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ZEBRAFISH AS A MODEL SYSTEM TO STUDY DOPAMINE-RELATED NEUROLOGICAL DISORDERS

A Thesis in

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By

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ABSTRACT

The mechanism by which brain circuitry is wired to produce various functional outputs such as emotion, locomotion, learning and memory remains poorly understood. Dysregulation of these processes can lead to neuropsychiatric and neurodegenerative disorders such as schizophrenia and Parkinson's disease. While both of these diseases are related to dysfunction in dopamine signaling, genetic analyses have shown no alterations in the genes encoding dopamine receptors in patients with these diseases. In addition, the mechanisms underlying aberrant dopaminergic signaling in these neuropyschiatric disorders remain enigmatic. Available treatments relieve the primary symptoms of these neurological disorders, but introduce a new host of unwanted side effects. Therefore, it is important to identify new targets new treatments for these patients. In order to determine the molecular mechanisms involved in these neuropathogies, it will be crucial to have a valuable animal model system.

Zebrafish have emerged as an important vertebrate model system not only to identify new genes and determine their function, but also as a high throughput model to identify new drugs and their targets. The main goal of our research is to identify and characterize the D2-like dopamine receptors in zebrafish. Furthermore, we hypothesize that the identification and characterization of the molecular components of the DA system in zebrafish will lead to new insights regarding regulation of dopamine signaling.

The zebrafish model system provides several advantages for identifying and characterizing genes and their function. The zebrafish embryo develops ex utero and is transparent, which allows for gene expression analysis using whole-mount *in situ* hybridization. Zebrafish are amenable to rapid forward and reverse genetic techniques. Mutant zebrafish can be screened morphologically and behaviorally to identify new genes and their function. Antisense morpholino oligonucleotides make it possible to knock down expression of any specified gene during the first several days of development, in order to determine its function. Zebrafish display a cohort of behaviors related to DA signaling, including locomotion and conditioned place preference. Additionally, chemicals and drugs can be added directly to the water, which makes zebrafish an attractive model system for toxicology and drug screening.

This work has focused on two families of G-protein-coupled receptors; the D2-like dopamine receptors and the A2-like adenosine receptors. Both families of receptors have been implicated in dopamine-related neurological disease. To better understand the function of these genes, we have cloned and characterized these genes in zebrafish.

In order to identify dopamine receptor genes, we performed searches of the zebrafish genomic sequence database, which yielded contigs containing segments of several D2-like dopamine receptor genes. From these sequences, we amplified full-length cDNAs encoding three D2, one D3, and three D4 receptor subtypes via RT-PCR. The predicted proteins ranged from 57-72% amino acid identity when compared to the human dopamine receptors. Zebrafish dopamine receptor genes were mapped by using the T51

radiation hybrid panel. With the exception of *drd2b* and *drd4b*, the remaining dopamine receptor genes mapped to positions found to be syntenic to regions of human chromosomes containing the orthologs of these dopamine receptor genes. To further characterize these genes, whole-mount *in situ* hybridization was used to investigate their expression during development. All of the receptor genes were found predominantly throughout the central nervous system and exhibit distinct but overlapping expression patterns during embryogenesis.

In order to analyze the function of the dopamine receptors identified, we examined the effect of the atypical antipsychotic clozapine (a D4R antagonist) on swimming behavior of zebrafish larvae. Clozapine produced a rapid and profound sedative-like effect on the fish. This response was dose-dependent and reversed by ABT 724 (a selective D4R agonist), but was not reversed by quinpirole (a D2/D3R agonist). The response of zebrafish to clozapine provides a novel platform for performing genetic screens designed to identify genetic components involved in D4-mediated dopaminergic signaling.

In the final chapter, we explore the mechanism of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is commonly used to model Parkinson's disease in various laboratory animals. Zebrafish are susceptible to MPTP, which induces the selective loss of dopaminergic neurons. Caffeine, an A1/A2a adenosine receptor antagonist, has neuroprotective properties against the toxicity of MPTP in mammals. In order to determine whether caffeine is neuroprotective in zebrafish, embryos were co-incubated with MPTP and caffeine. The presence of the dopamine transporter gene,

which specifically labels dopaminergic neurons, suggests a neuroprotective effect of caffeine against MPTP in zebrafish embryos. This assay suggests zebrafish can be used to screen drugs with potential neuroprotective activity. Neuroprotection of caffeine is believed to be mediated through the direct antagonism of the A2a adenosine receptors. In order to determine whether these receptors exist in zebrafish, we performed searches of the zebrafish genome database which lead to the identification and cloning of two A2a and one A2b AR genes. These studies suggest that a similar mechanism of dopaminergic neuroprotection may exist in zebrafish.

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List of Abbreviations

AC	adenylyl cyclase
ADHD	attention deficit hyperactivity disorder
ANOVA	analysis of variance
AR	adenosine receptor
bp	base pairs
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
DA	dopamine
DAT	dopamine transporter
DM	distance matrix
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpf	days post fertilization
DR	dopamine receptor
EPS	extrapyramidal symptoms
EST	expressed sequence tag
GPCR	G protein coupled receptor
hpf	hours post fertilization
IL	intracellular loop
μM	micromolar
MP	maximum parsimony

mRNA	messenger ribonucleic acid
ORF	open reading frame
PCR	polymerase chain reaction
PD	Parkinson's disease
RH	radiation hybrid
RNA	ribonucleic acid
RT	reverse transcriptase
SZ	schizophrenia
TM	transmembrane

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Chapter One

Literature Review

1.1 Mammalian Dopaminergic Neurotransmission

Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain and mediates a wide variety of functions including locomotion, cognition, emotion, and neuroendocrine secretion (Missale et al., 1998). In the mammalian brain, the central dopaminergic system is comprised of four main pathways: the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular. The mesolimbic pathway originates from the ventral tegmental area of the midbrain and projects to the nucleus accumbens and amygdala. This pathway is primarily involved in cognitive functions, memory, and emotion. The mesocortical pathway also projects from the ventral tegmental area to cortical structures where it is involved in motivation and reward. The nigrostriatal pathway originates in the substantia nigra and projects to the striatum. This pathway is involved in extrapyramidal motor function. Lastly, the tuberoinfundibular pathway projects from the hypothalamus to the hypophysis where it is involved in neuroendocrine regulation, specifically prolactin secretion (Missale et al., 1998).

