placed in the electrophoresis chamber containing 1X TBE with 50µl of 10 mg/ml Ethidium Bromide Solution (Invitrogen) per L of 1X TBE. The samples, including a φX174 RF DNA Hae III Fragments (Invitrogen) or λDNA/Hind III Fragments (Invitrogen) DNA ladder, were loaded and current applied at 100 D.C. Volts for 4 hr. The gel was checked under UV light to confirm DNA integrity and a photograph taken with a FOTODYNE camera.

2.2.7 Cloning of full length Neuronal Nicotinic Acetylcholine cDNA with RACE

Rapid amplification of cDNA ends (RACE) is a PCR-based technique which aids in the cloning of full-length cDNA sequences when only a partial cDNA sequence is available. RNA was isolated from 7 dpf (days post fertilization) embryos using TRIZol Reagent (Invitrogen). For the 3’ and 5’ RACE reaction, 7 dpf RNA was processed using the First Choice RLM-RACE kit (Ambion) with provided RACE primers and α4 nAChR specific primers.
3' RACE was performed with the 3’ RLM-RACE protocol where 1 µg of total RNA was reverse transcribed by: dNTP (2.5mM each dNTP) mix, 3’ RACE adapter, 10X RT buffer, RNase inhibitor, M-MLV Reverse Transcriptase, and DEPC H$_2$O were incubated at 42 ºC for 1 hr and stored at -20 ºC. Two Platinum Taq PCR reactions were assembled for 40 cycles with a 56 ºC annealing temperature. For 3’ RACE outer, a α4 nAChR-specific primer (α4 5’outer- GAGTACAAACACCGTGATT) and the 3’ RACE Outer primer (5’ GCGAGCACAGAATTAATACGACT-3’) were used. For the 3’ inner segment the α4 nAChR-specific primer (α4 3’ inner- TAATCTCCTGCTGACAGTC) and 3’ RACE Inner primer (5’-CGCGGATCCGAATTAATACGACTCCTATATAGG-3’) were amplified using the platinum taq PCR protocol mentioned earlier. The products were run on a 1% agarose gel and a single PCR reaction was enough to amplify product from the 3’RACE. The products were cloned into the vector pCRII TOPO and sequenced to ensure that α4 products were indeed amplified.

5' RACE was performed with the 5’ RLM-RACE protocol where 7 dpf total RNA was reverse transcribed and assembled in the following manner: 10 µg of total RNA, 10X CIP buffer, Calf Intesinal Phosphatase, and DEPC H$_2$O and incubated at 37 ºC for 1 hr. The reaction was terminated and cleaned up by phenol/chloroform EtOH precipitation. Next, the Tobacco Acid Pyrophosphatase (TAP) treatment to remove the CAP was assembled: CIP’d RNA, 10X TAP buffer, TAP, and DEPC H$_2$O and incubated at 37 ºC for 1 hr and stored at -20 ºC. During the 5’ RACE adapter ligation step, the CIP/TAP RNA was combined with 5’ RACE adapter, 10X RNA Ligase buffer, T4 RNA ligase, DEPC H$_2$O and incubated at 37 ºC for 1 hr and stored at -20 ºC. cDNA was synthesized using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). Subsequently,
PCR with platinum taq was performed with 2X MgCl and an annealing temperature of 56 ºC. α4 nAChR specific primer (α4 5’ inner- CAATCAGTGGGATGACCAAT) and the 5’ RACE inner primer (5’-CGCGGATCCGAACACTGCGTTTGCTGGCTTTTGATG-3’). The resulting amplification products were cloned into pCRII Topo vector (Invitrogen) and sequenced.

Based on the 3’ and 5’ RACE amplification results and sequencing, new primers were designed (α4 up: TATGATCAAAACTGGACGAAGA and α4 down: CCATGCCAGCGGATGTATTAATCA) to amplify a full length 2264 bp α4 cDNA. Again, 7 dpf RNA was reverse transcribed (Superscript III kit, Invitrogen), and amplified by Platinum Taq PCR with 2X MgCl2, 40 cycles, and an annealing temperature of 56 ºC. The product was resolved on a 1% agarose gel, TOPO cloned, and sequenced.

2.2.8 DNA Agarose Gel Extraction

The QIAEX II Agarose Gel Extraction Protocol is intended for use to extract DNA from low melt agarose gels and instructions were followed according to the manufacturer’s protocol. Once a PCR product was examined on an agarose gel, the cDNA band of interest was excised from the gel with a razor blade, taking care to cut the band with as little extra agarose as possible. The gel slice was placed into a pre-weighed 1.5ml microcentrifuge tube with 3 X vol of Buffer QX1 (added because our DNA fragments are less than 4 kb in size). The QIAEX II beads were resuspended by vortexing for 30 sec and then 10 µl of QIAEX II was added to the sample (because I excised less than 2 ug of DNA). The sample was then incubated at 50 ºC for 10 min to solubilize the agarose and bind the DNA. While at 50 ºC, the sample was vortexed every 2 min to aid
in dissolving the agarose. Next, the sample was centrifuged for 30 sec at room
temperature at 12,000 rpm, the supernatant was removed, the beads/DNA washed with
500 µl of Buffer QX1, briefly vortexed, and again centrifuged for 30 sec at room
temperature at 12,000 rpm. The 500 µl Buffer QX1 wash step was repeated. Next, the
sample was air dried until white. Finally, the DNA was eluded with 20 µl of DEPC H₂O,
suspended by vortexing, incubated at room temperature for 5 min (again because the
fragment was less than 4 Kb), centrifuged, and then supernatant was saved and placed
into a clean 1.5 ml micro centrifuge tube. The eluding step was repeated to increase yield
and the product was stored at -20 °C.

2.2.9 TOPO Cloning

TOPO sub-cloning provides a fast and efficient cloning method for PCR products
to be sequenced and analyzed. The plasmid pCR II-TOPO dual promoter (Invitrogen)
was used to clone PCR products according to the manufacturer’s instructions. The
plasmid contains a cloning site that supports bi-directional TA-cloning, combined with
topoisomerases that are able to synthesize covalent junctions of single stranded DNA.
Any PCR product, amplified by Taq polymerase can be incorporated into this plasmid.
For cloning, the freshly amplified PCR product (1 µl to 4 µl), 1 µl of salt solution (1.2 M
NaCl and 0.06 M MgCl₂), and 2 µl of H₂O were incubated with 1 µl pCR II-TOPO at
room temperature for 15 min. The reaction was then stopped by placing on ice.

Transformation refers to a direct uptake of foreign plasmid DNA by bacteria. For
selection and multiplication of the cloned plasmids, 2 µl of the TOPO vector containing
the inserted DNA was transformed into 50 µl chemically competent E. coli TOP10 One
Shot cells (Invitrogen). The sample was then mixed by tapping and incubated on ice for 30 min. Next, the cells were heat shocked for exactly 30 sec at 42 ºC and immediately placed on ice. Finally, in a sterile environment, 250 µl of S.O.C media (Invitrogen) was added to the transformed cells and incubated at 37 ºC for 1 hr while shaking horizontally at 200 rpm. After incubation 50-150 µl of positively transformed colonies were spread on pre-warmed selective LB-agar plates containing ampicillin (50 µg/ml). For some of the studies, the PCR generated DNA was cloned into TOPO TA blunt. The same protocol was followed except, the transformations were selected on LB-agar plates that were Zeocin resistant. All plates were incubated at 37 ºC overnight.

Liquid bacteria cultures were used for the multiplication of recombinant plasmid DNA. For each culture, a single bacteria colony was transferred into LB-medium (5 ml) containing the appropriate antibiotic. Cells were grown over night (37 °C, 250 rpm) until quiescent.

2.2.10 Purification of Plasmid DNA

For purification of the DNA, the FastPlasmid Mini kit (Eppendorf) was used. An overnight culture (3.0 ml) was centrifuged in an eppendorf bacterial collection tube at 12,000 rpm for 2 min. The bacterial pellet was resuspended in 400 µl of ice cold Lysis Buffer (includes Lysis soln, and RNase/Lysozyme mix), vortexed for 30 sec until it was a homogenous solution, and was allowed to incubate at room temperature for 3 minutes. The lysate was transferred to a spin column apparatus by decanting. Following a 60 sec centrifugation at 12,000 rpm, the DNA bound to the column was washed with 400 µl of Wash Buffer (composed of 100% isopropanol alcohol), and centrifuged one more time.
The column was removed, the liquid disposed of and the column placed back into the column apparatus, and centrifuged for 30 sec to remove any residual fluid. The column was then removed and placed into a collection tube where 50 µl of Elution Buffer (10mM Tris-Cl, 0.1 mM EDTA, pH 8.5) was applied to the center of the column and the DNA was eluted by centrifugation at 12,000 rpm for 30 sec at room temperature. The concentration of the recovered plasmid DNA was determined by spectrophotometry.

### 2.2.11 Identification of Appropriate Recombination of Clones

The TOPO vectors were engineered with EcoR1 sites flanking the inserted PCR product; therefore an enzymatic restriction digest of the plasmid with EcoR1 will result in the release of the PCR product. The Eco R1 digest reaction of the TOPO plasmid (at least 6 separate clones) was set up in the following manner:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>REact 3 buffer</td>
<td>3µl</td>
<td>3µl</td>
<td>3µl</td>
<td>3µl</td>
<td>3µl</td>
<td>3µl</td>
<td>3µl</td>
</tr>
<tr>
<td>DNA ladder</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3µl</td>
</tr>
<tr>
<td>EcoRI enzyme</td>
<td>-</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>-</td>
</tr>
<tr>
<td>Gibco H2O</td>
<td>24µl</td>
<td>15µl</td>
<td>15µl</td>
<td>15µl</td>
<td>15µl</td>
<td>15µl</td>
<td>24µl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>-</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
<td>-</td>
</tr>
</tbody>
</table>

The clones were incubated at 37 °C for 2 hr while the DNA ladder standards were placed on ice. 3 ul of 10X Blue Juice was added to each reaction and the DNA was resolved in a 1% agarose gel. The size of the PCR product was then informative as to whether a clone
was recombinant or empty. The plasmids were sequenced at the Ohio State University Plant-Microbe Genomics Facility using an automated 3730 DNA Analyzer from Applied Biosystems, Inc. and BigDye Terminator Cycle Sequencing chemistry. The M13F and M13R primers bind to regions of homology within the pTOPO vector and amplify the region across the cloning site of the vector. All sequence data were analyzed using the BLAST algorithm available at NCBI to GenBank (www.ncbi.nlm.nih.gov) or The Welcome Trust Sanger Institute (http://www.sanger.ac.uk) against the zebrafish genome.

2.2.12 In situ Hybridization: Restriction Enzyme Digests of Plasmids for synthesis of RiboProbe

Plasmid vectors (pCR II TOPO) containing DNA sequence insert of the individual subunits were linearized with an appropriate restriction enzyme to generate a 750 bp to 1 kb product. Table 3 includes the names of genes with the appropriate restriction enzyme, REact Buffer, and the approximate size of the product. The plasmid digests were set up with 10 µg of DNA, 10 µl of REact Buffer, 2 µl of enzyme, and DEPC H₂O to a final volume of 100 µl. The reaction was then incubated at 37 °C for 2 hr, stopped on ice, and 5 µl of DNA was run on an agarose gel to check for complete linearization.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Restriction Enzyme</th>
<th>REact Buffer</th>
<th>Polymerase</th>
<th>Approx. Size of Probe (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2</td>
<td>Not I</td>
<td>REact 3</td>
<td>SP6</td>
<td>1000</td>
</tr>
<tr>
<td>α4</td>
<td>PVU II</td>
<td>REact 6</td>
<td>T7</td>
<td>800</td>
</tr>
<tr>
<td>α6</td>
<td>PVU II or Bgl II</td>
<td>REact 6</td>
<td>SP6</td>
<td>750</td>
</tr>
<tr>
<td>krox 20</td>
<td>PstI</td>
<td>REact 2</td>
<td>T3</td>
<td>2100</td>
</tr>
<tr>
<td>TH</td>
<td>Sal I</td>
<td>REact 10</td>
<td>T7</td>
<td>2100</td>
</tr>
<tr>
<td>dlx-2</td>
<td>BAM H1</td>
<td>REact 3</td>
<td>T7</td>
<td>2100</td>
</tr>
</tbody>
</table>

Table 3: Riboprobe synthesis components: Genes are listed with the appropriate restriction enzyme, REact Buffer, and approximate size of the product.

2.2.13 In situ hybridization: Phenol Chloroform Extraction and Precipitation with EtOH

The linearized DNA was purified by phenol-chloroform extraction. To the plasmid DNA preparation (95μl), 105 μl of DEPC H₂O and 200 μl of Phenol:Chloroform: Isoamyl alcohol was added. The sample was vortexed for 10 sec. to mix and centrifuged at room temperature for 30 sec at 12,000 rpm. The aqueous upper phase was transferred to a fresh microcentrifuge tube and 1/10th the volume (approximately 18μl) of 5.2 M NH₄OAc was added, the sample briefly vortexed, and 2X volume of ice cold 100% EtOH (440μl) was added for DNA or 2.5X the volume (360μl) for RNA. Next, the samples were vortexed and placed at -70 for at least 30 min and sometimes over night. After sufficient freezing to aid in precipitation, the sample was centrifuged at 12,000 rpm for 15 min at 4°C, the supernatant removed, and the pellet
washed with and 1 ml of 70% EtOH, and centrifuged again at 12,000 rpm for 10 min at 4 °C. The EtOH was removed and the pellet air dried for 5-10 min at room temperature if RNA or placed in a Speed Vac if DNA for 3-5 min. The sample was suspended into 20 µl of DEPC H₂O and the concentration determined by spectrometry. Template DNA was stored at -20 °C.

2.2.14 In situ hybridization: Synthesis of DIG probes

Whole embryo in situ hybridization (Beattie, 1997, Thisse,1993) with digoxigenin or fluorescein labeled antisense RNA probes was used to determine the spatial expression pattern of the nAChRs in brain and spinal cord. RNA probes were synthesized using the DIG RNA Labeling Kit SP6/T7 (Roche) or the Fluorescein Labeling Mixture (Roche). The following components were added in order on ice in a biohood according to the manufacturers instructions:

<table>
<thead>
<tr>
<th>DIG-labeling</th>
<th>Fluorescein-labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg linearized, clean plasmid</td>
<td>1 µg linearized, clean plasmid</td>
</tr>
<tr>
<td>Up to 13 µl DEPC water</td>
<td>2 µl 10X Fluorescein RNA labeling mix</td>
</tr>
<tr>
<td>2 µl 10X Transcription buffer</td>
<td>2 µl 10X Transcription buffer</td>
</tr>
<tr>
<td>2 µl DIG-NTPs</td>
<td>DEPC up to a final vol. of 18 ul</td>
</tr>
<tr>
<td>1 µl RNase inhibitor</td>
<td>1 µl RNA polymerase (T3, T7, or SP6)</td>
</tr>
<tr>
<td>2 µl polymerase (T3,T7, or SP6)</td>
<td>1 µl RNase inhibitor</td>
</tr>
<tr>
<td>20 µl total</td>
<td>20 µl total</td>
</tr>
</tbody>
</table>

The reagents were incubated at 37 °C for 2 hr and then 2µl of DNase 1 was added and incubated at 37 °C for 15 min. To stop the reaction 2µl of EDTA, 1/10th ammonium acetate, and 2X ice cold 100 % EtOH were added. The sample was frozen at -70 °C for at least 15 min, centrifuged at 12,000 rpm for 15 min at 4 °C. The precipitate solution was
decanted off and the pellet washed with 70 % EtOH, spinning at 4 °C for 10 min. The pellet was air dried and suspended in 30 µl of DEPC H₂O on ice for approximately 30 min. The concentration of the RNA was determined by spectrophotometry and 2-3 µg of RNA was resolved in a 1% Northern gel. The probe was stored at -20 °C. When using RNA probes for in situ hybridization there is a chance of non-specific binding. I controlled for this by creating a sense strand RNA probe as a negative control in conjunction with the antisense strand and comparisons were made. Additionally, probe was omitted from the hybridization buffer of one group to control for non specific and endogenous phosphatase labeling and to ensure specificity. In our laboratory the α2, α4 and α6 nAChR were cloned and we also received some specific brain markers (krox 20, TH, and dlx-2) from Dr. Christine Beattie’s and Dr. Paul Henion’s laboratories at The Ohio State University in plasmid form. Table 3 depicts the polymerase used to synthesize the riboprobe, along with the size of the probe, and the restriction enzymes/REact buffers used to linearize the TOPO vector containing each gene.

2.2.15 Whole Mount Single Labeling In situ Hybridization

Staged embryos at various ages corresponding with major events in development as outlined by Kimmel et al (8 hpf, 12 hpf, 18 hpf, 24 hpf, 48 hpf, 72 hpf, 96 hpf) were used to determine the temporal and anatomical expression of each nAChR RNA by in situ hybridization. I used procedures outlined by Thisse et al (1993) and Beattie, et al (1997) with some modifications. All room temperature washes were rotated on a GryoTwister at 30 rpm. 30 whole embryos were placed in a 1.5 ml eppendorf tubes and fixed in fresh 4% paraformaldehyde (PFA)/1x phosphate buffered saline (PBS- NaCL: 49
80 g/L, KCl: 2 g/L, Na₂HPO₄: 14.4 g/L, NaH₂PO₄: 2.4 g/L, pH to 7.30) overnight if 72 hpf or 96 hpf, 2 days if 48 hpf, and 3 days if 24 hpf, making the PFA fresh each day.