Dysfunction in dopaminergic signaling is believed to underly several neuropathologies including schizophrenia (SZ) and Parkinson's disease (PD). It is hypothesized that schizophrenia is due to dopamine hyperactivity in the brain (Matthysse, 1974), which may be due to the dysregulation of dopamine receptor signaling. Much of what is known about schizophrenia has been driven by the development of antipyschotics which antagonize D2-like dopamine receptors. Even though antipsychotic drugs effectively treat the positive symptoms of schizophrenia, the drugs can cause discomforting side

effects such as unwanted sedation and Parkinsonian-like movements. In contrast to schizophrenia, PD displays a decrease in dopaminergic signaling due to the degeneration of dopaminergic neurons in the substantia nigra (Fahn, 2003). The lack of dopamine signaling in PD patients results in symptoms that include tremors, rigidity and bradykinesia (slowed movement).

In the periphery, dopamine also been implicated in the regulation of renal, cardiovascular, and neuroendocrine function. In the kidney, dopamine has been shown to increase the filtration rate and inhibit sodium reabsorption leading to an increase in the excretion of sodium and water (Missale et al., 1998). It has been suggested that deficiencies in dopaminergic responses may be associated with high blood pressure and hypertension. In the cardiovascular system, dopamine signaling is involved in inhibiting norepinephrine release and which indirectly causes vasodilatation and decreases cardiac contractility (Goldberg and Rajfer, 1985). Dopamine has also been found to stimulate and inhibit epinephrine and norepinephrine release and to regulate the secretion of aldosterone in the adrenal cortex (Porter et al., 1992; Vizi et al., 1993).

1.2 The Mammalian Dopamine Receptors

1.2.1 Classification of Dopamine Receptors

Dopamine mediates its diverse effects by activating dopamine receptors, a small family of G-protein coupled receptors (GPCRs). In mammals, five distinct dopamine receptors

(D1, D2, D3, D4, and D5) have been identified. All of the receptors exhibit the typical seven-transmembrane domain α-helical GPCR structure, including an extracellular N-terminus and an intracellular C-terminus. The five receptors are divided into two families based on structural similarities, pharmacological profiles, and signaling properties. The D1-like family consists of D1 and D5 and the D2-like receptor family consists of D2, D3 and D4 (Table 1.1) (Missale et al., 1998).

Analysis of the primary structure of the D1-like receptors reveals a shorter third intracellular loop (IC3) and a longer carboxyl-terminal tail compared to the D2-like receptors (Civelli et al., 1993). These structural differences are reflected in the functional differences between the D1-like and D2-like families. It is hypothesized that the long C-terminal tail of the D1-like family is the site of G-protein coupling, whereas the long IC3 of the D2-like family is the site of the G-protein coupling (Missale et al., 1998). D1-like receptors couple to stimulatory subsets of G-proteins (Gs) to activate adenlyl cyclase (AC), which leads to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP) (Kimura et al., 1995; Missale et al., 1998). The D2-like subfamily couples to inhibitory subsets of G-proteins, which inhibits AC and decreases levels of intracellular cAMP (Albert et al., 1990; Plug et al., 1992).

The activation of dopamine receptors and subsequent alterations in cAMP levels act, in part, to modulate intracellular calcium levels. The D1-like receptors can regulate the release of calcium by affecting calcium stores and calcium channels (Missale et al., 1998). The first mechanism is via the activation of phospholipase C (PLC) and

Table 1.1. Characteristics of Mammalian D2-like Dopamine Receptors

Receptor Subtype	Structure	Expression	Chromosomal location
D1-like			
D1	short IC3 long C-terminal tail	most highly expressed limbic system, hypothalamus, thalamus	5q35.1
D5	short IC3 long C-terminal tail	poorly expressed limbic system	4p16.1
D2-like			
D2	long IC3 short C-terminal tail	striatum, olfactory tubercle, VTA* nucleus accumbens, substantia nigra	11q23
D3	long IC3 short C-terminal tail	limbic system, striatum, substantia nigra VTA*, hippocampus, cerebellum	3q13.3
D4	long IC3 short C-terminal tail	frontal cortex, amygdala, hippocampus hypothalamus, midbrain, retina	11p15.5

*VTA, ventral tegmental area

production of inositol 1,4,5-triphosphate (IP_3), which mobilizes intracellular calcium stores (Jose et al., 1995). In addition, it has been shown that D1-like receptor activation can stimulate the release of intracellular calcium stores via the activation of protein kinase A (PKA). The second mechanism involves the regulatory phosphorylation of P-, N-, and L-type calcium channels (Lin et al., 1995). Finally, D1-like receptor signaling has been implicated in inhibitory regulation of the Na^+/H^+ exchanger and Na^+-K^+-ATPase (Felder et al., 1993; Felder et al., 1990).

D2-like receptors typically cause a decrease in intracellular calcium levels by inhibition of inward calcium currents (Seabrook et al., 1994a; Seabrook et al., 1994b). This subfamily of receptors has also been shown to activate potassium currents, leading to neuronal cell hyperpolarization (Castelletti et al., 1989; Greif et al., 1995; Williams et al., 1989). As opposed to the inhibitory regulation of the Na^+/H^+ exchanger by D1-like receptors, the D2-like receptors can activate this ion exchanger in many cells (Felder et al., 1993).

1.2.2 Gene Structure and Receptor Variants

The genomic organization of the DA receptors supports the idea that they are derived from the divergence of two ancestral gene families according to the absence or presence of introns in their coding sequence. The D1 and D5 receptor do not contain intron sequences which is a common characteristic of most GPCRs (Dohlman et al., 1987). In contrast, the D2-like receptor genes are interrupted by introns. The D2 receptor coding

region contains six introns, the D3 receptor coding region five, and the D4 receptor coding region three (Missale et al., 1998). The presence of introns in the D2-like dopamine receptor genes allows for the generation of receptor variants.

1.2.2.1 D2 Receptor

Alternative splicing generates two isoforms of the D2 receptor called D2S (short) and D2L (long). The shorter isoform of D2 is created by the splicing of an 87-bp exon between introns 4 and 5 which corresponds to a shorter IC3 domain. Because it is proposed that the IC3 is important in G protein recognition and coupling, it was hypothesized that D2S and D2L could display differential roles. The isoforms have been shown to display differential affinity for specific G proteins. Studies conducted in JEG3 cells suggest the D2L can specifically interact with Gαi2 (Montmayeur et al., 1993). In mammals, the D2S receptor exhibits a predominant presynaptic localization whereas the D2L receptor is postsynaptically situated (Khan et al., 1998). In addition, the isoforms display differential subcellular localization in NG108-15 cells. In this experimental system D2S was located at the plasma membrane while D2L was predominantly found in the Golgi apparatus. This differential trafficking of the D2 receptor is hypothesized to be due to the interaction of D2L with heart-type fatty acid binding protein (H-FABP) (Takeuchi and Fukunaga, 2003).

1.2.2.2 D3 Receptor

Multiple splice variants of the D3 receptor have been identified and all are truncated versions of the full-length D3 transcript. The longest and most notable variant, D3nf, contains only 5 of the 7 transmembrane domains due to a premature stop site (Liu et al., 1994). D3nf can bind to D3 and cause mislocalization of D3 receptors to the cytoplasm (Karpa et al., 2000).

1.2.2.3 D4 Receptor

The D4R gene contains a variable number of tandem repeats (VNTR) of 48 base pairs in exon 3 that codes for a variation in the third intracellular loop of the D4R. The number of repeats varies from 2 to 10 and is indicated as D4.2 through D4.10 (Helmeste and Tang, 2000). Globally, the most common variant is the D4.4 receptor followed by the D4.7 receptor. Initial efforts were aimed at linking these polymorphisms in the IC3 to neurological disorders such as schizophrenia and attention-deficit hyperactivity disorder however, these findings could not be substantiated by later findings (Helmeste and Tang, 2000). Interestingly, individuals with non-functional D4 receptors have been identified and there have been no obvious psychiatric disorders in these individuals (Di Bella et al., 1996; Nothen et al., 1994).

1.3 Dopamine Receptor Distribution

All of the dopamine receptor subtypes are differentially expressed throughout the mammalian brain. Immunostaining with subtype specific antibodies and *in situ* hybridization have been used to localize each receptor subtype. Of the five dopamine receptor subtypes, the D1 receptor is the most highly expressed and displays the most widespread expression (Missale et al., 1998). D1 mRNA transcripts have been localized to the striatum, nucleus accumbens, and olfactory tubercle. In addition, D1 mRNA has been found in the limbic system, hypothalamus, and thalamus. In contrast to the widespread expression of the D1 receptor, the D5 receptor is poorly expressed and is restricted to the limbic structures of the brain (Tiberi et al., 1991). Weak signals for D5 transcripts are detected in the thalamus, hippocampus, striatum, and cerebellum (Gingrich and Caron, 1993). D1 and D5 are differentially expressed at the cellular and subcellular levels shown by coexpression studies suggesting that D1 and D5 are not functionally redundant (Missale et al., 1998).

Within the D2-like subfamily, the D2 receptor displays the most abundant and diverse expression. The D2 receptor is found predominantly in the striatum, olfactory tubercle, and nucleus accumbens (Missale et al., 1998). Transcripts of the D2 receptor are also found in the substantia nigra and in the ventral tegmental area, which are the areas that give rise to the major dopaminergic pathways of the brain (Gingrich and Caron, 1993). The long and short splice variants of D2 are differentially expressed (Khan et al., 1998). Of the two isoforms, D2L is more abundantly expressed in the brain, including the

striatum, nucleus accumbens, and cortex (Usiello et al., 2000). D2S is most highly expressed in the midbrain where it is found at the presynaptic membrane (Khan et al., 1998). The localization of D2S at presynaptic sites supports the function of D2S as an autoreceptor.

The D3 receptor has a much more restricted expression than D2. The D3 receptor is expressed primarily in the limbic structures of the brain such as the nucleus accumbens and olfactory tubercle. A low abundance of D3 transcripts can also be found in the striatum, substantia nigra, in the ventral tegmental area, and hippocampus. D3 mRNA expression is also found in the cerebellum, however no dopaminergic projections are present in this area. This suggests the D3 receptor found in the cerebellum can respond to extrasynaptic dopamine (Missale et al., 1998).