Embryos were then washed at room temperature twice with 100% methanol for 5 minutes and one time for 10 minutes. Embryos were stored in fresh 100% methanol at -20°C. 24-48 hpf embryos were stored at -20°C for up to six months, but 72-96 hpf were used within a couple days for optimal expression. On day 1, all washes were performed with 750 μl of solution for 5 minutes at room temperature unless noted otherwise. Embryos were taken through graded methanol/1x PBS (75%, 50%, 25% methanol), washed four times with PBS-Tween (PBT:1X PBS + 500 μl Tween) and then treated with 1 ml of 10 μg/ml proteinase K in 1x PBS-Tween for 5-6 minutes if 24 hpf, 10-12 minutes for 48 hpf, 20 minutes for 72 hpf, and 30 minutes for 96 hpf. Following permeabilization, embryos were post-fixed in fresh 4% PFA for 20 min, rinsed five times with PBS-Tween, prehybridized in hybridization buffer (Heparin concentration at 100 mg/ml) for 2 hours and 15 minutes at 68°C, and finally hybridized with 200 ng RNA probe overnight at 68°C for at least 15 hours. Hybridization buffer was fixed in the following manner:

<table>
<thead>
<tr>
<th>Hybridization Buffer:</th>
<th>50% formamide (5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>25 ml 2.5 ml</td>
</tr>
<tr>
<td>20X SSC</td>
<td>12.5 ml 1.25 ml</td>
</tr>
<tr>
<td>Heparin (100 mg/ml)</td>
<td>500 μl 2.5 μl</td>
</tr>
<tr>
<td>tRNA(50 mg/ml)</td>
<td>500 μl 50 ul</td>
</tr>
<tr>
<td>citric acid (1M)</td>
<td>460 μl 46 ul</td>
</tr>
<tr>
<td>Gibco H₂O</td>
<td>10.7 ml 1.13 ml</td>
</tr>
<tr>
<td>20% Tween-20</td>
<td>250 ul 25 μl</td>
</tr>
</tbody>
</table>
In addition 30 embryos were not used to hybridize probe, but instead incubated in 2 ul of anti-DIG antibody (ROCHE) in 1 ml of blocking buffer (PBS-Tween, 2% bovine serum albumin and 3% normal goat serum) overnight at 4°C. On day 2, the embryos hybridized with probe were then washed in a graded series of 75%, 50%, 25% hybridization buffer/2xSSC (SSC- 3M NaCl, 0.3M Na3 Citrate . 2 H2O pH to 7.0 with 1M HCl), 2x SSC for 15 minutes at 68°C, 0.2x SSC for 30 minutes at 68°C, followed by a graded series of .02x SSC/PBS-Tween (25%, 50%, 75% 0.2x PBS-Tween) for 5 minutes at room temperature. The embryos were then incubated in blocking buffer (2mg/ml BSA, 2% normal goat serum and PBT) for 1.5 hours at room temperature. The anti-DIG antibody/blocking buffer solution incubated the previous night was diluted 1:5000 with blocking buffer. Embryos were incubated overnight at 4°C for at least 15 hours. On day 3, the embryos were washed with PBS-Tween six times for 15 minutes at room temperature. The alkaline phosphate substrates were diluted in AP buffer (100mM Tris pH 9.5, 50mM MgCl2, 100 mM NaCl, 0.1% Tween-20, and ultraPURE Distilled Water Gibco): 5 ml of AP buffer, 22.5 µl of NBT and 17.5 µl of BCIP. After adding NBT-BCIP (Promega), a color change occurred at the location where the mRNA matching the anti-sense probe is expressed, leading to a visual pattern of dark purple expression in the embryos. The developing stain was changed every 6-8 hours for up to 3 days. The reaction was halted with PBS-Tween and the embryos were post-fixed in 4% paraformaldehyde overnight at 4°C. To mount, the embryos were washed in 1:1 mixture of 100% glycerol: 2X PBS for 30 min at room temperature and then transferred to 100% glycerol for 30 min at room temperature. The embryos were placed in a drop of 100% glycerol on hanging drop slides (Fisher) and photographs were taken on a Zeiss
Axioscope Widefield LM in the Ohio State University Campus Microscopy and Imaging Facility.

2.2.16 Double Labeling In situ hybridization with Whole Embryos

After the single probe in situ hybridizations were completed, double labeling in situ hybridization was performed on whole embryo. Co-localization of our nAChR subunits (digoxigenin labeled nAChR RNA stained purple) and several brain region specific markers (fluorescein labeled markers stained orange) were easily identifiable by light microscopy. For our brain region specific markers, we used fluorescein conjugated distal-less homeobox 2 gene (dlx-2) to highlight forebrain and migrating neural crest cells in the pharyngeal arches (Akimenko et al., 1994), tyrosine hydroxylase (TH) to label catecholamnergic nuclei in the midbrain and diencephalon (Holzschuh, et al, 2001)), or the krox 20 gene to hindbrain rhombomeres 3 and 5 (Oxtoby and Jowett, 1993). Day 1-Day 3 of the single labeling in situ hybridization protocol was followed using NBT/BCIP to have dark purple expression of nAChR RNA and followed by a post fix with 4% paraformaldehyde over night at 4°C. Day 4 of the protocol began with a quick wash with 1 X MABT (100mM maleic acid, 150mM NaCl, 0.1% Tween, pH 7.5 with NaOH) at room temperature, followed by 2X washes with MABT for 20 min at room temperature, and then any residual DIG antibody was inactivated by heating the embryos at 68 °C for 10 min with 1 X MABT/10mM EDTA. Next, the embryos were rehydrated in a graded series of MetOH/MABT: 75% MetOH/ 25% MABT, 50% MetOH/ 50% MABT, 25% MetOH/ 75% MABT for 10 min at room temperature and finally washed in 4X in 100% MABT for 15 min at room temperature. After the washes, the embryos were pre-blocked
in blocking buffer (2 % Blocking Reagent, Roche, with 1X MAB (100mM maleic acid, 150mM NaCl, pH 7.5 with NaOH) and 20 % normal goat serum) for 2.5 hr at room temperature, then anti-fluorescein Fab fragments were diluted 1:5000 in blocking buffer and incubated overnight at 4°C. Day 5 of the *in situ* hybridization protocol involved a quick wash at room temperature with MABT, 6X washes with MABT for 15 min at room temperature, and 3 X washes with AP buffer for 5 min at room temperature. The embryos were stained in developing buffer (AP buffer, 10% PVA) with 17.5 µl of INT and 17.5 ul of BCIP. The developing stain was changed every 4-6 hours to avoid a red sticky precipitate from forming. The embryo stained for 18 hr-3 days, depending on the probe. The reaction was stopped with 3X quick washes of AP buffer, 3X washes with AP for 5 min at room temperature, 2X quick washes with PBT, and finally 3X washes with PBT for 5min at room temperature. The embryos were fixed overnight and prepared to mount similarly to the single labeled *in situ* hybridization protocol.

### 2.2.17 Single Fluorescent Labeling by *In situ* Hybridization with Whole Embryos

The protocol for the single labeled *in situ* hybridization was followed, except that detection of the AP conjugated anti-digoxign antibody was detected with Fast Red (Sigma). Thus, the wash steps on Day 3 differed, in that, there were 4X PBT washes for 30 min at room temperature and 3X washes in 100 mM Tris, pH 8.3 at room temperature for 5 min. Also the Fast Red (0.5 naphthol substrate, 2 mg fast red, 0.4 mg levamisole) was in tablet form and was brought to room temperature from -20 ºC and reconstituted in 1 ml distilled water with 1 Tris tablet .01 M Tris-HCl pH 8.2 (Sigma). The embryos stained for a couple of hours to 2 days depending on the probe used.
2.2.18 Immunohistochemistry in Whole Embryos

We performed fluorescent *in situ* hybridization in combination with immunocytochemistry (Novak and Ribera, 2003). After the Fast Red signal was developed in the nAChR RNA, the embryos were washed once quickly with PBT, washed 4X with PBT for 15 min, and the immunocytochemistry protocol followed immediately. To label Rohon Beard sensory neurons, a blocking solution of 2.5% normal goat serum (NGS) and PBT was added to the embryos for 1.5 hr at room temperature while rotating. The primary antibody Zn12 was diluted 1:100 in the blocking buffer and the embryos incubated over night at 4 °C for more than 15 hr. The next day, the embryos were washed once quickly with PBT and then 4X with PBT for 30 min at room temperature. Following the wash steps, the secondary antibody, Oregon green (Molecular Probes) was diluted 1:200 in blocking buffer and the embryos were incubated over night at 4 °C in the dark. To label motor neurons HBP-GFP transgenic embryos were used. The blocking solution was 10% NGS in PBT, the primary antibody anti-GFP (Molecular Probes) diluted to 1:750 and the secondary 1:500. The next day, for both sets of antibodies, the samples were washed with PBT while being protected from direct light at room temperature 4X for 30 min.

2.3 Histology/Sectioning/Imaging

Frozen sectioning of zebrafish embryos was performed as outlined (Passini, et al, 1997). Embryos were fixed overnight at 4 °C in 4% PFA made in 0.1 M phosphate buffer at pH 7.4 with 5% sucrose. The next day embryos were quickly washed five times
with 1 ml of 0.1 M phosphate buffer at pH 7.4 with 5% sucrose. The embryos were then incubated in a graded series of 0.1 M phosphate buffer to sucrose (5%, 15% and 30% sucrose) washes for 45 minutes at 4°C. The embryos were then placed in fresh 30% sucrose/0.1 M phosphate buffer overnight at 4°C. The next day embryos were brought to room temperature and incubated in 1 part OTC to 2 parts 30% sucrose/0.1 M phosphate buffer for at least 30 minutes with 1.5 hours generating optimal results. The embryos were then transferred to Biopsy Cryomolds (Tissue-Tek) with fresh 30% sucrose/0.1 M phosphate buffer and stored at -80°C overnight and up to 3 months. Embryos were mounted with OTC, allowed to equilibrate to -25°C, and then sectioned on a cryostat at 8-12 microns.

2.4 Cell Culture

2.4.1 ZEM2c Background and Maintainence

Zebrafish cells grow at room temperature, are viable for long periods at confluence, and do not require a CO₂-enriched atmosphere, greatly simplifying culture conditions. The ZEM2S cell line (zebrafish embryo-derived fibroblasts from diploid blastula) was obtained for the ATCC (Manassas, VA) and maintained in a 28.5 °C ambient air incubator without CO₂. ZEM2S embryonic zebrafish cells were maintained in LDF medium which was 50% Leibowitzs L-15, 35% Dulbeccos’s modified Eagle’s media, and 15 % Ham’s F-12. One liter of each medium was prepared separately by dissolving the powder in distilled water and HEPES buffer to a final concentration of 15 mM, pH 7.2, penicillin (100 ug/ml, ampicillin (25 ug/ml), and streptomycin sulfate (200
ug/ml) were added. The LDF medium at 50:35:15 was then supplemented with sodium bicarbonate (.180 g/l) and 10% FBS and filtered. The cells were maintained at 28.5 ºC with no CO₂ in 60mm dishes.

The cells were checked microscopically for infections and confluency, while the general state of the cells was recorded before splitting the cell line population. The medium was removed, the cell layer washed once with fresh media, and then the media removed again. 1.0 ml of the trypsin/EDTA solution was added to the cells, the dish swirled to distribute the solution over all cells, and incubated at room temperature for 10-15 minutes. Once the cells were detached from the substrate the solution was pipetted up and down vigorously with the pipet tip pressed to the bottom of the dish to generate higher shear forces. The cells were generally split 1:2, 1:3, or 1:4 depending on desired use. Therefore, an appropriate volume of LDF media without FBS was added, mixed well, and redistributed to the new plates. The plates were then incubated at 28.5 C for 30 minutes until the cells reattached. After reattachment, 10% FBS (400 ul) was added to the dish and mixed by swirling. Cells were passaged weekly.

2.4.2 Preparation of ZEM2c for nicotine treatment

Depending on confluence of the cells, cells were passaged and distributed onto new dishes. Once cells reached 80% confluence, cholinergic agents were added to the media. Media with treatment was exchanged once a day for up to 3 days of treatment, after which, RNA was isolated using standard Trizol Methods.
CHAPTER 3

Results and Discussion

α4 Nicotinic Acetylcholine Receptors

3.1 α4 Nicotinic Acetylcholine Receptors Background

The α4 nAChR is the most highly expressed α nAChR subunit in the brain. The chromosomal expression of the human α4, is located on chromosome 20. Studies indicate that the protein encoded by this gene is a heteromeric membrane receptor subunit that most commonly interacts with either a β2 nAChR or a β4 nAChR subunits to form a functional receptor. The subunits of the neuronal nAChR are localized in an extremely diverse number of places throughout the nervous system. The distribution of specific nAChRs can be detected and visualized using in situ hybridization and the binding of high affinity ligands. Radioligand binding, using L-[3H] nicotine, in mice show that the highest density of nicotine binding was in the interpeduncular nucleus, the medial habenula, many thalamic nuclei, ventral geniculate nuclei, and optic tract nucleus. Moderate binding is seen in the parietal cortex, cingulate cortex, substantia nigra, superior colliculus, medial geniculate nucleus, optic nerve dorsal raphe, and the laterodorsal tegmental nucleus. Finally, low levels of nicotine binding include the medial septum, nucleus accumbens, caudate putamen, hippocampus, and olfactory tubercle. Virtually no binding was found in the cerebellum. There appears to be very similar, though not exact, correspondence between the distribution of L-[3H] nicotine binding and
α4 mRNA expression in the CNS (Wada et al., 1989; Marks et al., 1992). In *Macaca mulatta* brain (Han, et al, 2000), both α4 and β2 mRNA signals are widely distributed in the brain, being stronger in the thalamus and in the dopaminergic cells of the mesencephalon. Most brain nuclei displayed both α4 and β2 signals with the exception of some basal ganglia regions and the reticular thalamic nucleus which are devoid of α4 signal. The distribution α4 and β2 subunit mRNAs in the monkey is substantially similar to that observed in rodent brain. Functionally, mutations in the α4 gene appear to account for a small proportion of the cases of nocturnal frontal lobe epilepsy.

### 3.2 Zebrafish α4 nAChR Cloning

The *chrna4* cDNA (2264 bp) encoded a 627 amino acid protein and included a 192 bp 5' untranslated region and a 191 bp 3' untranslated region. The 3' end of the zebrafish *chrna4* cDNA did not have a consensus polyadenylation sequence and possibly did not represent the actual 3' end of the native *chrna4* RNA. However, a complete coding region was present. Genomic analysis using the zebrafish genomic assembly version 7 (www.sanger.ac.uk) indicated that there were two tightly linked copies of *chrna4* on chromosome 11. However, the coding regions of these copies are 99-100% identical and the sequences 2000 bp upstream of the first exon were 96% identical, with the differences being some small deletions or insertions. Due to the high sequence identity it is possible that only one copy exists and that the duplicate is due to an error in the genomic assembly. The final resolution will be forthcoming upon completion of the zebrafish genomic sequencing. It should be noted that only one nAChR α4 subunit gene has been identified in another teleost, the pufferfish (*Fugu rubripes*) (Jones et al., 2003).
The zebrafish \textit{chrna4} DNA and translated protein sequences were used for BLAST searches of Genbank. The \textit{chrna4} protein was most similar to the chick nAChR $\alpha 4$ subunit with 63% identity. The zebrafish \textit{chrna4} protein sequence also had significant levels of protein sequence homology (56-63% identity) with chick, human, mouse, rat and \textit{Fugu} $\alpha 4$ nAChRs (Figure 11 A,B,C). High DNA sequence identity with other \textit{\alpha 4} nAChR subunits was also present. Interestingly, the percent of DNA identity was higher than the percent protein identity for many of the $\alpha 4$ subunits. The levels of sequence similarity were comparable to the similarities of previously cloned zebrafish nAChRs and had 56% identity to the previously cloned zebrafish \textit{chrna2} (Zirger et al, 2003). Sequence similarities with other nAChRs were maintained throughout the sequence with the exception of the cytoplasmic loop (Figure 11 A). The \textit{chrna4} cDNA also had sequence features characteristic of all nAChR subunits including cysteines at amino acids 155 and 169 as well as vicinal cysteines at amino acids 219-220 typical of nAChR alpha subunits. The \textit{chrna4} sequence was submitted to GenBank (accession number DQ822508).
Figure 11. ClustalW2 Alignments of *chrna4* and cDNAs sequence alignments to nAChRs.

A: The Clustal W2 alignment program was used on the EMBL-EBI site (http://www.ebi.ac.uk/Tools/clustalw2/). "*" identical residues in all sequences, " : " conserved substitutions, and " . " semiconserved substitutions. Zebrafish subunit transmembrane residues are in red and conserved cysteines are in green. B: Phylogenetic analysis of *chrna4*. Each tree search was conducted for 100 replicates in the RAXML web server (Stamatakis et al.,2008) using tree building followed by branch swapping replicates under the JTT model of amino acid substitution.
<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>MANSGPAPFPLLILLPLLLLGTGTLLPASSHIETRAAERRLLKRLFSGYNKMSRVPVN</td>
<td>60</td>
</tr>
<tr>
<td>Mouse</td>
<td>MEIGSGAPPFPLLILLPLLLLGTGTLLPASSHIETRAAERRLLKRLFSGYNKMSRVPV</td>
<td>60</td>
</tr>
<tr>
<td>Human</td>
<td>MELEGPGAPR---LPPFKLPTTTLGLGADVHTEATRAAERRLLKRLFSGYNKMSRVPV</td>
<td>58</td>
</tr>
<tr>
<td>Chick</td>
<td>MGFVLS--------KGNLLLNCASIPFGVHETRAAERRLLKRLFSGYNKMSRVPV</td>
<td>53</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>------------------KRAAWPVFELLCVHLQLAYFSGMPAHAAERRLLQSLFINYKLSRVPV</td>
<td>52</td>
</tr>
<tr>
<td>Fugu</td>
<td>Fugu-------------FSQVAFRAAERRLLQQLFPAHYNKLSRFVENT</td>
<td>52</td>
</tr>
<tr>
<td>Rat</td>
<td>SDVVLVRFGLSIALQTVDEKNNMTTNNVQWYKLDQPDGNYTVTSRIPSEELI</td>
<td>120</td>
</tr>
<tr>
<td>Mouse</td>
<td>SDVVLVRFGLSIALQTVDEKNNMTTNNVQWYKLDQPDGNYTVTSRIPSEELI</td>
<td>120</td>
</tr>
<tr>
<td>Human</td>
<td>SDVVLVRFGLSIALQTVDEKNNMTTNNVQWYKLDQPDGNYTVTSRIPSEELI</td>
<td>118</td>
</tr>
<tr>
<td>Chick</td>
<td>SDVVLVNLGSIALQTVDEKNNMTTNNVQWYKLDQPDGNYTVTSRIPSEELI</td>
<td>113</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>TDTVLVHFGLSIALQTVDEKNNMTTNNVQWYKLDQPDGNYTVTSRIPSEELI</td>
<td>112</td>
</tr>
<tr>
<td>Fugu</td>
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<td>180</td>
</tr>
<tr>
<td>Mouse</td>
<td>WRPAVLYNNAEGDFAVTHLTKAHLYDGRVQWTPPAIYKCESCIDVTFFFDQQCNMK</td>
<td>180</td>
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3.3 Zebrafish α4 nAChR Temporal Expression by PCR

RT-PCR was used with subunit-specific primers to determine the time-course of chrna4 RNA expression during development (Figure 12). Equal amounts of cDNA from each stage were used for the RT-PCR. β-actin transcripts were also co-amplified to allow semi-quantitative comparisons and to ensure that the RNA was intact at all stages examined. The chrna4 RNA was first expressed at 3 hpf in blastula staged embryos and was transiently expressed at all stages (except 16 hpf) examined. 2.5 hpf is approximately when zygotic transcription begins during the midblastula transition (Kimmel et al., 1995). However, chrna4 expression was not present in maternal RNA (data not shown). The low expression of chrna4 RNA at 16 hpf was observed several times and the coamplification of zebrafish β-actin indicated that the cDNA derived from 16 hpf RNA was intact.
RT-PCR was used to determine when chrna4 nAChR subunit RNAs were expressed in embryos and larvae. 3 μgs of RNA from each age zebrafish (h=hpf, d=dpf) were reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed using zebrafish RNA without reverse transcription (-r) or without cDNA (-d). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker.