Low levels of D4 mRNA transcripts have been localized to the basal ganglia. In contrast, the D4 receptor is highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, midbrain, and retina (Cohen et al., 1992; Missale et al., 1998; Van Tol et al., 1991). Antibody staining has revealed D4 receptors in GABAergic neurons of the cerebral cortex and hippocampus. Based on these expression patterns, it has been proposed that the D4 receptors modulate GABAergic transmission (Mrzljak et al., 1996).

1.4 Dopamine Receptor Functions: Knockout Mice

The *in vivo* functional specificities of the five DR subtypes are not completely understood partly due to the lack of selective ligands for the individual DRs (Holmes et al., 2004). Knockout mice have thus been an instrumental tool in identifying the contributions of DR subtypes on brain functions. Knockout mice deficient for each of the five DR subtypes have been generated and the phenotypes of these mice have been characterized providing a better understanding of the functions of each subtype (Table 1.2).

D1-deficient mice display a normal appearance and no apparent neurological defects, but do exhibit developmental delay and an increased mortality after weaning (Drago et al., 1994; Xu et al., 1994). Interestingly, the mice do not show an increased locomotor response after the administration of psychostimulants unlike wildtype mice suggesting that the D1 receptor is required for spontaneous motor behaviors caused by these drugs. D1-deficient mice have shown a reduction in voluntary ethanol consumption and have less motivation to work for a sucrose reward suggesting a significant role for D1 receptors in reward-related behaviors (El-Ghundi et al., 1998; El-Ghundi et al., 2003). This mutation is also believed to be related to deficits in prefrontal cortex-mediated behaviors such as working memory, response inhibition, and extinction of learned fear responses (Chudasama and Robbins, 2003; Goldman-Rakic, 1995; Milad and Quirk, 2002). Phenotypic analyses of D5R KO mice show much fewer abnormalities than shown in D1R KO mice. D5-deficient mice are healthy, develop normally, and can

Table 1.2. Phenotypes of Dopamine Receptor Knockout Mice

Receptor Knockout	Behavioral Phenotype
D1	No locomotor response to pyschostimulants Reduced ethanol consumption
D2	Postural abnormalities Delayed initiation of movement Prolonged periods of immobility Decreased drug seeking
D3	Increased drug seeking
D4	Superior rotarod motor coordination Aversion to novelty Locomotor supersensitivity to ethanol and pyschostimulants
D5	Enhanced motor coordination

reproduce normally. Because the hyperactivity-inducing effects of D1-like agonists and cocaine are attenuated in D5 KO mice, it is hypothesized that the D5 receptor normally contributes to the hyperactivity-inducing effects of dopamine mimetics (Centonze et al., 2003; Elliot et al., 2003; Holmes et al., 2001).

D2-deficient mice are viable, however they weigh less than wild-type mice, they are developmentally delayed, and they are less fertile than their WT littermates. One of the most striking features of D2 KO is postural abnormalities (Baik et al., 1995; Jung et al., 1999). These mice exhibit a hunched posture, paw flattening, and sprawling of the hind legs. In addition, locomotor functions are altered in D2 KO mice, which include delayed initiation of movement, decreased spontaneous locomotor activity, and prolonged periods of immobility (Baik et al., 1995). Reward behavior is affected in these mice as well. In a drug place preference paradigm, D2 mutant mice no longer show a preference for the drug-associated compartment suggesting the rewarding properties of drug require D2R (Glickstein and Schmauss, 2001). Because D2R are localized both pre- and post-synaptically on dopamine terminals, this receptor can function as an autoreceptor to inhibit dopamine release. Consistent with this functional role, D2 null mice can no longer control the inhibition of dopamine release in the midbrain. Additionally, isoform specific D2 null mice have been generated to study the distinct functional properties of this receptor. D2 autoreceptor function is absent in D2S/D2L deficient mice, but retained in D2L KO mice suggesting D2S major function as the D2 autoreceptor in the mammalian brain (Centonze et al., 2002).

D3 KO mice develop normally, are fertile, and show minimal neurological abnormalities. D3-deficient mice have increased body fat when maintained on a high-fat diet and exhibit blunted hyperphagic responses to leptin and insulin suggesting a role for D3R in body weight regulation (McQuade et al., 2004). The dense localization of D3R to the mesocortical pathway has led to the hypothesis that this receptor may play a role in mediating reward behavior. Indeed, D3R KO mice show increased conditioned place preference to amphetamine and morphine (Narita et al., 2003; Xu et al., 1997).

Similarly to D3 KO mice, D4-deficient mice develop normally, are viable, and show no gross neurological abnormalities. Interestingly, D4 KO mice show superior rotarod motor coordination compared to their WT littermates and exhibit supersensitivity to ethanol and psychostimulants (Katz et al., 2003; Rubinstein et al., 1997). D4 KO mice also exhibit a high aversion to novelty. There is some evidence of a similar finding in humans. Humans with the D4R variant that has decreased sensitivity to ligands show significantly lower levels of novelty seeking (Paterson et al., 1999). The link between the D4R and decreased novelty seeking is yet to be fully understood.

1.5 Disorders Relating to the Dysfunction of Dopaminergic Signaling

1.5.1 Schizophrenia

1.5.1.1 Pathology of Schizophrenia

Schizophrenia is a neuropsychiatric disorder that affects approximately 1% of the general population. Onset of the disease ranges from late adolescence through early adulthood, occurring more often in males. Schizophrenia, like most psychiatric disorders, is complex in its origins. Schizophrenia is diagnosed by the presentation of two classes of symptoms. The positive symptoms include delusions, hallucinations, and bizarre thoughts, and negative symptoms include blunted affect, apathy, social withdrawal, and decreased motivation (Lewis and Lieberman, 2000). From a societal perspective, the cost of schizophrenia has a large economic impact. It is estimated that the cost of this debilitating disease reaches 62.7 billion dollars in the US alone (Wu et al., 2005).

1.5.1.2 Pharmacology of Schizophrenia

There was virtually no effective treatment for SZ before the 1950s until the discovery of the beneficial effects of chlorpromazine. This drug, originally used for its antihistamine properties, caused unwanted drowsiness in patients. Because of the drug's sleep-inducing property, it was tested in psychiatric patients. Chlorpromazine effectively treated the positive symptoms of SZ and allowing patients to return to society (Shen,

1999). This drug and its successor drugs were termed neuroleptics. Through various studies it was determined that neuroleptics block the D2 dopamine receptor subtype in the nucleus accumbens and prefrontal cortex and that this phenomenon was found to account for the drugs' antipsychotic effects. Administration of indirect dopamine agonists such as amphetamines was found to worsen SZ symptoms (Carlsson, 1988). It was therefore hypothesized that schizophrenia is due to dopamine hyperactivity in the brain. This hypothesis was coined the "dopamine hypothesis" (van Rossum, 1966).

Therapeutic concentrations of typical antipsychotics also effectively block D2 receptors in the basal ganglia, a region of the brain that controls motor function and in most cases causes severe extrapyramidal side effects (EPS) and tardive dyskinesia (Sanyal and Van Tol, 1997). Typical antipyschotics can also elicit other unwanted side effects such as akinesia, akathisia and hyperprolactinemia. Furthermore, these conventional antipyschotics only treat the positive symptoms of SZ and are much less effective at treating the negative symptoms.

A breakthrough in antipsychotic treatment emerged with the introduction of clozapine and led to a new class of drugs in the treatment of SZ, the atypical antipsychotics. Both classes of antipsychotics act as competitive inhibitors of D2-like dopamine receptors. In contrast the atypical antipsychotics act on other receptors such as the D4 dopamine receptors and specific subtypes of the serotonin, adrenergic, and histamine receptors. Clozapine was the first drug that had clinical effectiveness for both positive and negative symptoms of SZ with less severe side effects. Unfortunately, the use of this drug has

been restricted to refractory SZ patients due to a 1-2% incidence of potentially fatal agranulocytosis (Naheed and Green, 2001). Unraveling the mechanism of action of atypical antipsychotics like clozapine may facilitate the design of better drugs.

1.5.1.3 Genetics of Schizophrenia

The genetic cause(s) of schizophrenia remains unknown. Evidence from family, twin, and adoption studies has strongly suggested that genetics plays a large role in the etiology of schizophrenia and the risk of illness increases with affected family members. In addition, antipyschotics used to treat schizophrenia effectively block D2 receptors but there has been no evidence of mutations in the dopamine receptors are present in patients with this disorder. Genetic studies have determined several chromosomal loci implicated in this disease which has yielded the identification of several candidate genes.

Chromosome 22q11 deletions result in a genetic anomaly known as DiGeorge syndrome (Maynard et al., 2002). Patients with this deletion exhibit developmental defects such as craniofacial abnormalities. One out of four of these patients are diagnosed with schizophrenia. Currently, no genes within this region have been identified as the sole cause of schizophrenia. Catechol-O-methyl transferase (COMT) is one of the genes found on this locus. COMT encodes an enzyme that is responsible for the breakdown of catecholamines including dopamine. Therefore, it is hypothesized that mutations in this gene could result in increased dopamine levels, which may lead to psychiatric symptoms (Harrison and Weinberger, 2005).

Family linkage studies have also suggested chromosome 8p12-22 as a schizophrenia-susceptibility locus (Harrison and Weinberger, 2005). This linkage revealed an association with the disease and the gene for neuregulin 1 (NRG1). The function of NRG1 remains unclear, but it is believed to play a role in the mediation of glutamatergic mechanisms (Stefansson et al., 2002). The gene has also been proposed to play roles in various functions of the brain including axon guidance and synaptogenesis (Corfas et al., 2004).

Chromosome 1q42 has also been linked to psychopathology including schizophrenia. Two genes have been cloned from this locus, DISC1 and DISC2 (disrupted in schizophrenia). DISC2 contains no open reading frame, but it is hypothesized to regulate DISC1 expression via antisense RNA. Although the functions of DISC1 are not completely understood, initial studies suggest it plays a role in neuronal migration, neurite outgrowth, and intracellular transport (Miyoshi et al., 2003; Ozeki et al., 2003).

1.5.2 Parkinson's Disease

1.5.2.1 Pathology of Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder affecting 1% of the population over the age of 65. It is characterized by the degeneration of dopaminergic neurons in the substantia nigra resulting in a range of extrapyramidal motor deficits (Gandhi and Wood, 2005). Due to the lack of dopamine in the brain, levodopa, the metabolic precursor, is

used as a replacement therapy to alleviate the symptoms of PD. The cause of this disease is unknown, however genetic studies of patients with familial PD has uncovered mutations in several proteins, such as alpha-synuclein, parkin, DJ-1, and Pink1 (Gandhi and Wood, 2005). The identification of these genes has uncovered major pathways that may precipitate the neurodegeneration in PD. These pathways include dysfunction in the ubiquitin-proteasome system (UPS), mitochondrial dysfunction, oxidative stress, protein aggregation, and kinase signaling (Gandhi and Wood, 2005). It is unknown whether these pathways converge to cause nigral cell death or if one pathway is more significant in PD. The familial forms of PD are rare and only account for 5-10% of PD (Bossy-Wetzel et al., 2004; Bove et al., 2005). Therefore, it is hypothesized that environmental toxins may contribute to the development of sporadic PD in genetically predisposed individuals. Rotenone, paraquat, and MPTP (methyl-4-phenyl-1,2,3,6-tetrahydropyridine) reproduce parkinsonism by selectively destroying dopaminergic neurons in the substantia nigra of mammals (Bove et al., 2005).