### 3.4 Zebrafish α4 nAChR Expression by In Situ Hybridization

To reveal the temporal and spatial expression pattern of chrna4 during embryogenesis, I performed whole-mount RNA *in situ* hybridization at 24, 48, 72, and 96 hpf. *krox20*, also known as *erg2*, was used as a hindbrain marker of rhombomeres 3 and 5 (Oxtoby and Jowett, 1993) at 24hpf. *dlx2* was used for a marker at 24 and 48 hpf and is expressed in multiple domains including telencephalon, diencephalon, and the hypothalamus of embryonic forebrain and in cranial neural crest cells at the pharyngeal arches (Akimenko et al., 1994). The zebrafish chrna4 expression pattern was distinct from that of the chrna6 nAChR subunit which is detailed later in the document. In 24 hpf embryos (Figure 13 A-E) chrna4 was detected in a subset of neurons in rhombomeres 4-7 (Figure 13 A-C) and possibly overlaps with *dlx2* expressing cells of cranial neural crest cells migrating posterior to the pharyngeal mandibular arch and along the hyoid arch and brachial arch (Figure 13 D,E) (Albertson et al., 2005). Limited
expression was observed in forebrain and midbrain structures. At 48 hpf significant bilateral expression was seen in both midbrain and hindbrain (Figure 13 F,G) consistent with the nucleus of the medial longitudinal fascicle and reticulospinal neurons (Metcalf et al., 1986), with no expression detected in the spinal cord. At 72 (Figure 13 H,I) and 96 hpf (Figure 13 J,K) *chrna4* continued to be highly expressed in specific midbrain and hindbrain areas. No brain region-specific probes were used at these stages due to the obvious widespread expression in the midbrain.

The zebrafish *chrna4* RNA expression patterns differ from that characterized in other species. It is possible that multiple *chrna4* nAChR genes are expressed in zebrafish and that we cloned a variant with a more restricted pattern of expression. As noted above, the two apparent copies of *chrna4* contain identical coding regions and thus our probe should have detected RNA transcribed from either gene. We previously demonstrated the presence of high affinity epibatidine binding sites in 2 day embryos (Zirger et al., 2003) consistent with the presence of α4 containing receptors and *chrna4* RNA in 48 hpf embryos. Interestingly we detected no *chrna4* RNA in the zebrafish retina, although α4 nAChRs are expressed as early as E7 in chick retina (Gotti and Clementi, 2004) and α4 RNA was present in E15 rat retina (Hoover and Goldman, 1992; Zoli et al, 1995). Zebrafish *chrna4* RNA was not detected in the optic tectum, in contrast to chick optic tectum which expresses α4β2 nAChRs (Gotti and Clementi, 2004). In addition zebrafish *chrna4* was also not highly observed in the forebrain, in contrast to chick forebrain which contains α4α5β2 and α4β2 subtypes (Conroy and Berg, 1998).
Figure 13: Whole-mount in situ hybridization analysis of chrna4 RNA expression in 24hpf-96hpf zebrafish embryos

The purple stain represents chrna4 nAChR subunit mRNA in all panels and orange labeling denotes either krox20 RNA in rhombomeres 3 and 5 or dlx2 RNA in telencephalon, diencephalon, and the pharyngeal arches. All images are lateral views with the anterior to the left unless otherwise noted. Arrows point to specific brain regions and arrowheads point to spinal neurons. **A:** 24 hpf, chrna4 expression was localized to a limited area of forebrain, a subset of cells along the yolk sac consistent with neural crest cells, and in a subset of hindbrain neurons with expression beginning in rhombomere 4. **B:** 24 hpf, magnification of A, expression in forebrain, hindbrain neurons, and in areas consistent with neural crest. **C:** 24 hpf, dorsal view whole embryo, neural crest expression at the level of, but outside of the rhombomeres with expression also evident in spinal neurons. **D:** 24 hpf, chrna4 localization in neural crest cells in the dlx2 expressing domain of the mandibular (1), hyoid (2), and brachial (3) pharyngeal arches. **E:** 24 hpf, higher magnification of D, chrna4 expression was evident in the dlx2 expression domain of the hyoid (2) pharyngeal arch and brachial (3) arch. **F:** 48hpf, dorsal view, localization consistent with the nucleus of the medial longitudinal fascicle and reticulospinal neurons of the hindbrain. **G:** 48 hpf, chrna4 expression was not present in the telencephalon or hypothalamus as evident by using dlx2 expression to localize these structures. There was midbrain and hindbrain expression consistent with the localization pattern of the nucleus of the medial longitudinal fascicle and reticulospinal neurons. **H:** 72 hpf, dorsal view & **I:** 72 hpf, chrna4 expression was extensive in midbrain and limited in hindbrain. **J:** 96 hpf, dorsal view & **K:** 96 hpf, chrna4 expression was extensive in midbrain and limited in hindbrain similar to the pattern that seen in 72 hpf embryos (I).

Anatomical abbreviations: diencephalon (dien), forebrain (fb), hindbrain (hb), hindbrain neurons (hn), midbrain (mb), midbrain hindbrain boundary (mhb), neural crest (nc), otic vesicle (otic), pharyngeal arches- mandibular (1), hyoid (2), and brachial arch (3), nucleus of the medial longitudinal fascicle (nmlf), reticulospinal neurons (rs), rhombomeres 3 (r3) and 5 (r5), telencephalon (te).
Figure 13: Whole-mount *in situ* hybridization analysis of *chrna4* RNA expression in 24hpf-96hpf zebrafish embryos
CHAPTER 4

Results and Discussion

α6 Nicotinic Acetylcholine Receptors

4.1 α6 Nicotinic Acetylcholine Receptors Background

Genomic analysis indicates that the α6 nAChR gene is composed of 6 exons and
is mapped to chromosome 8 (Ebihara, et al, 2002) where it is tightly linked to the β3
nAChR in humans. Studies indicate that the receptor composition is heteromeric most
commonly composed of α6β3 and α6β2, but can also be much more complicated in
trimeric and tetrameric combinations α6β3∗ (α6β2β3 & α6α4β2β3). The presence of
distinct nicotinic acetylcholine receptor (nAChR) subtypes in specific central nervous
system (CNS) areas offers the possibility of developing targeted therapies for diseases
involving the affected brain region. Parkinson's disease is a neurodegenerative movement
disorder characterized by a progressive degeneration of the nigrostriatal system. α6-
containing nAChRs have a relatively selective localization to the nigrostriatal pathway
and a more limited number of other CNS regions. The α6 subunit is specifically and
highly expressed in catecholaminergic neurons and retina (Le Novere, et, 1996).
Specifically, the α6 subunit of the nAChR is expressed at very high levels in
dopaminergic (DA) neurons. However, α6 null mutant mice, there are no developmental
malformations, most notably in the dopaminergic areas and visual system (Champtiaux,
et al, 2002).
Although nAChRs are expressed in many CNS regions, nicotine’s major addictive effects are thought to be mediated through the dopaminergic mesocorticolimbic pathways. Dopaminergic neurons originating in the VTA project to nucleus accumbens and prefrontal cortex. Three cell types in the VTA (dopaminergic neurons, GABAergic neurons, and glutamatergic axonal terminals from the prefrontal cortex) all contain nicotinic receptors (Mansvelder and McGehee, 2000; Mansvelder et al., 2002) implicating a modular role for all of the transmitter systems.

4.2 Zebrafish α6 nAChR Cloning

The chrna6 cDNA (1940 bp) encoded a 512 amino acid protein and included a 161 bp 5' untranslated region and a 243 bp 3' untranslated region. The 3' end of the zebrafish cDNA did not have a consensus polyadenylation sequence and possibly did not represent the actual 3' end of the native chrna6 RNA. However, a complete coding region was present and a consensus polyadenylation sequence (AATAAA) was present in the genomic sequence approximately 270 base pairs from the 3' end of the chrna6 cDNA (R.T.Boyd, data not shown). Genomic analysis using the zebrafish genomic assembly version 7 (www.sanger.ac.uk) indicates that there is one copy of the chrna6 on chromosome 1.

The zebrafish chrna6 DNA and translated protein sequences were used for BLAST searches of Genbank. The chrna6 protein was most similar to a nAChR designated α3 from goldfish (Hieber et al., 1990) with a 95% identity at the protein level. Despite the homology to the goldfish nAChR α3 subunit, I have designated the zebrafish cDNA chrna6 because of several observations (Figure 14 A and B). First, another
zebrafish nAChR cDNA with closer sequence identity to nAChR α3 subunits from other species has been cloned that we have designated chrna3 (Figure 14 A and B). Besides the goldfish α3, the zebrafish chrna6 cDNA has a highest homology to the Fugu rubripes nAChR α6a subunit (Figure 14) than to any other nAChR subunits. In addition, and similar to other species, zebrafish chrna6 is closely linked to zebrafish chrnb3 (Zirger et al., 2003; www.sanger.ac.uk). This linkage of the zebrafish chrna6 and chrnb3 nAChR genes and the highest sequence identify to the Fugu α6a supports our conclusion that a zebrafish nAChR α6 subunit orthologue was cloned. The zebrafish chrna6 protein sequence also had significant levels of protein homology (64-81% identity) with chick, human, mouse, rat, and Fugu nAChRs. The high sequence identity was also maintained at the DNA level. The levels of sequence similarity were comparable to the similarities between previously cloned zebrafish nAChRs and nAChRs from other species (Zirger et al, 2003). The homology to the other nAChRs was high throughout the sequence, especially in the 4 TM regions, including the comparison with Fugu α6 cDNA (Figure 14 A), but not in the cytoplasmic loop. The high degree of DNA sequence identity of the zebrafish chrna6 cDNA with the goldfish α3 subunit cDNA sequence was maintained throughout the cytoplasmic loop between transmembrane regions 3 and 4 (TM3-TM4), even though this sequence is known to be most variable between nAChR subunits (Figure 14 A). Analysis of chrna6 and the comparison to the goldfish α3 lead to the proposal that the previously cloned goldfish α3 was actually an α6 nAChR orthologue. The chrna6 cDNA also had sequence features characteristic of all nAChR subunits including cysteines at amino acids 151 and 165 as well as vicinal cysteines at amino acids 215-216
typical of nAChR alpha subunits. The *chrna6* sequence was submitted to GenBank (accession # DQ822507).
Figure 14. ClustalW2 Alignments of *chrna6* and cDNAs sequence alignments to nAChR

A: The Clustal W2 alignment program was used on the EMBL-EBI site (http://www.ebi.ac.uk/Tools/clustalw2/). "*" identical residues in all sequences, " : " conserved substitutions, and " . " semiconserved substitutions. Zebrafish subunit transmembrane residues are in red and conserved cysteines are in green. B: Phylogenetic analysis of *chrna4*. Each tree search was conducted for 100 replicates in the RAXML web server (Stamatakis et al., 2008) using tree building followed by branch swapping replicates under the JTT model of amino acid substitution.
Figure 14 Continued

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Figure 14: Continued

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Fugub           TYDKAKIDLVLIGSKVNLKDFWESGEWEIIDAPGYKHDIKYNCCEEIYPDITYSFYIRRL 232
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Fugua           QSQQ--GAMNSLEFGEGK-AALE---------GKKGGCPCHPLKEATEG----------- 422
Fugub           QSQQ--GAMNSLEFGEGK-AALE---------GKKGGCPCHPLKEATEG----------- 422
Humana3         --S-----------------------NEGNAQKPRPLYGAELSN------------------ 379
Rata3           --S-----------------------NEGNAQKPRPLYGAELSN------------------ 379
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Chicka6         --S-----------------------NEGNAQKPRPLYGAELSN------------------ 379
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Fugub           GSKQ--GAMSLEFGEGK-AALE-------------------GKKGGPCPSPLKEATEG-------- 422
Humana3         --S-----------------------NEGNAQKPRPLYGAELSN------------------ 379
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Figure 14 continued

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3 Zebrafish α6 nAChR Temporal Expression by PCR

Reverse transcription polymerase chain reaction (RT-PCR) was used with subunit-specific primers to determine the time-course of chrna6 RNA expression during development (Figure 15). Equal amounts of cDNA from each stage were used for the RT-PCR. β-actin transcripts were also co-amplified to allow semi-quantitative comparisons and to ensure that the RNA was intact at all stages examined. The chrna6 RNA was first detected at 10 hpf, the onset of neurogenesis and somitogenesis. Low chrna6 RNA expression was apparent until 24 hpf with an increase until 60 hpf. The chrna6 RNA expression was maintained at 7 days (168 hpf). chrna6 RNA was not detected in embryos younger than 10 hpf including maternal RNA after 40 cycles of PCR (data not shown).
RT-PCR was used to determine when chRNA4 nAChR subunit RNAs were expressed in embryos and larvae. 3 µgs of RNA from each age zebrafish (h=hpf, d=dpf) were reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed using zebrafish RNA without reverse transcription (-r) or without cDNA (-d). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker.

**4.4 Zebrafish α6 nAChR Expression by In Situ Hybridization**

To reveal the temporal and spatial expression pattern of chRNA6 during embryogenesis, we performed whole-mount RNA in situ hybridization at 24, 48, 72, and 96 hpf. We used krox20, also known as erg2, as a hindbrain marker of rhombomeres 3 and 5 (Oxtoby and Jowett, 1993) at 24hpf. dlx2 was used for a marker at 24 and 48 hpf and is expressed in multiple domains including telencephalon, diencephalon, and the hypothalamus of embryonic forebrain and in cranial neural crest cells at the pharyngeal arches (Akimenko et al., 1994). A probe for tyrosine hydroxylase (TH) (Holzschuh et al., 2001) was used to colocalize expression of α6 RNA with catecholaminergic neurons in 48, 72, and 96 hpf embryos.

In 24 hpf embryos, chRNA6 RNA was present in a subset of Rohon Beard neurons as well as spinal cord neurons (Figure 16 A-D). Expression was also observed in ventral
forebrain (Figure 16 B,D), specifically in \textit{dlx2} labeled diencephalon (Figure 16 E),
trigeminal ganglion (Figure 16 C), pineal (Figure 16 B,D,E), and in the first hindbrain
rhombomere (Figure 16 D,E) ventral to the cerebellum. In 48 hpf embryos \textit{chrna6} was
no longer observed in spinal neurons (data not shown), but was still expressed in
trigeminal ganglion (Figure 16 F-I), pineal (Figure 16 F-J), and was now co-localized to
the \textit{TH} + locus coeruleus (Figure 16 H,I) and the \textit{TH}+ diencephalic catecholaminergic
cluster (Holzschuh et al, 2001) (Figure 16 H, J-N). A small amount of \textit{chrna6} expression
was detected in tectum at 48 hpf (Figure 16 H). \textit{chrna6} RNA was also highly expressed
in the 48 hpf retina (Figure 16 F-J), but was not detected in 24 hpf retina.
Figure 16. In situ hybridization analysis of chrna6 RNA expression in 24hpf and 48hpf zebrafish embryos

The purple stain represents chrna6 nAChR subunit mRNA in all panels and the orange labeling is either krox20 in rhombomeres 3 and 5, dlx-2 in telencephalon, diencephalon and pharyngeal arches, or tyrosine hydroxylase (TH) in catecholaminergic neurons. Use of these additional probes is indicated in each panel. All images are lateral views with the anterior to the left unless otherwise noted. Arrows point to specific brain regions and arrows with an asterisk denote co-labeling with TH.  

A: In 24 hpf embryos, the chrna6 transcript was seen in a subset of spinal neurons and Rohon Beard sensory neurons in the trunk. 

B: 24hpf, chrna6 expression was present in pineal, ventral forebrain, and Rohon Beard sensory neurons. 

C: 24hpf, dorsal view, chrna6 expression in trigeminal ganglion and Rohon Beard sensory neurons. 

D: 24hpf, chrna6 expression was seen in rhombomere 1 as well as forebrain, pineal, and spinal neurons. The krox 20 probe (orange) labeled rhombomeres 3 and 5. 

E: 24 hpf, chrna6 expression was identified in diencephalon as shown by the co-localization with dlx2, a marker for telencephalon, diencephalon, and hypothalamus. Additionally, chrna6 is labeled in pineal and rhombomere 1. 