1.5.2.2 MPTP Model of Parkinson's Disease

MPTP-induced Parkinsonism is a well-accepted and currently the most popular model to study Parkinson's disease. It was accidentally discovered in the early 1980s as a contaminant of meperidine (synthetic heroin). Individuals that were exposed to this compound developed Parkinsonian-like symptoms, including bradykinesia, tremor, and postural imbalance (Bretaud et al., 2004). Since its discovery, MPTP has been used in

the laboratory to understand how neuronal degeneration is mediated. It has been used to model PD in a variety of species ranging from worms to nonhuman primates.

The hydrophobicity of MPTP allows it to readily cross the blood brain barrier where it gets metabolized by monoamine oxidase-B (MAO-B) to 1-methyl-4-phenylpyridine (MPP+). MPP+ is selectively taken up into DA neurons via the dopamine transporter (DAT) for which it has a high affinity. Inside the cell, it exerts its toxic effects by inhibiting mitochondrial electron transport complex I which leads to an increase in reactive oxygen species and an impairment of energy metabolism leading to the degeneration of DA neurons (Smeyne and Jackson-Lewis, 2005).

Several compounds have been shown to be neuroprotective against MPTP including inhibitors of MAO-B, iron chelators, antioxidants, and caffeine. Unfortunately, there has been little success with these drugs in the clinical treatment of PD with perhaps the exception of caffeine. Epidemiological evidence suggests that caffeine intake is associated with a reduced risk of developing PD but the mechanism of neuroprotection is unclear (Ascherio et al., 2001; Ross et al., 2000). The hypothesis that caffeine represents a neuroprotective agent in PD is also supported by studies that show caffeine can protect against MPTP-induced DA neuron depletion in mice (Chen et al., 2001). Caffeine is an A1/A2a adenosine receptor antagonist. A2a receptor knockout mice show neuroprotection against MPTP suggesting the neuroprotective properties of caffeine may be thought the antagonistic actions at this receptor (Chen et al., 2001). Additionally, the A2a receptor has been found to directly interact with D2 and D3 receptors (Canals et al.,

2003; Hillion et al., 2002; Torvinen et al., 2005). These findings have important implications for the treatment of basal ganglia disorders, including PD and SZ.

1.6 A2a/D2 Receptor Interaction: Implications for Neuropsychiatric Disorders

Dopamine and adenosine signaling exert antagonistic effects in the basal ganglia, the brain region involved in neuropsychiatric disorders. Adenosine agonists promote sleep while adenosine antagonists, such as caffeine, induce motor activation. In contrast, dopamine agonists stimulate motor activation while dopamine antagonists are sedative. These opposing effects are a result of antagonistic interactions of specific subtypes of adenosine receptors and dopamine receptors in the striatum (Ferre, 1997).

The striatum is the main input structure of the basal ganglia. GABAergic efferent neurons constitute 90% of the striatal neuronal population and can be divided into two subtypes based their neurochemical profiles. The striatonigro-striatoentopeduncular neurons contain dynorphin, substance P, and D1 DRs. Striatopallidal neurons contain the peptide enkephalin, D2 receptors, and A2a receptors. In addition, striatopallidal neuron dysfunction is believed to be a main cause of the symptoms of PD and these neurons are the main target for antipsychotic drugs (Ferre et al., 1997).

Antagonistic actions between the A2a and D2 receptor was studied initially at the biochemical level. In crude membrane preparations, stimulation of A2a receptors produces a decreased affinity for agonists of the D2 receptor (Ferre et al., 1991). Thus,

A2a adenosine agonists produce a similar effect to the administration of D2 receptor antagonists (antipyschotics). These findings have led to the hypothesis that adenosine agonists or uptake inhibitors have therapeutic potential in the treatment of schizophrenia. In fact, dipyridamole, an uptake inhibitor of adenosine, in combination with the antipsychotic haloperidol has been shown to be more effective than haloperidol alone at decreasing the positive symptoms (Akhondzadeh et al., 2000). On the other hand, A2a specific antagonists are currently being developed and studied as novel therapies for the treatment of PD. Clinical trials have shown that the administration of a low dose of L-DOPA in combination with a selective A2a receptor antagonist produced symptomatic relief with reduced side-effects, such as dyskinesia (Chase et al., 2003). In addition, it has been recently determined that the adenosine A2a and dopamine D2 receptors directly interact. Colocalization and coimmunoprecipitation of the two receptors has been demonstrated in the SH-SY5Y human neuroblastoma cell line (Hillion et al., 2002). Clearly, it will be of great importance to understand the functional mechanism of this interaction and the role it may play in the neuropathology of SZ and PD.

1.7 Adenosine Signaling

1.7.1 Formation of Endogenous Adenosine

The purine nucleoside adenosine is the primary agonist of the adenosine receptor class. It is found in all living cells as part of the normal metabolic pathway that runs the cell. Adenosine is continuously formed intracellularly and extracellularly. The production of

intracellular adenosine is mediated via two distinct pathways. The first is the hydrolysis of AMP 5'-nucleotidase and the second is the hydrolysis of S-adenosyl-homocysteine. Intracellular adenosine can then be transported into the extracellular space via specific bi-directional transporters through facilitated diffusion. Extracellular adenosine is produced by the dephosphorylation of extracellular AMP which is mediated by ecto-5'-nucleotidase. When extracellular adenosine levels are high, adenosine is transported into cells via transporters. Once inside the cells, adenosine is then phosphorylated to AMP by adenosine kinase or degraded into inosine by adenosine deaminase. Physiologically, adenosine can regulate a variety of functions including, cardiac rate and contractility, wakefulness, release of neurotransmitters, platelet and white blood cell function, lipolysis, and renal hemodynamics (Fredholm et al., 2001).

1.7.2 Adenosine Receptor Subtypes

The adenosine receptor family is comprised of four receptor subtypes (A1, A2a, A2b, and A3) and all are putative seven transmembrane G-protein coupled receptors. Adenosine receptors can be differentiated based on mechanism of signal transduction. A1 and A3 couple to Gi proteins to inhibit adenylyl cyclase. A2a and A2b couple to Gs to increase cAMP (Fredholm et al., 2001). All of the receptor subtypes have been knocked out in mice with the exception of A2b. The knockout models suggest functions of adenosine receptors are related to the physiologies of the cardiovascular and nervous systems. These systems are either enhanced or reduced depending upon which the specific receptor gene has been deleted (Yaar et al., 2005).

1.7.3 A2a Adenosine Receptor and Neuroprotection

In mice, it has been shown that A2a receptor blockade or genetic depletion of the A2a receptor is neuroprotective against the selective dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is commonly used to model PD (Chen et al., 2001). In addition, due to the primary expression of the A2a receptor in the striatum, A2a antagonists were explored as potential therapeutics in PD. It has been shown that administration of the A1 and A2a antagonist, caffeine, reduces the dopaminergic toxicity caused by MPTP in mammalian systems (Chen et al., 2001). The neuroprotective mechanism of genetic depletion or blockade of the A2a receptor has yet to be determined.

1.8 Zebrafish: A Model System for Dopamine-related Neurological Disorders

There are exceptional genetic and genomic tools available in zebrafish that make it an attractive model system to further examine DA receptor mediated function in a vertebrate. Rapid forward and reverse genetic manipulations in this organism are greatly facilitated by properties that include small size, fecundity, rapid development, transparency of embryo and larvae and an ongoing genome sequencing project. Due to the transparency of the zebrafish embryo, gene expression can be analyzed using *in situ* hybridization. Zebrafish can be mutagenized and screened for morphological or behavioral phenotypes which allows for the identification of novel genes. Complementing forward genetics is the use of morpholino antisense knockdown, which

is a method used to inhibit translation of known genes during early embryonic development. However, this technology is not a permanent knockdown and repeated injections for each experiment are required. In addition, nonspecific side effects, such as widespread cell death and neuronal degeneration, have been reported (Heasman, 2002). Recently, TILLING (Targeted-Induced Local Lesions IN Genomes) has been adapted to zebrafish. TILLING can identify targeted knockouts that can be used to study gene function (Wienholds et al., 2003). This method is based on the use of CEL-1 which enzymatically cleaves mismatched DNA heteroduplexes (Oleykowski et al., 1998).

1.8.1 Dopamine System in Zebrafish

Recently, there have been several studies on the organization and function of the dopamine system in zebrafish. The ontogeny of dopaminergic neurons in zebrafish has been characterized (Bellipanni et al., 2002; Guo et al., 1999; Ma, 2003; Rink and Wullimann, 2001). The majority of DA neurons are localized to the diencephalon as opposed to the midbrain. Immunocytochemical analysis suggests the zebrafish DA neurons send ascending projection pathways to the telencephalon. These ascending pathways may represent homologous mammalian mesostriatal, mesolimbic and mesocortical pathways (Rink and Wullimann, 2002a). Immunohistochemical screens for mutants with deficits in the development of dopaminergic neurons have been identified. The *too few* and *foggy* mutant both exhibit a reduction of DA neurons in the forebrain (Guo et al., 1999). The *foggy* mutant has a disruption in a regulator of transcription

elongation while the *too few* mutant has a disruption in a zinc-finger containing protein (Tof/Fezl) (Guo et al., 2000).

1.8.2 Zebrafish as a Behavioral Model

Dopaminergic neurotransmission regulates a wide variety of functions such as locomotion, learning, memory, and reward, therefore behavioral studies are desirable in elucidating this monoamine system. Zebrafish have a large repertoire of behaviors associated with dopaminergic signaling including locomotion, visual thresholds, and conditioned place preference. The primary motor behavior exhibited by zebrafish is a spontaneous contraction of the trunk at 17 hpf. By 27 hpf, zebrafish embryos can respond to a gentle touch, particularly of the tail, and swim a short distance in response to the touch (Guo, 2004; Saint-Amant and Drapeau, 2001). At 5 dpf, zebrafish develop a more frequent swimming behavior that can be induced by a sensory cue such as escape from a predator or a goal-directed motion such as feeding (Guo, 2004). The visual thresholds of zebrafish have also been studied behaviorally. When the retinal dopaminergic interplexiform cells (DA-IPCs) of a zebrafish are destroyed the visual thresholds are shown to be elevated in a predator-escape response paradigm (Li and Dowling, 2000).

Addictive substances such as alcohol and cocaine possess powerful reinforcing properties. In mammals, the brain dopamine system is heavily implicated in this process. Zebrafish have been used as an animal model to measure motivation and reward by

examining preference behavior (Darland and Dowling, 2001). By using conditioned place preference, a common assay for studying reward-related behavior, zebrafish were shown to exhibit a preference for cocaine with only a single trial of drug exposure paired with a visual cue to elicit the preference (Darland and Dowling, 2001). In addition to cocaine, the effects of alcohol on locomotor activity have been examined in zebrafish. Adult and larval zebrafish produce a hyperlocomotor activity when exposed to low doses of alcohol and exhibit a sedative phenotype at higher doses of alcohol (Gerlai et al., 2000; Lockwood et al., 2004). These findings provide the framework for the development of rapid and robust assays to subsequently screen mutant zebrafish to potentially identify new genes underlying addiction.