F: 48 hpf, dorsal view, chrna6 transcript was heavily expressed in the retina with limited expression in midbrain and continued expression in pineal and trigeminal ganglion. 

G: 48 hpf, dorsal view, expression in midbrain is not co-localized with dlx2 and is dorsal to the dlx2 expression domain (orange labeling) in telencephalon, diencephalon, and hypothalamus. Additionally, expression in retina and trigeminal ganglion was present. 

H: 48 hpf, chrna6 was expressed in tectum, retina, pineal, and trigeminal ganglion. The diencephalic catecholaminergic cluster and locus coeruleus are detected by the TH probe (orange). As heavy retinal expression is evident in H that impedes with visualizing midbrain, I-N are 8-12 micron sagittal sections though the diencephalic regions marked by a line in H and L. 

I: 48 hpf, chrna6 expression was clear in the pineal, retinal ganglion cells, trigeminal ganglion, and co-localization with TH in the locus coeruleus was evident. The insert is a magnification of locus coeruleus. 

J: 48 hpf, indicates chrna6 expression in the most lateral regions of the diencephalic catecholaminergic cluster co-localizing with TH. Additionally, chrna6 expression in the pineal and retinal ganglion cells was present. 

K: 48 hpf, higher magnification of K, co-localization of TH and chrna6 transcript in the lateral most region of the diencephalic catecholaminergic cluster in the midbrain. 

L: 48 hpf, a midsagittal section through the diencephalic catecholaminergic cluster indicates chrna6 co-localization with TH. 

M,N: 48 hpf, medial sagittal sections, indicate areas of co-localization with TH and areas solely expressing chrna6 RNA.

Anatomical abbreviations: diencephalon (dien), diencephalic catecholaminergic cluster (dcc), forebrain (fb), hypothalamus (hypo), lateral (lat), locus coeruleus (le), mandibular pharyngeal arch (1), midbrain (mb), olfactory cluster in olfactory bulbs (obc) pineal (pin), retinal ganglion cells (rgc), rhombomere 1 (r1), Rohon Beard sensory neurons (rb), spinal neurons (sn), tectum (tec), and trigeminal ganglion (tg).
Figure 16: *In situ* hybridization analysis of *chrna6* RNA expression in 24hpf and 48hpf zebrafish embryos
Zebrafish *chrna6* continued to be expressed in trigeminal ganglion (Figure 17 A,D,E,F,H,J,L), retina (Figure 17 A,D,F,H,J), and pineal (Figure 17 A,B,F,H,I,J) at 72 and 96 hpf. *chrna6* RNA was also expressed in tectum (Figure 17, A, B, H,I) in 72 and 96 hpf zebrafish, at robust levels with more widespread distribution than at 48 hpf. At 96 hpf *chrna6* expression was detected for the first time with a pattern consistent with cranial sensory neurons (Figure 17 L) in the hindbrain. At 72 hpf and 96 hpf *chrna6* RNA continued to be expressed in the diencephalic catecholaminergic cluster (Figure 17 A-H,J,K), but was also present in non-catecholaminergic cells in both midbrain and hindbrain (Figure 17 B-E,G,H,K).
Figure 17: In situ hybridization analysis of chrna6 RNA expression in 72 hpf and 96 hpf zebrafish embryos.

The purple stain represents chrna6 nAChR subunit mRNA in all panels and the orange labeling is tyrosine hydroxylase (TH) mRNA. All images are lateral views with the anterior to the left unless otherwise noted. Arrows point to specific brain regions, arrowheads point to amacrine cells in the retina, and arrows with an asterisk denote co-labeling with TH. Line denotes dcc in A-G. A: 72 hpf, chrna6 was now localized heavily to the eye and tectum, pineal, trigeminal ganglion, and co-localization with TH in the diencephalic catecholaminergic cluster and pre-tectal catecholaminergic cluster. B: 72 hpf, midsagittal section, shows chrna6 expression in pineal, tectum, and co-localization with TH in a subset of cells in the diencephalic catecholaminergic cluster, and no expression in the olfactory bulb catecholaminergic cluster. C: 72 hpf, midsagittal section, higher magnification of B, co-localization of chrna6 with TH in the diencephalic catecholaminergic cluster. D: 72 hpf, sagittal section, chrna6 expression in amacrine cells of the retina, retinal ganglion cells, trigeminal ganglion, and co-localization with TH in a subset of cells in the diencephalic catecholaminergic cluster. E: 72 hpf, sagittal section higher magnification of D, co-localization of chrna6 with TH in the diencephalic catecholaminergic cluster and expression in trigeminal ganglion. F: 72 hpf, dorsal view of whole animal, showed chrna6 expression in retina, trigeminal ganglion, pineal, and co-localization with TH in the diencephalic catecholaminergic cluster and locus coeruleus. G: 72 hpf, longitudinal section, showed chrna6 expression in a subset of TH+ cells in the diencephalic catecholaminergic cluster. H: 96 hpf, expression was evident in retina, pineal, tectum, trigeminal ganglion, a subset of cells in the hindbrain consistent with visceral sensory neurons, and co-localization with TH in the diencephalic catecholaminergic cluster, locus coeruleus, and pretectal catecholaminergic cluster. I: 96 hpf, longitudinal section, demonstrates co-localization of TH and chrna6 expression in the pretectal area, with pineal and tectum labeled. J: 96 hpf, dorsal view whole mount, chrna6 expression shown in retina, pineal, trigeminal ganglion, diencephalic catecholaminergic cluster, and locus coeruleus. K: 96 hpf, dorsal view whole embryo, chrna6 co-localization with TH in the diencephalic catecholaminergic cluster. L: 96 hpf, dorsal view whole embryo, chrna6 expression in hindbrain neurons consistent with the localization of visceral sensory neurons.

Anatomical abbreviations: amacrine cells in the retina (ac), diencephalic catecholaminergic cluster (dcc), hindbrain nuclei (hb), locus coeruleus (lc), olfactory bulb catecholaminergic cluster (obc), pretectal catecholaminergic cluster (ptc), pineal (pin), tectum (tec), trigeminal ganglion (tg).
Figure 17: In situ hybridization analysis of *chrna6* RNA expression in 72 hpf and 96 hpf zebrafish embryos
The localization of chrna6 in catecholaminergic neurons advances the use of zebrafish as a model to study the role of nAChRs in dopaminergic transmission. chrna6 expression in several catecholaminergic regions of the zebrafish nervous system is consistent with studies in mammals where α6 nAChRs have been localized to locus coeruleus, mesostriatal dopaminergic neurons, substantia nigra and VTA (Champtiaux et al., 2003; Gotti and Clementi, 2004). α6 and β3 expression colocalizes to retina and dopaminergic neurons (SN, VTA) in several species (Gotti and Clementi, 2004). This study in conjunction with our previous work (Zirger et al., 2003) indicates that chrna6 and chrnb3 RNA are also colocalized in zebrafish retinal ganglion. Zebrafish also express chrna6 RNA in trigeminal ganglion consistent with the expression pattern in adult rats. Zebrafish lack chrna6 expression in the olfactory bulb of 48, 72, and 96 hpf embryos, although the olfactory bulb catecholaminergic cluster (Holzschuh et al., 2001) was clearly detected by the TH probe in 72 and 96 hpf embryos. This is also consistent with rats in that only 17% of rats express α6 in adult olfactory bulbs (Liu et al., 1998; Keiger and Walker, 1999). Zebrafish chrna6 expression occurs in retina, consistent with α6 expression after embryonic day 14 (E14) in chick retina. However, while chrna6 RNA was detected in developing optic tectum in zebrafish, α6 was not detected in chick optic tectum (Gotti and Clementi, 2004).
CHAPTER 5

Results, Discussion, and Proposed Future Experiments

\( \alpha_2 \) Nicotinic Acetylcholine Receptors

5.1 \( \alpha_2 \) Nicotinic Acetylcholine Receptors Background

The \( \alpha_2 \) nAChR was one of the first subunits to be cloned; yet, to date there is relatively little known about the properties of the subunit. The chromosomal expression of the human \( \alpha_2 \), along with the \( \beta_4 \) gene, is tightly linked to chromosome 8 (Anand and Lindstrom, 1992). Studies in cell culture and expression studies in Xenopus oocytes, indicate that \( \alpha_2 \) partners with \( \beta_2 \) (Wada, et al, 1988, Deneris, et al, 1989) or \( \beta_4 \) (Cachelin and Rust, 1994) nAChR subunits to form functional receptors. There is strong distribution of \( \alpha_2 \) mRNA in developing rodent which includes olfactory bulb, interpeduncular nuclei of midbrain, tegmental nuclei, raphe nucleus, medullary reticular nuclei, and spinal cord. Meanwhile, moderate expression in rodent is limited to cortex, subiculum, hippocampus CA1, medial septum substantia innominata, and amygdala (Ishii, et al, 2005). In monkey, \textit{Macaca mulatta}, the \( \alpha_2 \) subunit is very highly expressed throughout the brain with levels similar to \( \alpha_4 \) and \( \beta_2 \). Site of expression in the monkey correlates with the rodent data, but is more extensive: cortex, subcortical forebrain, hippocampus, thalamus, hypothalamus, pineal, substantia niagra, ralphe nucleus, etc. However, numerous studies investigating the developmental expression of nAChRs and subunit mRNAs have shown that the expression is often developmentally regulated. In
chick developmental expression is limited to the lateral spiriform nucleus (Daubas, 1990), and the habenula (Brussaard, et al 1994). Additionally, postnatal developmental expression of α2 mRNA in rat cortex and hippocampus was first detected at P3 in cortex and hippocampus (Son and Winzer-Serhan, 2006). During postnatal development the distribution of α2 subunit mRNA expression was spatially similar to the one found in adult, exhibiting highly restricted expression in scattered cells mostly in cortex and stratum. However, by P7 the expression intensity and number of α2 positive cells reached peak levels in both cortex and hippocampus and then decreased to moderate to low to levels. Thus, similar to other nAChR subunits, α2 mRNA expression is transiently upregulated during postnatal development. To date no developmental functions have been assigned to the α2 subunit, as gene knockout studies have not been performed.

But, a clinical and molecular genetic study of sleep-related epilepsies identified a new genetic locus and sequencing of the candidate gene indicated a missense mutation in the neuronal cholinergic receptor alpha2 subunit gene (CHRNA2). CHRNA2 is the third neuronal cholinergic receptor gene (chrna4 and chrnb2) to be associated with familial sleep-related epilepsies (Aridon P, et al, 2006).

5.2 Zebrafish α2 nAChR Expression

The zebrafish α2 nAChR subunit ortholog was first cloned in 2003 (Zirger, et al) and encoded a full length cDNA of 2365 bp with a predicted protein size of 520 amino acids. The zebrafish chrna2 cDNA showed highest sequence homology with mouse, rat, and chick α2. The chrna2 RNA was first detected by RT-PCR at 2.5 hpf, around the time period when zygotic transcription begins (Kimmel, 1995), and continued heavily thru 48 hpf. In situ hybridization localization of the RNA between 18-20 hpf indicated that
expression was very low, but at 24 hpf a distinct pattern appeared. *chrna*2 was detected in the first 7 anterior segments of the spine, consistent with interneurons, that continued to 72 hpf. *chrna*2 was also detected in the olfactory bulb/forebrain until 60 hpf. At 60 hpf, the forebrain expression diminished with an increase in midbrain expression (not included in manuscript) that was still apparent at 72 hpf.

Because *chrna*2 RNA was expressed at 2.5 hpf, the midblastula transition, the mRNA could have been transcribed by the embryo or have been provided by the mother as a set of maternal RNAs. I isolated unfertilized embryos by squeezing methods, performed RT-PCR, and found that *chrna*2 RNA was supplied as a maternal RNA (Figure 18) deposited in the egg and then continued to be expressed zygotically during early embryogenesis. (data not shown). To control against contamination, a sample without DNA (-dna), a sample without reverse transcriptase enzyme (-rt), and the housekeeping gene 18s to ensure that the RNA was intact was included. Secondly, I also used RNA extracted from the unfertilized embryos and attempted to amplify the nAChR *chrna*7 gene, which was previously shown to be first expressed at 8 hpf (Zirger, et al 2003). The gene was not amplified as predicted.
In zebrafish molecular and genetic analysis has shown that maternal mRNAs play a major role during early development of the embryo (reviewed in Pelegri, 2003). Furthermore, it has been demonstrated that basic developmental processes, like embryonic axes formation and cell fate determination, depend upon localization of maternal transcripts in both the inactive and activated egg. Even after the initiation of zygotic gene expression maternal products continue performing essential functions, either together with other maternal factors or through interactions with newly expressed zygotic products. To our knowledge, the zebrafish α2 nAChR was the first ion channel subunit to be described as being a maternal factor. Following the procedures outlined for the chrna2 RNA maternal amplification, I set out to determine if there was a β-subunit counterpart expressed in unfertilized zebrafish eggs. In addition to chrna2 RNA being expressed maternally, chrnb2 (β2) and chrnb3 (β3) nAChR transcripts were present, but not chrnb4 (β4) (Figure 19).
RT-PCR was used to determine if the α2 subunit RNA also had a β subunit partner that was expressed as a maternal factor. 3 µgs of RNA from unfertilized embryos was reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed using zebrafish RNA amplified with subunit specific primers for α6 nAChR which is first expressed at 10 hpf, without reverse transcription (-rt), without cDNA (-dna), and RNA from 72 hpf embryos, a timepoint when α2, β2, β3 and β4 nAChR RNA are all expressed. The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker.

To identify the role that the chrna2 gene plays during development of the embryonic vertebrate nervous system, I first determined its mRNA expression pattern in the zebrafish embryo. Between 10 and 96 hpf, chrna2 transcripts were detected in several populations in the brain and spinal cord. To extend on the knowledge gained from previous in situ hybridization studies, I repeated the expression studies with 10 hpf, 18 hpf, 24 hpf, 48 hpf, 72 hpf, and 96 hpf embryos. At 10 hpf, around the time of somitogenesis, chrna2 expression was heavy in the anterior head region with some labeling in the dorsal spine. By 18hpf, there was diffuse labeling in the brain, but now a well defined pattern in the anterior spine (Figure 20) was evident. Similar to Zirger, et al,
2003, at 24 hpf there was expression in the forebrain consistent with the olfactory bulb and interneuron labeling in the first 1-7 hemisegments of the spinal cord. In contrast, at 24 hpf there was an additional *chrna2* expression pattern in a neuronal population down the entire length of the spinal cord and also a population of cells in the hindbrain (HB). At 48 hpf (Figure 21), forebrain expression had disappeared, and midbrain (MB) expression was prominent with very limited expression in hindbrain. The distinct pattern in the anterior spine remained intact; meanwhile, the expression had lessened slightly as compared to 24 hpf. By 72 hpf (Figure 22) all labeling in the spinal cord had disappeared, as previously reported in Zirger, et al, but robust expression remained in the midbrain with limited expression in the hindbrain. By 96 hpf (Figure 23) *chrna2* expression was still evident in the midbrain, but reduced as compared to the 72 hpf. Our expression studies closely mimicked those found in other species. Zhang, et al, 1998 described the postnatal changes of α2 nAChR in rat as having multiple developmental expression patterns, including 1) transient expression in some populations with 2) constant expression in other brain regions. In additions there were brain areas with 3) no expression through out development and expression that 4) increased with age, etc. The zebrafish expression pattern highly mimiced these categories of developmental patterns with: 1) constant expression in midbrain areas after 48 hpf, 2) transient expression in the spinal neurons, olfactory area, and hindbrain, 3) no expression in the eye or tectum and 4) a general increase in expression in the brain from embryo to early larvae stage, specifically in the midbrain.
Figure 20: Whole-mount in situ hybridization analysis of *chrna2* RNA expression in 10, 18 and 24 hpf zebrafish embryos. At 10 hpf cells along the dorsal somites were α2 positive. At 18 hpf there was diffuse labeling in the brain with a very distinct population of cells in the spinal cord. At 24 hpf there was limited expression in forebrain, in a cell population spanning the midbrain hindbrain boundary and heavy expression in the anterior spinal cord.

Figure 21: Whole-mount in situ hybridization analysis of *chrna2* RNA expression in 48 hpf zebrafish embryos. There was a reduction in forebrain expression with an upregulation of midbrain expression as evident. The anterior spinal population was maintained.
Figure 22: Whole-mount in situ hybridization analysis of *chrna2* RNA expression in 72 hpf zebrafish embryos. There was a robust increase in midbrain expression with limited expression in the hindbrain and no expression in the spinal cord.

Figure 23: Whole-mount in situ hybridization analysis of *chrna2* RNA expression in 96 hpf zebrafish embryos. Similarly to the 72 hpf expression pattern, α2 was highly expressed in the midbrain with limited expression in the hindbrain and no expression in the spinal cord.
The most interesting *chrna2*nAChR expression pattern was the transient expression of *chrna2* in the spinal cord between 18 hpf and 72 hpf. Double *in situ* hybridization with *chrna2* RNA labeled in purple and *islet 1* RNA, a reliable marker of primary motor neurons before 24 hpf, labeled in orange (Figure 24 A-E) was used for localization. At 24 hpf there was heavy *chrna2* RNA expression dorsal to *Islet 1* expressing motor neurons in the first 7 anterior hemisegments (Figure 24 A) of the spinal cord. Additionally at 24hpf, there was a more posterior *chrna2* expressing cell population localized down the entire ventral spinal cord at the level of *Islet 1* expressing motor neurons (Figure 24 A, B). By 48 hpf both the anterior and posterior cell populations were localized dorsal to all *Islet 1* expressing motor neurons, consistent with an interneuron population (Figure 24 E, F). To elucidate which ventral spinal neuron population were *chrna2* expressing at 24 hpf, I used fluorescent *in situ* hybridization in conjunction with fluorescent immunocytochemistry (Figure 24 E-H). At 24 and 48 hpf *chrna2* expressing cells in wild type embryos were labeled with Fast Red. Rohon Beard sensory neurons, which are the most dorsal cell type in the spinal cord, were labeled with Zn-12 antibody conjugated to Oregon Green. Clearly, there was no co-localization of *chrna2* with Zn-12, indicating that Rohon Beard sensory neurons were not *chrna2* nAChR expressing (Figure 24 C, G). Furthermore, I used the HB9 transgenic zebrafish line that labeled motor neurons at 24 and 48 hpf (Figure 24 D,H). HB9 transgenic fish were first characterized by Flanagan-Street, et al, 2005. HB9 is a transcription factor that is expressed in motoneurons and required for early stages of their development (Jessell, 2000). Primary motor axons are intensely labeled in HB9:GFP fish with caudal primary motor neurons (CaPs) being labeled by 14 hpf, before their axons extended in the
myotomes. Between 14 and 24 hpf, GFP labeled CaP ventral axons are marked at least as effectively with a well-characterized neuron-specific antibody, znp-1. At 24 hpf the three primary motor neuron nuclei and ventral projections (CaP) were clearly labeled in the HB9 transgenic embryos (Figure 24 D). *chrna2* RNA was not expressed in motor neurons, but was expressed at the same level in the spinal cord rostral to motor neurons (Figure 24 D). In conclusion, *chrna2* RNA was localized to two subset populations of interneurons in the spinal cord and was not expressed in Rohon Beard sensory neurons nor primary motor neurons at 24 hpf or 48 hpf. This was the first study in zebrafish to identify a subset of cholinergic interneurons. To date studies have localized many other members of the LGIC family of receptors to spinal interneurons including glutamate, GABA, and glycine.
Figure 24: Expression of chrna2 RNA in spinal interneurons. (A) Double in situ hybridization of 24 hpf embryo with purple chrna2 nAChR RNA labeling in the spine and orange Islet 1 staining in motor nuclei in the brain and in motor neurons of the spine. (B) Magnification of A, 24 hpf embryo with purple chrna2 labeling in the spine and orange Islet 1 in motor neurons of the spine. There were two subset populations of chrna2 expressing cells. One was in the first 7 anterior segments of the spine and dorsal to Islet 1 expressing cells. The other chrna2 population extended down the entire spine at the level of ventral Islet 1 expressing cells. (C) 24 hpf, labeling with chrna2 RNA (red) and Zn12 antibody (green) to label Rohon Beard Sensory neurons. Rohon Beard sensory neurons were not chrna2 expressing. (D) 24 hpf, labeling with chrna2 RNA (red) in a transgenic HB9 embryo in which primary motor neurons are labeled (green). chrna2 RNA was not expressed in motor neurons, but was localized at the same level in the somite and rostral to the motor nuclei. (E) Double in situ hybridization of 48 hpf embryo with purple chrna2 nAChR RNA labeling in the spine and orange Islet 1 staining in motor nuclei in the brain and in motor neurons of the spine. (F) Magnification of E, 48 hpf embryo with purple chrna2 labeling in the spine and orange Islet 1 in motor neurons of the spine. (G) 48 hpf, labeling with chrna2 RNA (red) and Zn12 antibody (green) to label Rohon Beard Sensory neurons. Rohon Beard sensory neurons were not α2 expressing. (H) 48 hpf, labeling with chrna2 RNA (red) in a transgenic HB9 embryo in which primary motor neurons were labeled (green). chrna2 RNA was not expressed in motor neurons.
5.3 α2 nAChR Gene Function

Reverse genetics, the ability to inactivate a given gene in an entire animal, is a popular assay in the zebrafish. Two approaches have been attempted in order to develop somatic gene inactivation in zebrafish embryos. The use of morpholino oligonucleotides (oligos) has consistently phenocopied gene mutations, whereas RNAi has not been as successful. Morpholino oligos, first developed for clinical applications, have been successful in inducing transient gene knock down effects in zebrafish embryos. These 25 bp DNA analogs operate by blocking mRNA translation or splicing. They only operate when complementary to a sequence between the 5’ UTR through the first 25 bases 3’ of the AUG start site or to splice junctions. The principal difference between the two is that AUG morpholinos also affect maternal transcripts, and can therefore create more severe phenotypes. Morpholinos are typically injected into zebrafish embryos at the 1–4 cell stage at a final concentration range of 0.1–1.0 nM. The DNA analogs are immune to DNAse degradation and are thus stable in the embryo for extended periods (up to 5 days or even longer). Furthermore their small size allows for even distribution to all cells in the developing embryo at concentrations sufficient for inhibition. The main disadvantages of morpholinos are the variations in injections and genetic variation between individual zebrafish, both leading to the possibility of inconsistent results, non-specific toxicity, or off target effects. As zebrafish specific nAChR antibodies are needed to detect a decrease in protein levels after a start site morpholino knockdown, splice site directed morpholinos were designed for these studies.
To reveal the function of the chrna2, the the intron-exon (splicing acceptor site)-target was designed to block the splicing of the chrna2 exon 5 and the efficacy of the splice blocking morpholinos was tested by RT-PCR. Chrna2 contains 6 exons (Figure 25). The initial primer set was designed to amplify a full length chrna2 product of approximately 2000 bp. With efficient splicing, a 2000 bp DNA fragment was detected in wild type embryos and control morpholino embryos and a 900 fragment was detected in morphant embryos. Exon 5 was chosen because it contains most of the transmembrane domains of nAChRs and is critical for a function receptor. Direct sequencing of the PCR product confirmed that exon 5 had been deleted (data not included), resulting in a premature STOP codon (results not shown). By contrast, in control-injected embryos, normal splicing occurred. The spliced mRNA was reduced or almost undetectable in morphants.

A cause for concern in the use of morpholinos is the potential for "off target" effects. Up to 18% of morpholinos appear to have non-target related phenotypes including cell death in the central nervous system and somite tissues of zebrafish embryos (Ekker and Larson, 2001). To filter out off-target effects, we sought a control strategy that included the used of a standard control, RNA rescue experiments, and the co-injection of p53 morpholino. The co-injection of a standard control morpholino (5’-3’: CCTCTTACCTCAGTTACAATTATA-3’), designed by Gene Tools, Inc. was used at 6 ng or 9 ng and did not replicate the morphological and behavioral deficits induced by chrna2 morpholino; thereby, indicating that the mere injection of morpholino was not cause for these disruptions. However, a 5 base pair mismatch control morpholino was not used in these experiments. For RNA rescue experiments, synthetic capped zebrafish
chrna2 mRNA was produced using mMMESSAGE mMACHINE kit (Ambion, Inc.) according to manufacturer’s instructions. Approximately 100 pg of mRNA co-injected with 4 ng of chrna2 morpholino was sufficient to partially rescue the phenotype. Finally, off target effects have been shown to be due to activation of p53-mediated apoptosis, and can be suppressed by co-injection of an anti-p53 morpholino along with the experimental morpholino. The co-injection of 4 ng of chrna2 mopholino with 6 ng of p53 morpholino did not rescue the phenotype.

Figure 25: Genomic structure and sequence of α2 nAChR gene.
A: The α2 nAChR gene contains 6 exons. Approximate size of exon in bp: Exon 2 (152), Exon 3(45), Exon 4(100), Exon 5(1054 bp), and finally Exon 6.

Embryos were first injected with 8 ng, 6ng, 4 ng, or 2 ng of chrna2 morpholino. After embryos were injected with morpholino and allowed to develop to the desired stage, RNA was isolated and reverse transcribed. Amplification with gene specific primers allowed for amplification of the desired product and knockdown was confirmed
by PCR. 8 ng was extremely lethal, while 2 ng did not produce a phenotype nor exon deletion as evident by PCR (data not shown). Both 4 ng and 6 ng produced visible changes in gross morphology at 72 hpf, along with a knock down of exon 5 as confirmed by PCR analysis. Although extensive data was collected after the injection of 6 ng, the data represented here is the lowest concentration of morpholino, 4 ng, to produce a phenotype (Figure 26) and deletion of exon 5 (Figures 27, 29, 30).

Figure 26: Dose response of α2 antisense oligonucleotide morpholino gene knockdown
Four concentrations of α2 morpholino were used in these experiments: 2ng, 4 ng, 6 ng, and 8 ng. Additionally, a variety of phenotypes were present after gene knock down termed none (blue), mild (purple), moderate (yellow), or severe (green). 4 ng of morpholino was chosen as the appropriate dose because it was the lowest concentration of morpholion to induce a phenotype that coincided with an exon deletion as evident by PCR.
After injection of 4 ng of α2 nAChR morpholino, 24 hpf and 48 hpf embryos were nearly indistinguishable from their wild type control mates. Of those embryos injected with α2 morpholino, some embryos looked completely normal (NORM), while others were slightly shorter with no differences in gross morphology (ODD) such as brain atrophy. Upon division of these phenotypes, the embryos that displayed a normal gross morphology contained a full α2 product of 2000 bp, while the embryos with the slightly odd morphology showed a complete knock down of the gene with a 900 bp product visible (Figure 27).
Figure 27: RT-PCR of knockdown of $\alpha_2$ exon 5 in 48 hpf embryos.

RT-PCR was used to determine if efficiency of the $\alpha_2$ morpholino-mediated gene knockdown was sufficient to delete exon 5. Using subunit-specific primers, the full length $\alpha_2$ products was approximately 2000 bp and an exon 5 deletion product was approximately 900 bp. 3 µgs of RNA from the gene knock down embryos was reverse-transcribed and amplified using subunit-specific primers. $\beta$-actin was also amplified for each age. As controls, PCR was performed without reverse transcription (-rt) or without cDNA (-dna) (data not shown). The size of each PCR product was consistent with the size predicted by the cDNA sequences. $\phi$X 174 HaeIII DNA ($\phi\chi$) was used as a size marker. Wild type (WT) or uninjected embryos had a strong level of 2000 bp product. There was a group $\alpha_2$ morpholino injected embryos that were indistinguishable from the WT ($\alpha_2$ Norm) with a 2000 bp product. Finally, there was a group of $\alpha_2$ morpholino injected embryos with slightly odd morphology (ODD) with a significant loss of a full length 2000 bp product and only amplified a 900 bp exon 5 deletion product.

By 72 hpf there were distinct phenotypes which were divided into groups termed mild, moderate, or severe based on visible differences in gross morphology and deficits in behavior as assayed by swimming behavior (Figure 28 A-E). A typical motor behavior at 72 hpf includes the abolishment of spontaneous motor activity, with the presence of tactile stimulation induced swimming. An escape touch response is one in which after the embryo is probed, the embryo swims rapidly forward and then takes a 180 degree escape turn. Additionally, at 72 hpf the embryo displays intermittent swimming in which
the embryo burst forward and then stops. Embryos included in the Mild group (Figure 28 B) consisted of 7.14% of the embryos and were only distinguishable from the controls because of smaller size, short trunks, and erratic swim patterns. These were designated as “normal short swim” or NSS. When probed these embryos did not take a characteristic escape response with a 180 degree turn, but swam in an unpredictable direction of short forward bursts. Embryos in the Moderate group (Figure 28 C) consisted of 11.01% of the α2 morphants and were indistinguishable in gross morphology from the controls, but were paralyzed upon tactile stimulation. These were designated “normal paralyzed” or NP. Embryos in the Severe group (Figure 28 D and E) consisted of 31.85% of the embryos which displayed shorten trunks and prominent atrophy of the brain, but maintained the ability for short forward swim bursts. These were termed “Odd Straight Swim” or OSS (Figure 28 D). Additionally, 49.78% of the embryos displayed a curved trunk and mounted a swim response, but only in a circular pattern and were labeled as “Odd Straight Curved” or OCS (Figure 28 E). Both the OSS and OCS were included in the Severe group.
Figure 28: α2 morphant embryos at 72 hpf
After gene knockdown, prominent phenotypes including changes in gross morphology and swim behavior did not emerge until 72 hpf. Embryos injected with control morpholino did not display any abnormalities. The mild phenotype was smaller in size with short trunks and erratic swim patterns than control embryos. The moderate phenotype had normal gross morphology, but were paralyzed with no contraction evident after tactile simulation. The severe phenotype had shortened trunks and prominent brain atrophy. We do not believe that these phenotypes are a result of toxic or off target effects of morpholino injections, as the changes in gross morphology do not emerge until 72 hpf and coincide with the functional or behavior changes.

Most of the α2 morphants, despite the differences in morphology or motor behavior were smaller than their control mates, indicating that α2 may be important in overall development. As I realized that some of the α2 morphant embryos looked as though they were displaying toxic morpholino phenotypes, I believe that these were real phenotypes and not off target effects for the following reasons: 1) cell death in the brain was not observed until 72 hpf. 2) injection of a control morpholinos produced no

N=952
phenotype. 3) co-injection of p53 morpholino did not rescue the phenotype. 4) co-injection of chrna2 RNA partially rescued the phenotype. 5) only deficits in dorsal motor neuron pathfinding were observed with no differences in ventral neuron projections which demonstrated that there was not a generalized defect in development, but some selectivity.

The efficiency of the morpholino-mediated gene knockdown was again determined by RT-PCR at 72 hpf and 96 hpf (Figure 29 and Figure 30, respectively) and variable penetrance of gene knockdown was observed. At 72 hpf (Figure 29) PCR analysis of the mild phenotype (smaller in size, short trunks and erratic swim patterns) showed at least a 50% decrease in the full length 2000 bp α2 product. The moderate group of paralyzed embryos contained even less transcript than the mild phenotype with an approximate 75% reduction of full length 2000 bp product from control morpholino injected embryos and non injected wild type embryos. The severe group of embryos (brain atrophy, malformations of the trunk, and ability to swim) contained a full knock down of exon 5 as evident by the 900 bp product.
Figure 29: RT-PCR of knock down of $\alpha_2$ exon 5 in 72 hpf embryos

RT-PCR was used to determine if efficiency of the $\alpha_2$ morpholino-mediated gene knockdown was sufficient to delete exon 5. Using subunit-specific primers, full length $\alpha_2$ products was approximately 2000 bp and an exon 5 deletion product was approximately 900 bp. 3 µgs of RNA from the gene knock down embryos was reverse-transcribed and amplified using subunit-specific primers. $\beta$-actin was also amplified for each age. As controls, PCR was performed without reverse transcription (-rt) or without cDNA (-dna) (data not shown). The size of each PCR product was consistent with the size predicted by the cDNA sequences. $\phi$x 174 HaeIII DNA ($\phi\chi$) was used as a size marker. Control morpholino (Ctrl) injected embryos displayed a strong level of a full length $\alpha_2$ 2000 bp product. The mild phenotype (NSS) had a reduction in full length $\alpha_2$, while the moderate paralyzed group (NP) displayed even less full length $\alpha_2$ than the mild group. The severe phenotype (OSS and OCS) amplified only an exon 5 deletion product of 900 bp.

As predicted the loss of full length 2000 bp product in the mild and moderate group was also accompanied by an increase in the exon 5 deleted product of 900 bp, but this was not the case for the severe group (Figure 29). There was concern that after gene knock down in the severe group (OSS or OCS) that the RNA was not stable enough to amplify a full length product and thereby not a true representation of a full knock down. A new smaller primer set was designed that flanked and included exon 5: full length $\alpha_2$ product was approximately 1420bp and knock down of exon 5 resulted in a 370 bp
product. Again the moderate paralyzed embryos (NP) at 96 hpf (Figure 30) displayed at least a 50% reduction in the amount of full length product, while the other severe phenotypes (OSS and OCS) had full knock down of the gene. Importantly, PCR analysis at 96 hpf showed that our morpholino was still fully active.

Figure 30: RT-PCR of knock down of α2 exon 5 in 96 hpf embryos

RT-PCR was used to determine if efficiency of the α2 morpholino-mediated gene knockdown was sufficient to delete exon 5. Using subunit-specific primers, full length α2 products was approximately 1420 bp and an exon 5 deletion product was approximately 370 bp. 3 µgs of RNA from the gene knock down embryos was reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed without reverse transcription (-rt) or without cDNA (-dna) (data not shown). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker. Control morpholino (Ctrl) injected embryos displayed a strong level of a full length α2 1420 bp product. The moderate paralyzed group (NP) displayed less full length α2 than the control group. The severe phenotype (OSS and OCS) amplified only an exon 5 deletion product of 370 bp.

For these studies a total of 952 embryos over four experiments were analyzed and the percent of each phenotype observed was consistent throughout these three experiments. One of the most prominent phenotypes observed in all of the morphants
was the deficit in motor behavior. Although a majority (88.77%) of the embryos still elicted a stimulus evoked swim response, the pattern was not normal. None of the embryos took a full escape response turn of 180 degrees, but rather swam forward only in a short straight burst. Not only was the swim pattern abnormal, but so was the response rate after stimulation (Figure 31). Repeated taps with forceps to the spine eventually evoked a response, but it was not a coordinated escape. After being probed 10 times in succession, wild type, sham injected and control morpholino injected embryos mounted a response between 95.20%-97.20% of the time. The mild embryos with a 50% reduction in $\alpha_2$ and unpredictable swim patterns elicited a response rate of 89.70%, the severe embryos with full knock down of $\alpha_2$ responded between 68.30% and 68.90%, while the paralyzed group obviously showed no response.
Figure 31: α2 morphants respond abnormally to touch stimuli

In this assay the embryos were divided into phenotypic categories: Mild (NSS), Moderate (NP) and Severe (OSS and OCS) and were then probed 10 times in succession while the number of times the embryo moved, not necessarily a normal swim response, was recorded. The wild type (WT), Sham injected (Sham), and control morpholino (Ctrl MO) injected embryos mounted a swim response between 95.20% and 97.20% of the time. The mild phenotype (NSS) responded with an 89.70% response rate, the severe group (OSS or OCS) mounted a response only 68.30%-68.90% of the time after touch stimulation. Finally, the moderate (NP) or paralyzed group obviously mounted no swim response.

Because of the obvious deficits in motor behavior, the expression of α2 in the ventral spine at 24 and 48 hpf, and that nicotine has been shown to affect motor neuron development and function in other systems (Svoboda, et al., 2002, Hory-Lee and Frank, 1995; Oppenheim et al., 2000), I first examined the α2 nAChR morphants for motor neurons abnormalities at 72 hpf (onset of paralysis) and 96 hpf. Among other neuronal types, this transgenic line has GFP-expressing secondary motor neuron cell bodies and dorsally projecting axons. Dorsally projecting motor neurons were missing or exhibited truncation and branching defects as a result of chrna2 knockdown (Figure 32)
Approximately 980 axons were counted over three experiments for each morphant group. In wild type and control morpholino injected embryo, the axons displayed a normal pattern of motor neuron growth. At 72 hpf, the dorsal extending axons first projected ventrally along the common path and then at the first checkpoint grew dorsally with a single branch point at the dorsal edge of the extending axon (Figure 32 A). At 96 hpf, there was more extensive branching, but the pattern remained with the single branch point at the dorsal myotome (Figure 32 B). With the injection of control morpholino, 98.61% of the axons extended in this stereotypical pattern. After $\alpha_2$ nAChR knock down, all of the morphants displayed varying degrees of abnormal axonal pathfinding to the dorsal myotome (Figure 32) at both 72 hpf and 96 hpf. At 72 hpf within the mild phenotype group (displayed overall small size, erratic swim patterns, and an approximate 50% reduction in $\alpha_2$), 81.75 % of the axon displayed the stereotypic extension and branch pattern, while 18.25% of the axons extended fully to the dorsal surface but had extra branching along the axon. (Figure 32 C). The moderate group (composed of the paralyzed (NP) embryos which had a significant reduction (75%) in $\alpha_2$ after gene knock down) contained a variety of abnormal axons (Figure 32 E). 66.4% of the axons displayed normal axonal extension, 14.11% were truncated, 10.18 % extended fully to the dorsal surface but did not take a branch point, and 9.31% of the axons were missing. The severe group (with full knock down of $\alpha_2$, despite the differences in gross morphology) contained only truncated or missing axons. In the embryos with atrophy in the brain and straight trunk (OSS), 77.35% of the axons were truncated and 22.65% were missing (Figure 32 G). In the embryos with atrophy in the brain and a curved trunk (OCS), 48.51% were truncated and 51.49% were missing (Figure 32 I).
By 96 hpf, a slight recovery in the axonal phenotypes was observed. Similar to 72 hpf in the control morpholino injected embryos, 99.11% of the axons were normal (Figure 32 B). In the mild phenotype of erratic swimmers (Figure 32 D), 82.67% of the axons were normal (81.75 % at 72 hpf) and 17.33% displayed extra branching that now have began to innervate the adjacent somite (18.25% at 72 hpf). In the moderate group of paralyzed embryos 86.37 % of the axons were normal (66.4% at 72 hpf), 9.04% displayed extra branching that extended into adjacent somites (did not exist at 72 hpf), and 4.59% extended to the dorsal surface with no branch points (10.18% at 72 hpf) (Figure 32 F). Unlike at 72 hpf, there were not truncated axons or missing axons in the paralyzed embryos. In the severe morphants there were no normal axons. In the OSS, 31.82% of the axons were truncated (77.35% at 72 hpf), 60.41% extended to the dorsal surface without branching and 7.77% were missing (22.65% at 72 hpf) (Figure 32 H). Additionally, in the OCS 82.49% of the axons were truncated (48.51% at 72 hpf) and 17.51% were missing (51.49% at 72 hpf) (Figure 32 J). In general the number of missing and truncated axons decreased from 72 hpf and 96 hpf, indicating that the cells were not undergoing cell death, but possibly a delay in axon extension.
Figure 32: α2 morphants display abnormal pathfinding of dorsal motor neurons
Islet 1 GFP transgenic zebrafish that label (MiP) dorsal extending axons were examined after α2 nAChR gene knockdown at 72 hpf and 96 hpf. **A and B: Control Morpholino** (Control MO) injected embryos displayed a normal pattern of axon extension. (A) At 72 hpf the axons first projected ventrally along the common path and then at the first checkpoint grew dorsally with a single branch point at the dorsal edge of the extending axon. (B) At 96 hpf there was more extensive branching, but the pattern remained with the single branch point at the dorsal myotome. **C and D: Mild Phenotype** displayed both normal patterns of axon extension and axons that extended fully to the dorsal surface but had extra branching along the axon. (C) At 72 hpf 81.75 % were normal while 18.25% of the axons extended fully to the dorsal surface but did not branch properly. (D) At 96 hpf 82.67 % of the axons were normal while 17.33% displayed extra branching that began to innervate adjacent somites. **E and F: Moderate Phenotype** displayed normal axonal extension, truncated axons, axons that extend fully to the dorsal surface but did not take a branch point, and finally axons were missing. (E) At 72 hpf 66.4% were normal, 14.11% were truncated, 10.18 % extend fully to the dorsal surface but did not take a branch point, and 9.31% of the axons were missing. (F) At 96 hpf 86.37 % of the axons were normal, 9.04% displayed extra branching that extended into adjacent somites, and 4.59% extended to the dorsal surface with no branch points. Unlike at 72 hpf, there were not truncated or missing axons. **G and H: Severe Phenotype (OSS)** had truncated and missing axons. (G) At 72 hpf 77.35% of the axons were truncated and 22.65% were missing. **I and J: Severe Phenotype (OCS)** had missing and truncated axons. (I) At 72 hpf 48.51% were truncated and 51.49% were missing. (J) At 96 hpf 82.49% of the axons were truncated and 17.51% were missing.
To determine whether other spinal motor axons were affected by decreased levels of chRNA2, I analyzed ventral motor neurons at 24 and 48 hpf in hb9-GFP transgenic zebrafish. Among other neuronal types, this transgenic line has GFP-expressing primary
motoneuron cell bodies and ventrally projecting axons. Approximately 421 axons were counted over three experiments for each morphant group. In wild type and control morpholino injected embryo, the axons displayed a normal pattern of ventral motor neuron extension. After α2 knockdown was verified by PCR, there was not a significant difference in axon number, morphology, or axon extension the of the ventral axons at 24 hpf (data not shown) or 48 hpf (Figure 33). Unlike the dorsal motor axons no axons were missing. In conclusion, abolishing the α2 nAChR subunit-containing receptors had selective effects on dorsal motor neurons and did not effect the ventral projections, indicating that a generalized defect of overall development in the spinal cord did not occur.

![Figure 33: α2 morphants display normal patterns of ventral motor neuron growth](image)

HB9 GFP transgenic zebrafish that labels (CaP) ventral extending axons were examined after α2 nAChR gene knockdown at 24 hpf (data not shown) and 48 hpf. There is not a difference in ventral axon number, morphology, or extension in the control morpholino (Ctrl MO), α2 morpholino (α2 MO), or α2 morpholino with p53 morpholino (α2 p53 MO) injected embryos.
To confirm the specificity of the morpholino function, RNA rescue experiments were performed using zebrafish \textit{chrna2} mRNA. 1-4 cell \textit{islet 1-GFP} embryos were injected with a mixture of \textit{chrna2} morpholino (4 ng) and \textit{chrna2} mRNA (6 ng). When compared to \textit{chrna2} morpholino alone, the co-injection of zebrafish \textit{chrna2} mRNA and \textit{chrna2} morpholino partially rescued the phenotype (Figure 34 A). In the embryos injected with only \textit{α2} morpholino there were 7.14\% of the morphants in the mild group, 11.23 \% were moderate, and 81.63 \% were severe, numbers which were similarly replicated in the \textit{α2 p53} co-injected groups. The addition of \textit{chrna2} RNA shifted the severity of the phenotype to 9.82 \% mild, 3.16 \% moderate, 23.84 \% severe, and 63.18 \% had a normal phenotype. I also demonstrated by PCR that the \textit{α2} morpholino knocked down exon 5 and that the addition of \textit{α2} RNA along with \textit{α2} morpholino allowed for the re-amplification of full length \textit{chrna2} transcript (Figure 34 B).
Figure 34: α2 RNA partially rescues the α2 morphant phenotypes at 72 hpf
(A). Graph indicates that the addition of α2 RNA with gene knockdown significantly rescued the severe phenotype. In the embryos injected with only α2 morpholino there were 7.14% mild (purple), 11.23% moderate (yellow), and 81.63% severe (green), which was similarly replicated in the α2 p53 co-injected groups. The addition of α2 RNA shifted the severity of the phenotype to 9.82% mild (purple), 3.16% moderate (yellow), 23.84% severe (green) and 63.18% with a normal phenotype (blue). (B.) RT-PCR was used to determine if the efficiency of the α2 morpholino-mediated delete exon 5 was complete and to see if the addition of α2 RNA rescue is also amplified in the rescued phenotypes. Using subunit-specific primers, full length α2 product was approximately 1420 bp and an exon 5 deletion product was approximately 370 bp. 3 μg of RNA from the gene knock down embryos was reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed without reverse transcription (-rt) or without cDNA (-dna) (data not included). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker. Uninjected wild type (WT) and Control morpholino (Ctrl) injected embryos displayed a strong level of a full length α2 1420 bp product. The moderate α2 morpholino injected paralyzed (α2 P) displayed a reduction in full length α2, the severe group (α2) displayed a full knockdown of the gene, and the two lanes with different concentrations of α2 rescue RNA (α2 RNA) with α2 morpholino displayed the reappearance of full length α2 product.
Figure 34: α2 RNA partially rescues the α2 morphant phenotypes at 72 hpf
Finally, off target effects have been shown to be due to activation of p53-mediated apoptosis, and can be suppressed by co-injection of an anti-p53 morpholino along with the experimental morpholino. Embryos were examined at 96 hpf after a co-injection of 4 ng of α2 morpholino and 6 ng of p53 morpholino and little to no change was observed in the gross morphology or motor behaviors of the previously described morphants (Figure 35 A), indicating that the resulting phenotype was the result of α2 gene knock down and not the result of a non-targeted p53 mediated cell death phenotype. Additionally, the knock down of p53 did not effect the expression of the chrna2 transcript (Figure 35 B).
Figure 35: Co-injection of α2 morpholino with p53 morpholino does not rescue the phenotype. A. Graph indicates that there was not a significant difference in the percent of each phenotype: mild (norm swim), moderate (norm paralyzed) or severe (odd straight swim and odd curved swim) with only α2 morpholino (blue) as compared to the co-injection of α2 and p53 morpholino (purple). B. RT-PCR was used to determine if the efficiency of the α2 morpholino-mediated delete exon 5 was affected by the addition of p53 morpholino. Using subunit-specific primers, full length α2 products was approximately 1420 bp and an exon 5 deletion products was approximately 370 bp. 3 µgs of RNA from the gene knock down embryos was reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed without reverse transcription (-rt) (data not included) or without cDNA (-dna) The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker. Uninjected wild type (WT) and Control morpholino (Ctrl) injected embryos displayed a strong level of a full length α2 1420 bp product. The α2 injected embryos, the α2 injected paralyzed embryos and α2-p53 morpholino embryos all amplified only an exon 5 deletion product of 370 bp in equal amounts.
Phenotypes at 96 hpf resulting from $\alpha_2$p53 MO co-injection

Figure 35: Co-injection of $\alpha_2$ morpholino with p53 morpholino does not rescue the phenotype
5.4 Discussion:

As previously mentioned no developmental functions have been assigned to the \( \alpha_2 \) nAChR subunit as gene knockout studies have not been performed and until recently no adult function had been discovered. Now a clinical and molecular genetic study has linked a mutation in \textit{chrna2} to sleep-related epilepsies. The presence of both acetylcholine (ACh) and nAChRs during embryogenesis suggests that nAChRs play a significant role during development. Most notably because of the limited expression pattern of the \( \alpha_2 \) nAChR in the adult and narrow functional assignment, I hypothesized that the transiently expressed \( \alpha_2 \) nAChR gene may be playing a critical role in development and then may be downregulated in the adult and therefore not functionally relevant. A critical role of the \( \alpha_2 \) subunit during development was supported by the fact that so far it is the only nAChR \( \alpha \) subunit to have transcript expressed maternally. These studies are the first to define a developmental function for the \( \alpha_2 \) nAChR gene and indicate that its expression is important in dorsal motor neuron extension in the spinal cord and needed to maintain proper swim behaviors.

A strength of zebrafish model system is the ability to perform mutagenesis screens and identify animals with single gene disruptions that affect behavior, including motor behaviors. Granato et al., 1996 carried out the first large scale mutagenesis study in which mutants were identified with disrupted movements not associated with impaired muscle development. Many mutants with motor defects have problems with genes associated with the neuromuscular junction, or with the muscle itself. These include paralyzed mutants with abnormal acetylcholine receptor at the neuromuscular junction
(NMJ), such as Nic1 and sofa potato. Zebrafish normal muscle activity is not required for normal motor neuron development. Morphology and axonal trajectories of motor neurons in sofa potato embryos appear to be normal (Ono et al., 2001); they are not truncated or delayed in their development. In the paralytic mutant known as nic-1, ACh receptor function in the muscle is completely blocked. However, motor neuron innervation and the neuron muscular junction are normal (Westerfield et al., 1990). Thus, muscle inactivity in zebrafish appears to have no effect on secondary motor neuron development, morphology or axonal pathfinding. Therefore, it is possible that the paralysis in our α2 mutant is due to a nervous system problem and not because of disrupted muscle, as muscle paralysis alone would not be sufficient to disrupt the axonal growth.

The nAChR α2 knockdown paralysis phenotype can be pharmacologically mimicked by raising embryos in the cholinergic agonist nicotine. Svoboda, et al 2002, showed that zebrafish embryos exposed to 15 μM nicotine have abnormal swim behaviors and those treated with 33 μM nicotine were functionally paralyzed at 66 hpf. Most importantly they demonstrated that the dorsal axons of secondary motor neurons did not extend into properly into the periphery. Continued expression beyond 66 hpf caused a severe stunting of the dorsal axons. Furthermore, the addition of the nicotinic acetylcholine receptor antagonist Dihydro-β-erythroidine (DHβE) was sufficient to block the deficits in motor neuron pathfinding, indicating in part that the phenotype was nAChR mediated. DHβE is most selective to α4β2 receptor subtype, but can also act on α2β4 and α2β2 receptors.
To date, detailed pharmacological studies of zebrafish neuronal nAChR have not been performed. Using *Xenopus* oocyte whole-cell recording experiments with a hybrid zebrafish nAChR $\alpha_2$: human $\beta_4$ subunit and zebrafish $\alpha_2\beta_2$ were performed by Dr. Mark Levandoski. The hybrid zebrafish $\alpha_2$: human $\beta_4$ receptor complex responded normally when challenged with increasing concentrations of acetylcholine from 3 $\mu$M to 300 $\mu$M (Figure 36 A). Additionally, the receptor could be blocked with the antagonist DH$\beta$E (Figure 36 B), at concentrations used by Svoboda, et al 2002 to block the delay in motor neuron growth. Finally, a zebrafish $\alpha_2\beta_2$ subtype formed a functional receptor that responded normally when challenged with acetylcholine or nicotine (Figure 36 C and D) (Dr. Roger Papke, unpublished). These are the first pharmacological studies to indicate that the drug concentrations used in Svoboda, et al and our zebrafish studies were relevant and had the ability to activate or block zebrafish nAChR. Additionally, these studies provided evidence that the zebrafish nAChR $\alpha_2$ subunit that we had cloned was able to assemble as a heteromeric receptor and, upon activation, produced currents characteristic of $\alpha_2$ nAChRs in other species.
Figure 36: Pharmacological Profiles of α2 containing zebrafish nAChR subtypes

Xenopus oocyte whole-cell recording experiments were performed by Dr. Roger Papke expressing a hybrid zebrafish nAChR α2: human β4 subunit or zebrafish α2β2. The receptor subtypes were challenged with acetylcholine (Ach), nicotine, or Dihydro-β-erythroidine (DHβE) and all respond appropriately. (A) A hybrid zebrafish nAChR α2: human β4 subunit was challenged with increasing concentrations of ACh from 3 µM to 300 µM and displayed appropriate increasing downward currents as the drug concentration increased. (B) The hybrid zebrafish nAChR α2: human β4 subunit activity could be blocked by the nAChR antagonist DHβE. The channel was challenged with 30 nM Ach, then blocked with 30 mM DHβE, and after removal of the blocker the channel again responded to 30 nM Ach. (C and D) The zebrafish α2β2 did have the ability to form a functional channel and responded normally to increasing concentrations of ACh (C) and nicotine (D).
Finally in Svoboda’s study, treatment of the nicotine treated embryos with MLA, a homomeric $\alpha_7$ subtype selective antagonist failed to reverse the effects of the nicotine, indicating that a heteromeric receptor subtype (both $\alpha$ and $\beta$ subunits) was involved in developmental motor neuron deficits and abnormal motor behavior. In conclusion, our $\alpha_2$ nAChR knock down studies provide evidence that an $\alpha_2$ containing nAChR could be targeted or involved in the mechanism of nicotine induced paralysis, defects in swim behavior, and/or the dorsal motor neuron pathfinding deficits observed after nicotine treatment.

**Discussion with Future Directions**

Below I will list four explanations of how the loss of $\alpha_2$-containing receptors in brain or ventral spinal cord interneurons could cause motor deficits in swimming and/or effect the pathfinding of dorsal motor neurons.

1) In knock out studies using mice, the absence of a single nAChR subunit produced some adverse effects, but the knock down of multiple subunits resulted in death. It is possible that the absence of a single nAChR subunit can be compensated for during development. It is possible that a partial loss of zebrafish $\alpha_2$ results in paralysis, but a complete loss could be lethal; therefore, there is an upregulation of another nAChR subunit to recover this loss or there is an upregulation of a gene in the motor neuron pathway for delayed/recovered growth of the axons. I propose to use the cDNA
amplified from embryos after α2 knockdown, and will determine if there is a change in expression of α4, α6, α7, Islet 1, or Shh transcript levels. α6 RNA is localized to a subset of spinal neurons at only 24 hpf and α4 and α6 are highly expressed in brain by 72 hpf and may overlap α2 expression in the midbrain at that time. I will use α7 as a control, as α7 seems to only localize to retina in zebrafish and knockdown of α2 should not have an effect on this gene.

As the knockdown of α2 containing nAChR caused a phenotype of abnormal dorsal motor neuron projectories in the spinal cord and motor behavior impairments, it is relevant to determine if genes involved in the development of motor neurons are affected. Primary neurons in the zebrafish spinal cord have been identified and divided into three main classes: motor neurons, interneurons and sensory neurons (Bernhardt et al., 1990; Kuwada et al., 1990; Eisen, 1991). These neurons are repeated in the somites along the anteroposterior axis and are characterized based on the location of their cell body, the projection of the axon, and their position within the somite boundaries. By 18-20 hpf, each spinal cord hemisegment contains approximately 11 differentiated neurons: three primary motor neurons, five interneurons, and three primary sensory Rohon-Beard neurons (Kuwada and Bernhardt, 1990). The axon pathways of the primary motor neurons have been well characterized and shown to follow highly specific trajectories (Beattie, 2000): CaP axons innervate the ventral myotome, RoPs innervate the horizontal myotome, and MiPs extend to the dorsal myotome. The LIM homeobox genes are thought to dictate a combinatorial code leading to primary motor neuron development (Appel et al., 1995; Tokumoto et al., 1995; Segawa et al., 2001). Initially, all primary motor neurons express Islet 1, isl-1, but later in development they express different
combinations of LIM genes. The formation of primary motor neurons is dependent on Islet 1 expression, as when Islet 1 is absent then primary motor neurons are missing, with an upregulation of ventral interneurons (Hutchinson and Eisen, 2006). While this expression pattern may provide a molecular code for primary motor neuron differentiation there is no evidence that it is involved in axon guidance in zebrafish (Segawa et al., 2001). As in later development the dorsal extending RoP express Islet 1, I propose to use the cDNA amplified from embryos after $\alpha 2$ knockdown, and will determine if there is a change in expression of transcript levels of Islet 1.

The differentiation of floor plate cells and motor neurons in the vertebrate neural tube appears to be induced by signals from the notochord. The secreted protein encoded by the Sonic hedgehog ($shh$) gene is expressed by axial midline cells and can induce floor plate cells. A gradient of Shh specifies progenitor domains for motor neurons (MN) and four classes of interneurons (VO–V3). I propose to use the cDNA amplified from embryos after $\alpha 2$ knockdown, and will determine if there is a change in expression of transcript levels of Shh.

2) Again many mutants with motor defects have problems with genes associated with the neuromuscular junction, or with the muscle itself. Some immotile mutants have mutations that interfere with the calcium release needed for muscle contraction (Ono et al., 2001). The twitch once mutant, has a mutation in rapsyn, a protein that is involved in localization of acetylcholine receptors at the neuromuscular junction (Ono et al., 2002). These mutants show an inability to swim continuously after a stimulus and alpha
bungarotoxin labeling shows that acetylcholine receptors are diffusely distributed rather than localized at the synapses. The accordion class of mutants is a group showing bilateral muscle contractions. Studies of mutants have revealed that there are many ways to make a fish generate accordion-like movements. One is to disrupt the calcium transporter (SERCA) responsible for removal of the calcium from the cytoplasm after muscle contraction (Gleason et al., 2004; Hirata et al., 2004). When this is not working the muscles do not relax properly and the result is that the contraction on one side of the fish overlaps with that on the other, generating the phenotype. The accordion class mutant \textit{Zieharmonika} is a mutation in acetylcholinesterase, which would elevate acetylcholine at the synapses (Downes and Granato, 2004).

Early work proposed that central deficits in glycinergic transmission could also produce an accordion phenotype because glycinergic inhibition is known to play a critical role in the alternation of contractions on opposite side of the fish during bending. One of the mutants in the accordion class, called \textit{bandoneon}, indeed turned out to be a mutant of a beta subunit of the glycine receptor which disrupts proper inhibitory transmission and lead to contraction of opposite sides of the body (Hirata et al., 2005). Proper glycinergic transmission is essential to sustain motor behaviors as the mutant shocked shows an inability to sustain movement. The mutation is in the glial, glycine transporter-1, and the disruption appears to elevate glycine levels which may allow for an inappropriate rise in the level of inhibition during movements, thereby truncating the movements (Cui et al., 2005, 2004).

Other mutants with motor defects have problems with genes associated with neuron pathfinding errors that result in motor phenotypes. These can be informative
about cues used in axonal pathfinding as in the case of the mutants *unplugged* and *diwanka*, both of which involve molecules that play a role in guiding motor axons to their targets (Schneider and Granato, 2006; Zhang et al., 2004).

Finally, mutants have been identified which indicate that a disruption in central brain neurons can disrupt motor circuitry. The mutants *deadly seven* and *space cadet* have both offered circuit level insights. *Deadly seven*, a mutant of a notch gene, leads to extra Mauthner neurons in the hindbrain (Gray et al., 2001; Liu et al., 2003; van Eeden et al., 1996). Usually there is one on each side of the brain, but there can be as many as 5 in mutant fish. Interestingly, this has little impact on the motor output in the escape response (Liu et al., 2003). A close look at the morphology of the outputs of individual neurons indicates that the extra neurons divide up the synaptic targets of the neurons normally innervated by a single Mauthner cell. The space cadet mutant involves a wiring deficit (Lorent et al., 2001). The phenotype of this mutant is the generation of multiple, inappropriate escape bends, which are often repeated ones to the same side. Central commissures in the hindbrain of this animal are disrupted. Thus, it may be that an axon pathfinding deficit leads to a behavioral phenotype because of a miswiring of a neuron critical for the response.

Mutant fish have demonstrated the importance of proper axon guidance and neurotransmitter signaling not only in the brain, but also in the spinal cord. As the α2 nAChR subunit is localized to ventral interneurons, presumably knockdown of α2-containing receptors caused swimming impairment and axon pathfinding deficits, how are the interneurons involved in this circuitry? A diagram of the known existing interneurons in zebrafish is in Figure 37. To link neurons to movements, it is important
to know which movement is occurring when the neurons are active. To determine which behaviors are being produced in association with the neural activity, advancements in assay design by embedding embryos in agar now allows for active neurons in the fish to be identified while movements in the tail can be filmed. This was initially used to study which neurons were active in spinal cord during swimming versus escape responses (Ritter et al., 2001). This work showed that excitatory descending commissural neurons (MCoDs) are active during slow speed movements and a different descending excitatory interneuron class (CiDs) is active in fast escape responses. This partially moving preparation has allowed studies of the patterns of activation of a class of interneuron during escapes which show that the escape is graded largely by changes in the level of activity in the entire pool of interneurons rather than recruitment of inactive cells (Bhatt et al., 2007). A recent preliminary report indicates that there is actually a switching of spinal interneuron classes as the swimming speed is increased, with excitatory neurons active at slow speeds being inhibited at higher speeds, when another class of excitatory interneurons becomes active (Masino et al., 2005).

Higashijima, et al, 2004 defined the likely transmitter phenotypes of most of the known classes of spinal interneurons, but the contribution of the cholinergic system was not included in the study. Glutamatergic interneurons (positive for VGLUT) include the commissural CoPA, MCoD, UCoD, and some of the CoSA neurons. VGLUT-positive CiD interneurons have ipsilateral descending axons and ventrally located VeMe interneurons have descending axonal trajectory. Glycinergic interneurons (positive for GLYT2) include the commissural CoLAs, CoBL and CoSA neurons. The CiA cells were the only GLYT2-positive cells with an ipsilateral axon. GABAergic interneurons
(staining for GAD) included the dorsal longitudinal ascending (DoLA) and KA interneurons.

Figure 37: Identification of spinal interneurons in zebrafish embryos and larvae. A. Embryonic spinal interneurons. B. Spinal interneurons in larvae. For A and B dorsal is up and anterior to the left.

The inhibitory modulation of GABAergic and glycinergic are important developmental signals as evident by zebrafish mutant studies. At least to date, it is thought that the most ventrally localized interneurons (such as KA interneurons), which are in similar regions of the ventral spinal cord as chrna2 RNA, are primarily glycinergic and GAD+ (Higashijima S, et al, 2004). Then one could imagine that the knockdown of
α2 containing cholinergic receptors could decrease the excitatory circuitry and leave the inhibition of these interneurons unchecked; therefore, the local environment of the spinal cord is now that of increased inhibition.

Recently, it was demonstrated that Pax2a, Pax2b and Pax8 transcription factors were redundantly required to specify the glycinergic and GABAergic fates of Circumferential Ascending (CiA) and other Pax2/8-expressing spinal interneurons. This function of Pax2/8 is very specific: in triple knock-down embryos CiAs lose expression of glycinergic and GABAergic (inhibitory) neurotransmitters, but they do not become excitatory (glutamatergic or cholinergic) and their morphologies and axon trajectories are unchanged (Batista and Lewis, 2008). Therefore, the loss of inhibitory signals in CiA neurons does not affect axon pathfinding. In the another study, the effects of perturbing glycinergic transmission found that the predominant effect of the knockdown of Gly Receptor α2 subunit was to reduce spinal interneuron populations by altering differentiation with no effect on sensory or motor neuron number or axon outgrowth/pathfinding (McDearmid, et al 2006). It seems that the loss of inhibitory signal does not effect motor neuron development.

Unlike mature neurons, developing neurons have increased intracellular chloride concentrations. The immature neurons first express the sodium-potassium-chloride cotransporter 1 (KCC1). At time points during development when glutamatergic transmission is silent, chloride channels are responsible for depolarization (Liao and Malinow, 1996). Additionally, the sodium-potassium-chloride cotransporter 2 (KCC2) is expressed in mature neurons and involved in the switch of GABAergic and glycinergic excitation to that of an inhibitory role. KCC2 is first expressed in zebrafish by 48 hpf, a
timepoint in which *chrna2* RNA is expressed in the spinal cord. The over expression of a potassium-chloride cotransporter 2 (KCC2) in the zebrafish blastula, which is “too early” in development, causes glycine hyperpolarization and results in disrupted motor activity, less motor neurons and interneurons, loss of axonal tracts, smaller brain and spinal cords, an increase in immature neurons and a decrease in spontaneously active spinal neurons (Reynolds, et al 2008). It could be that the $\alpha_2$ nAChR plays a modulatory role in the spinal cord at 24 and 48 hpf. Liu, et al 2006, using ciliary ganglion showed that “endogenous nicotinic acetylcholine activity drives maturation of GABAergic signaling, determining when it becomes inhibitory” and that the mechanism was likely to involve a change in chloride transporter levels. The use of nicotinic acetylcholine receptor antagonists prevented the GABA developmental change. Electrophysiological studies determined that blockade of nAChR prevented the change in chloride gradient by maintaining high levels of KCC1. Additionally in hippocampal tissue slices, $\alpha_7$ nAChR knockout mice have higher levels of KCC1 and lower levels of KCC2 protein. It could be that the loss of $\alpha_2$ containing nAChR in spinal interneurons, mimicking pharmacological nAChR blockade seen by Liu, alters levels of KCC1 or KCC2 protein or changes the temporal developmental expression of these genes. The alteration in the chloride currents then cause the motor neuron abnormalities and disrupted motor behaviors.

3) Clearly, I have shown that the knock down of $\alpha_2$-containing nAChR exerts effects in the spinal cord, specifically, altering dorsal motor neuron development and swim
behavior. As previously mentioned, *in situ* hybridization expression of *chrna2* RNA indicated a prominent expression of α2 in the midbrain at 48 hpf, 72 hpf, and 96 hpf. Additionally, *chrna6* subunit RNA co-localizes with tyrosine hydroxylase (*TH*) in midbrain diencephalic catecholaminergic cluster at 48 hpf, 72 hpf, and 96 hpf. Although I have not completed co-localization studies of *chrna2* and *chrna6* or *chrna2* and *TH*, it is likely that these expression domains overlap. Midbrain dopaminergic (DA) nuclei comprising the substantia nigra (SN) and ventral tegmental area (VTA) in rodent play key roles in locomotion, reinforcement, and associative motor learning (Berke and Hyman, 2000) and nicotinic acetylcholine receptors (nAChRs) are densely distributed in the SN and VTA (Fallon and Loughlin, 1995). nAChRs are probably implicated in the regulation of the dopaminergic circuits in several pathophysiological conditions. In the α2 nAChR knock down embryos, approximately 10% of the embryos were paralyzed, despite the fact that the number of dorsal motor neurons and their pathfinding events were relatively normal as compared to the severe morphants that could still swim. By 96 hpf in the moderate phenotype group of paralyzed embryos 86.37% of the axons were normal (66.4% at 72 hpf), 9.04% displayed extra branching that extended into adjacent somites (did not exist at 72 hpf), and 4.59% extended to the dorsal surface with no branch points (10.18% at 72 hpf). At 72 hpf or 96 hpf none of the axons were missing or truncated. Even the severe group of morphants was able to mount a swim response despite the grossly abnormal number and morphology of the dorsal extending axons. It is possible that a difference in the levels of brain α2 or an α2 mediated reduction in α6 levels could be responsible for the paralysis. Therefore, I propose to perform transections or spinalization on the α2 nAChR knock down paralyzed morphants, in
which the spine would be removed from the brain. Literature indicates that transecting the spinal cord at the hindbrain border does not affect the rhythmic activity patterns of the motoneurons (Saint-Amant and Drapeau, 1998), suggesting that the brain is not needed for the trunk to move. Therefore, if the transected spinal cords in our paralyzed mutants can swim without attachment to the brain, then the spinal circuitry must be intact enough to mount a contraction and motor response. Thus, the signaling in the brain would be causing the paralysis.

4) The LIM homeodomain (LIM-HD) transcription factor expression is well-characterized and predicts axonal trajectories and ultimate muscle targets. Initially, all primary motor neurons express Islet 1, *isl-1*, but later in development they express different combinations of LIM genes. The CaP (ventral) neurons express *isl-2* and *lim3*, while the MiP (middle) and RoP (dorsal) neurons express *isl-1* and *lim3*. While these expression patterns may provide a molecular code for primary motor neuron differentiation there is no evidence that it is involved in axon guidance in zebrafish (Segawa et al., 2001). Can the LIM-HD code also predict specific properties of membrane excitability in primary motor neurons as electrical excitability differs between and identifies many neuronal subtypes? Moreno and Ribera, 2007 used the HB9 transgenic line of zebrafish to study firing properties of primary motor neurons at 24 hpf and 48 hpf. They found that the duration of the CaP (ventral projections) action potential (AP) decreased by almost 3 fold between 24 and 48 hpf. The decrease in AP duration resulted from developmental changes in inward and outward conductances. To study the conductances underlying the developmental changes in firing properties, whole cell net
outward and inward conductances were recorded under voltage clamp. Whole cell outward currents consist of both voltage gated potassium ($I_{Kv}$), and calcium dependent ($I_{KCa}$) so they isolated and recorded the voltage dependent potassium conductance ($I_{Kv}$). Their findings revealed that CaPs undergo a significant increase in $I_{Kv}$ density between 24 and 48hpf, consistent with the developmental decrease in the AP duration. Further, when compared to MiPs that express a different LIM-code, there was a significant difference between the CaP and MiP outward currents as early as 24 hpf. Moreover, because CaPs and MiPs have similar $I_{Kv}$ densities the difference in outward current densities and inactivation point to divergent $I_{KCa}$. The nAChR is a non-selective cation channel, meaning that several different positively charged ions can cross through, namely sodium, potassium and calcium. It is permeable to $\text{Na}^+$ and $\text{K}^+$, with some subunit combinations that are also permeable to $\text{Ca}^{2+}$. The amount of ion flow through the pore depends on the specific subunit composition. It is possible that the loss of $\alpha_2$ nAChR locally changed normal levels of $\text{Ca}^{2+}$, thereby dictating changes in the axonal pathfinding and extension.
CHAPTER 6
In Progress
Results

Effects of In vivo and In vitro Nicotine Treatment

6.1 In vivo Nicotine Treatment

Cohorts of embryonic zebrafish were chronically exposed to nicotine [(-)-nicotine bitartrate] (Sigma) at concentrations of 50, 100, or 200 µM diluted directly into fish water from 1-3 hpf (hours post fertilization) up to 96 hpf. The nicotine/fish water solution was changed daily. Over 5,000 embryos were used in 11 separate treatment days. At 24 hpf, there was general trend of protection with 100 µM nicotine treatment at 24 hpf, as there was a 27.12% decrease in embryonic death versus the non treated controls. Conversely, treatment with 200 µM nicotine at 24 hpf generated a 29.61% increase in embryonic death versus the non treated embryos. Regardless of nicotine concentration, the treated embryos displayed a delayed coiling response and looked morphologically normal indicating that the nicotine was not toxic to overall early morphological development. At 48 hpf, the embryos had typical motor behavior, normal morphological characteristics, and no increase in the number of embryos that died. Finally, at 72 hpf there was a significant increase in the number of embryos that died regardless of nicotine treatment at 10.40% and 14.84% over control levels for 100 µM and 200 µM nicotine, respectively.
Figure 38: Graph depicting changes in zebrafish embryonic death at 24 hpf and 72 hpf after nicotine treatment. At 24 hpf (blue), there was general trend of protection with 100 µM nicotine treatment, as there was a 27.12% decrease in embryonic death versus the controls. Conversely with treatment of 200 µM nicotine at 24 hpf there was a 29.61% increase in embryonic death versus the nontreated embryos. At 72 hpf (purple) there was a increase in the number of embryos that died regardless of nicotine treatment at 10.40% and 14.84% over control levels for 100 µM and 200 µM nicotine, respectively.

By 72 hpf, embryos treated with 100 µM nicotine experienced very limited motor movement. When probed at the head and trunk, they only twitched with no forward movement implying that the muscle could contract but there was no coordinated movement. The embryos treated with 200 µM displayed complete paralysis regardless of being probed at the head or trunk. At 72 hpf with 100 µM nicotine, the embryos were smaller and slightly shorter (Figure 39 B) than wild type non treated embryos (Figure 39 A) and display some brain atrophy (Figure 39 C). Treatment with 200 µM nicotine produced more distinct gross morphological changes with embryos clearly being shorter.
and having curved tails (Figure 39 D), cardiac edema (Figure 39 E), brain atrophy (Figure 39 F, G), and an overall toxic effect displayed in some of the embryos (Figure 39 F and G). At 96 hpf, the abnormal gross morphology and paralysis continued.

Figure 39: Light Micrographs of zebrafish embryos treated with 100 µM or 200 µM nicotine at 72 hpf.

As nicotine induced motor behavior deficits, paralysis, and changes in gross morphology, I set out to determine if nicotine treatment changed the transcript levels of a predetermined set of genes: *cfos* (early immediate gene), *p53* (proapoptotic gene), nAChRs subunits, *islet 1* and *shh* (both involved in motor neuron development).

Embryos were treated with no nicotine [(-)-nicotine bitartrate] (0), 100 µM nicotine (100) or 200 µM nicotine (200) for 3 days. After nicotine treatment, RNA was isolated and zebrafish gene specific primers were used to amplify the targeted transcripts.

First as a control measure, I used semi-quantitative RT-PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135 to determine if nicotine treatment changed transcript levels of the early immediate gene *cfos* (Figure 40). *Cfos*, is widely used as a marker for neural activity in vertebrates, including responses to noxious stimuli. In many other species the upregulation of *cfos* is seen after nicotine treatment. By using semi-quantitative RT PCR, I was able to observe an increase in *cfos* transcript after nicotine treatment. Control levels of *cfos*, in the non treated embryos (0), were nearly lost in the 1:45 cDNA dilution; meanwhile, robust levels of *cfos* in the embryos treated with 100 µM (100) nicotine and elevated levels after treatment with 200 µM (200) nicotine still remained. Thus this induction of *cfos* determined that these concentrations of nicotine were sufficient to penetrate the embryos, able to elicit a response at the transcript level, and is a first step in validating the zebrafish model to quickly screen changes in gene regulation after nicotine exposure.
Figure 40: Semi-quantitative RT PCR of cfos RNA expression in 72 hpf zebrafish embryos after nicotine treatment.

Semi-quantitative RT-PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135 was used to determine if nicotine treatment changed transcript levels of the early immediate gene, cfos. Embryos were treated for 3 days with 0, 100 or 200 µM nicotine, RNA was isolated, 3 µgs of RNA was reverse-transcribed and amplified using subunit-specific primers. As controls, PCR was performed without reverse transcription (-rt), without cDNA (-dna), and β-actin was also amplified (data not shown). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φx) was used as a size marker. Clearly there was an increase in cfos transcript after nicotine treatment regardless of the level of cDNA dilution.

Previous work in the laboratory provided evidence for nicotine induced apoptosis that could be blocked with the nicotinic antagonist DHβE (Zirger, unpublished). After repeating these studies, there was a qualitative increase in the number of apoptotic cells, most notably in the tectum and retina which could indicate an α6 nAChR-containing response. Qualitatively, the number of apoptotic cells increased with a dose increase in nicotine (Figure 41 A) and was subsequently absent when blocked with 20 µM DHβE (Figure 41 B). As there was nicotine induced apoptosis, I examined the expression of p53, a proapoptotic gene that is often induced in the cell death pathway. Again using semi-quantitative RT-PCR, I observed an increase in p53 transcript after nicotine treatment (Figure 41 C). Control levels of p53 in the non treated embryos (0) was lost in
the 1:135 cDNA dilution; meanwhile, elevated levels of p53 transcript remained in 100 µM (100) nicotine and 200 µM (200) nicotine treatment groups.

Figure 41: Nicotine induced apoptosis and RT PCR of p53 RNA expression after 100 µM and 200 µM nicotine treatment in 72 hpf embryos

A: TUNEL labeled whole zebrafish embryos after 3 days of nicotine treatment. Qualitatively there was an increase in the number of apoptotic cells in the tectum as the dose of nicotine increased from 0, 50, 100, or 200 µM. B: Cross sections of TUNEL labeled of zebrafish embryos after 3 days of nicotine treatment. Qualitatively there was an increase in the number of apoptotic cells in the tectum as the dose of nicotine increased from 0 to 50 µM. Additionally, apoptosis was blocked with treatment of 20 µM DHβE, a nicotinic antagonist. C: Semi-quantitative RT PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135 was used to determine if nicotine treatment changed levels of the proapoptotic gene, p53. Embryos were treated for 3 days with 0, 100 or 200 µM nicotine, RNA was isolated, 3 µgs of RNA was reverse-transcribed and amplified using subunit-specific primers. Clearly there was an increase in p53 RNA levels after nicotine treatment regardless of the level of cDNA dilution.
Nicotine, like other substances of abuse, such as cocaine and alcohol, induces the expression of immediate early genes such as c-fos and jun-B in various brain regions. Because these immediate early genes function as transcription factors, their nicotine-mediated upregulation suggests that nicotine may regulate the expression of additional genes. Treatment of zebrafish embryos from 2 hpf to 72 hpf with 100 µM and 200 µM nicotine altered transcript levels of the α2, α4, α6 and α7 nAChR subunit RNAs. Using semi-quantitative RT PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135, I determined that transcript levels of α2, α4, α6 and α7 nAChR subunits increased after nicotine treatment. In a 1:5 serial dilution of cDNA, there was an increase in α2 transcript with 100 µM or 200 µM nicotine treatment (Figure 42 A). In a 1:135 serial dilution of cDNA, there was a slight increase in α4 transcript with 100 µM and a more robust increase with 200 µM nicotine treatment (Figure 42 B). In a 1:135 serial dilution of cDNA, there was a robust increase in α6 transcript with both 100 µM and 200 µM nicotine treatment (Figure 42 C). Finally, in a 1:5 serial dilution of cDNA, there was a significant increase in α7 transcript with 100 µM nicotine or 200 µM nicotine treatment (Figure 42 D). Additionally, the subunits were differentially expressed at 72 hpf as evident by RT PCR. The α2 and α7 nAChR RNA, which have a more limited in situ hybridization expression pattern, were diluted out by a 1:5 dilution of the cDNA. In contrast, α4 and α6 transcript, which are expressed at high levels at 72 hpf, were not significantly altered until a 1:135 dilution of the cDNA. Overall, these results identify
gene expression changes in nAChR subunits after nicotine treatment that may contribute
to further defining how nicotine exerts its effects on development.

**Figure 42: Semi-quantitative RT PCR of α2, α4, α6, α7 nAChR RNA expression in 72 hpf zebrafish embryos after nicotine treatment**
Semi-quantitative RT PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135 was used to determine if nicotine treatment changed levels of α2, α4, α6 and α7 nAChR RNA. Embryos were treated for 3 days with 0, 100 or 200 µM nicotine, RNA was isolated, 3 μgs of RNA was reverse-transcribed and amplified using subunit-specific primers. **A:** in a 1:5 serial dilution of cDNA, there was an increase in α2 transcript with 100 µM or 200 µM nicotine treatment. **B:** in a 1:135 serial dilution of cDNA, there was an increase in α4 transcript with 100 µM and a more robust increase with 200 µM nicotine treatment. **C:** in a 1:135 serial dilution of cDNA, there was a robust increase in α6 transcript with both 100 µM and 200 µM nicotine treatment. **D:** in a 1:5 serial dilution of cDNA, there was a significant increase in α7 transcript with 100 µM nicotine or 200 µM nicotine treatment.
As nicotine treatment in zebrafish provoked swimming deficits and paralysis, I examined whether or not nicotine induced changes in Islet 1 and Shh transcript levels. Islet 1 and Shh are two genes that are involved in the development of motor neurons. Both Islet 1 and Shh transcript levels increased after nicotine treatment. In a 1:135 serial dilution of cDNA, there was a robust increase in Islet 1 transcript with 100 µM and a slight increase with 200 µM nicotine treatment. Conversely, in a 1:135 serial dilution of cDNA, there was an equal increase in Shh transcript with 100 µM and 200 µM nicotine treatment.

![Semi-quantitative RT PCR of Islet 1 and Shh RNA expression in 72 hpf zebrafish embryos after nicotine treatment](image)

**Figure 43: Semi-quantitative RT PCR of Islet 1 and Shh RNA expression in 72 hpf zebrafish embryos after nicotine treatment**

Semi-quantitative RT PCR and serial dilutions of cDNA from 1:5, 1:15, 1:45 and 1:135 was used to determine if nicotine treatment changed levels of Islet 1 and Shh RNA. Embryos were treated for 3 days with 0, 100 or 200 µM nicotine, RNA was isolated, 3 µgs of RNA was reverse-transcribed and amplified using subunit-specific primers. **A:** in a 1:135 serial dilution of cDNA, there was a robust increase in Islet 1 transcript with 100 µM nicotine treatment and an increase with 200 µM nicotine treatment. **B:** in a 1:135 serial dilution of cDNA, there was an equal increase in Shh transcript with 100 µM nicotine or 200 µM nicotine treatment.
6.2 In vitro Nicotine Treatment

In addition to the in vivo work using zebrafish embryos, I have begun using the ZEM2S cell line—derived from an embryonic stem cell culture. Cell culture would allow for a quick screening process to examine changes in transcript levels after drug treatments. Preliminary screening of the ZEM2S indicated that α2, α4, α6, α7, and β2 nAChR subunit RNAs, not α3 and α8, along with Shh, Islet 1, p53 and cfos were present (Figure 44 A). β2 and β4 nAChR RNAs were not screened. The expression of these genes in cell culture perfectly modeled the nAChR expression in 10 hpf zebrafish embryos (Figure 44 B) where α2, α4, α6, α7, β2, and β3 nAChRs, along with Shh, Islet 1, p53 and cfos were also expressed. Therefore, the ZEM2S cell line may be a valid model of examining nAChR during early developmental time points.
Figure 44: RT PCR of genes expressed in ZEM2S cell line versus 10 hpf zebrafish

RT-PCR was used to determine which nAChR subunit RNAs, along with cfos, p53, Islet 1 and shh, were expressed in the zebrafish cell line ZEM2S and then compared to the expression profile in 10 hpf zebrafish embryos. 3 µgs of RNA from each age zebrafish (h=hpf, d=dpf) were reverse-transcribed and amplified using subunit-specific primers. β-actin was also amplified for each age. As controls, PCR was performed using zebrafish RNA without reverse transcription (-r) or without cDNA (-d). The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker. **A:** α2, α4, α6, α7, and β2 nAChR subunit RNAs, not α3 and α8, along with Shh, Islet 1, p53 and cfos were present in ZEM2S. **B:** α2, α4, α6, α7, β2, and β3 nAChRs, along with Shh, Islet 1, p53 and cfos were expressed in 10 hpf zebrafish embryos.

Preliminary studies indicate that the paradigm for nicotine treatment in ZEM2S differs from that of nicotine treatment in whole zebrafish embryos. Changes in cfos transcript levels were not detected with 3 days of nicotine treatment as in the zebrafish (Figure 45). But transcript levels were effectively altered after 12 hr (Figure 45 A) and 24 hr (Figure
45 B) of nicotine treatment, with slight changes at 48 hr (Figure 45 C), and no changes after 72 hr (Figure 45 D) of drug treatment. Therefore, future studies involving the treatment of the ZEM2S zebrafish cell line with cholinergic drugs will allow for 12-24 hr of drug exposure.
Figure 45: Changes in cfos expression in ZEM2S treated with nicotine for 12, 24, 48 and 72 hours

RT PCR was used to determine if nicotine treatment changed transcript levels of the early immediate gene, cfos in a zebrafish cell line. ZEM2S cells were treated for 12, 24, 48, or 72 hours with 0, 50, 100 or 200 µM nicotine, RNA was isolated, 3 µgs of RNA was reverse-transcribed and amplified using subunit-specific primers. As controls, PCR was performed without reverse transcription (-rt) or without cDNA (-dna) (data not shown), and β-actin was also amplified. The size of each PCR product was consistent with the size predicted by the cDNA sequences. φx 174 HaeIII DNA (φχ) was used as a size marker. A and B: Clearly there was an increase in cfos transcript after nicotine treatment at 12 hours (A) and 24 hours (B) of drug exposure, specifically when treated with 100 µM nicotine. C: there was a slight change in transcript levels after 48 hours of 50 µM and 100 µM nicotine exposure. D: no changes in cfos transcript were visible after 72 hours of 50 µM, 100 µM, or 200 µM nicotine treatment.
Conclusions:
The results from our laboratory presented in this manuscript represent a continuation in the study of the neuronal nAChR in the vertebrate zebrafish model. Our research efforts have resulted in the cloning of the chrna4 and chrna6 zebrafish neuronal nAChR genes. Full-length cDNAs for these zebrafish neuronal nAChR subunit genes were determined and sequence analysis showed that these cDNAs demonstrated a high degree of homology to nAChR genes found in other species. To date, it does not appear that the α4 or α6 genes were duplicated in the zebrafish genome. Additionally, our laboratory has determined RNA expression patterns and examined the functional roles that nAChRs may play in nervous system development. Time course studies using RT-PCR and subunit-specific primers to each of the chrna2, chrna4, and chrna6 subunits have been determined. All of these subunits were expressed very early in development, with chrna2 RNA being expressed maternally in unfertilized embryos, chrna4 expressed by 3 hpf and chrn6 RNA present by 10 hpf.

In situ hybridization studies using sense and antisense digoxigenin-labeled RNA probes were also used to localize the temporal and spatial expression of nAChR RNA in developing embryos. Each of the subunits displayed unique expression patterns that were transiently regulated. The zebrafish chrna4 RNA was expressed in a subset of hindbrain neurons in rhombomeres 4-7 and in cells consistent with cranial neural crest cells migrating along the pharyngeal arch at 24 hpf. Additionally, limited expression was observed in forebrain and midbrain structures. At 48 hpf significant bilateral expression was seen in both midbrain and hindbrain consistent with the nucleus of the medial longitudinal fascicle and reticulospinal neurons, with no expression detected in the spinal
cord. At 72 and 96 hpf, *chrna4* continued to be highly expressed in specific midbrain and hindbrain areas.

At 24 hpf, zebrafish *chrna6* RNA was expressed in a subset of Rohon Beard sensory neurons, ventral neurons in the spine, diencephalon, trigeminal ganglion, pineal, and in the first hindbrain rhombomere ventral to the cerebellum. At 48 hpf *chrna6* was no longer observed in spinal neurons, but was still expressed in trigeminal ganglion, pineal, and was now co-localized to the *TH*+ locus coeruleus and the *TH*+ midbrain diencephalic catecholaminergic cluster. *chrna6* was highly expressed in retina at 48 hpf (not detected in 24 hpf retina) and expressed in tectum which was not detected 24 hpf. At 72 and 96 hpf, zebrafish *chrna6* continued to be expressed in trigeminal ganglion, retina, and pineal. *chrna6* RNA was highly expressed in tectum at 72 and 96 hpf zebrafish, at robust levels with more widespread distribution than at 48 hpf. Additionally, at 96 hpf *chrna6* expression was detected for the first time with a pattern consistent with cranial sensory neurons in the hindbrain. At 72 hpf and 96 hpf *chrna6* RNA continued to be expressed in the diencephalic catecholaminergic cluster, but was also present in non-catecholaminergic cells in both midbrain and hindbrain.

At 10 hpf, around the time of somitogenesis, *chrna2* expression was heavy in the anterior head region with some labeling in the dorsal spine. By 18hpf, there was diffuse labeling in the brain and a well defined pattern in the anterior spine. At 24 hpf *chrna2* was expressed in the forebrain consistent with the olfactory bulb, interneuron in the first 1-7 hemisegments of the spine, interneurons that were localizaed down the entire length of the spinal cord, and cells along the midbrain/hindbrain boundary. At 48 hpf *chrna2* forebrain expression had disappeared and midbrain expression was prominent with very
limited expression in hindbrain. The distinct pattern in the spine remained intact. By 72 hpf all the spinal labeling had disappeared, but robust expression remained in the midbrain with limited expression in the hindbrain. By 96 hpf chrna2 expression was still evident in the midbrain.

An antisense oligonucleotide morpholino gene knock down approach was used to determine the function of chrna2 during development. Knock down of the chrna2 resulted in swimming deficits, paralysis, and a disruption in the number, morphology, and extension of dorsal projecting motor neurons to the dorsal myotome. The loss of chrna2 did not affect ventral projecting motor neurons.

Expression of the zebrafish chrna2 subunit RNA in Xenopus oocytes provided preliminary evidence of functional nAChRs in the zebrafish model. Whole-cell recordings demonstrated that the zebrafish chrna2 subunit was able to assemble as a heteromeric receptor and, upon activation, produced currents characteristic of α2 nAChRs in other species.

Treatment of zebrafish with nAChR agonists and antagonists was done to further examine the effect of the nAChR on development. Treatment of zebrafish embryos with 50, 100 or 200 µM concentrations of the nAChR agonist nicotine during specific periods of development resulted in motor behavior deficits, paralysis, and the induction of apoptosis. This nicotine-induced cell death was blocked when the nAChR antagonist, DHβE, was co-applied with nicotine, confirming this mechanism involved nAChRs. Embryos treated with nicotine displayed a nicotine-mediated upregulation of chrna2, chrna4, chrna6, chrna7, cfos, p53, islet 1 and shh transcripts.
In addition to the in vivo work using zebrafish embryos, preliminary screening the zebrafish ZEM2S cell line indicated that α2, α4, α6, α7, and β2 nAChR subunit RNAs, not α3 and α8, along with Shh, Islet 1, p53 and cfos were present. β2 and β4 nAChR RNAs were not screened. The expression of these genes in cell culture perfectly modeled the nAChR expression in 10 hpf zebrafish embryos where α2, α4, α6, α7, β2, and β3 nAChRs, along with Shh, Islet 1, p53 and cfos were also expressed. Preliminary studies indicate the nicotine induced upregulation of cfos transcript is evident in the cell line after 12 or 24 hours of drug exposure.

Although this preliminary work has generated a substantial amount of data in several areas, much work remains to be done in the zebrafish model. Future plans using the zebrafish to study the nAChRs include the determination of expression patterns of the remaining nAChR subunits, the use of morpholino oligonucleotides to detect the functions of specific nAChRs subtypes, and the examination of the effect of other cholinergic agents on nervous system development. Our results indicate that zebrafish will provide an excellent model for the continued study of the role of cholinergic receptors in neural development.
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