1.8.3 Zebrafish and Neurotoxicity

Recently, it has been shown that larval and embryonic zebrafish are susceptible to the loss of dopaminergic neurons when exposed to the neurotoxin MPTP (Bretaud et al., 2004; Lam et al., 2005). In adult zebrafish, intramuscular injections of MPTP cause a significant reduction in the levels of dopamine in the brain (Anichtchik et al., 2004). In addition, zebrafish demonstrate behavioral changes in locomotion and swimming responses. Adult and 7 dpf larvae treated zebrafish exhibit significant reductions in locomotor activity (velocity) (Bretaud et al., 2004; Anichtchik et al., 2004). Embryos (72 hpf) treated with MPTP display reductions in response to a tactile stimuli measured by reflexive movements of the tail (Lam et al., 2005).

MPTP-induced neurotoxicity has been demonstrated to be mediated by the same pathways that have been described for mammalian species. McKinley et al. (2005) show that MPTP neurodegeneration can be inhibited by co-incubation with a MAO-B inhibitor (L-deprenyl), a DAT inhibitor (nomifensine), and gene knockdown of DAT. This study suggests MPTP is likely converted into MPP+ by MAO-B and selectively transported into dopaminergic neurons via the DAT (McKinley et al., 2005). Taken together, these studies suggest that zebrafish may be a valuable animal model for understanding the molecular mechanisms underlying MPTP-induced neurotoxicity and for testing compounds for neuroprotective properties.

1.9 Rationale and Hypothesis

Pharmacologically, neuropyschiatric disorders such as PD and SZ are treated primarily via the D2-like dopamine receptors. However, no genetic alterations in these receptors have been detected in patients. Furthermore, the current therapies used to treat patients with PD and SZ bring with them a cohort of unwanted side effects. Therefore, there is a need to identify novel gene targets involved in dopaminergic signaling and to discover new therapies for dopamine-related neurological disorders.

The newly established zebrafish vertebrate model provides the potential for exploring novel genes and drugs involved in dopamine signaling. The embryonic and adult zebrafish brain contains several clusters of dopaminergic neurons in the diencephalon. Zebrafish display behaviors implicated in dopamine signaling such as locomotion,

reward, and conditioned place preference. Additionally, chemical compounds can be added directly to the water of zebrafish embryos, larvae, and adults providing a high throughput model organism for drug discovery (Zon and Peterson, 2005). Therefore, genetic and behavioral analyses and drug screening in zebrafish may uncover important insights into the mechanisms causing neuropsychiatric disorders.

We hypothesize that the zebrafish genome encodes genes for D2-like dopamine receptors and these receptors are found in analogous brain regions to the mammalian counterpart. In addition, we hypothesize that zebrafish can be used to study the behavioral affects of inhibitors of dopamine receptors. Finally, we believe zebrafish can be used as a model system to identify compounds that protect dopaminergic neurons from the neurotoxin MPTP.

The work presented in this dissertation presents the identification and characterization of D2-like receptors in zebrafish. To date, we have amplified full-length cDNAs encoding three D2, one D3, and three D4 receptor subtypes. The ability to add drugs directly to the water allows us to potentially dissect the function of specific dopamine receptor subtypes using zebrafish. Furthermore, we propose that zebrafish can be used to identify compounds that provide neuroprotection from MPTP. Together, these studies suggest that zebrafish may be a well suited animal model system for understanding dopamine-related neurological disorders.

Chapter Two

Cloning of D2-like Dopamine Receptors in Zebrafish

2.1 Introduction

The importance of dopaminergic signaling in evolution is reflected by the fact that dopamine modulates movement and behavior in species as diverse as worms and man, and dopamine receptors have been identified in a variety of invertebrate and vertebrate species. In *Drosophila*, cDNAs encoding D1-like (Feng et al., 1996; Gotzes et al., 1994; Han et al., 1996; Sugamori et al., 1995) and D2-like (Hearn et al., 2002) dopamine receptors have been characterized. Although the *Drosophila* polypeptides are considerably divergent in sequence from their mammalian counterparts, they exhibit the signaling and pharmacologic properties characteristic of mammalian D1- and D2-like dopamine receptors (Feng et al., 1996; Gotzes et al., 1994; Han et al., 1996; Hearn et al., 2002; Sugamori et al., 1995). cDNAs encoding D1- and D2- like dopamine receptors have been identified recently in the nematode *Caenorhabditis elegans* (Suo et al., 2002; Suo et al., 2003). In the case of the putative *C. elegans* D2 receptor, two splice variants have been characterized that differ in the length of the predicted third intracellular loop (Suo et al., 2003), a situation analogous to the long and short forms of the mammalian D2 receptor (Giros et al., 1989; Monsma et al., 1989). Several D2-like dopamine receptors have been cloned and characterized from teleosts. For example, in the pufferfish *Fugu rubripes*, genes encoding the D2 and D3 receptor subtypes have been identified (Macrae and Brenner, 1995), whereas D2 receptor cDNAs have also been characterized in carp (Hirano et al., 1998) and trout (accession nos. AJ347728 and AJ347729).

Here we have initiated studies to identify and clone D2-like dopamine receptor genes in zebrafish. Mining the zebrafish genomic sequence database allowed us to identify contigs containing segments of several D2-like receptor genes. From these sequences, we were able to amplify full-length cDNAs encoding three D2, one D3 and three D4 receptor subtypes. Identification and characterization of these dopamine receptor genes provides a framework to investigate the functional properties of these genes using the powerful genetic tools and drug inhibitor studies in zebrafish.

2.2 Experimental Procedures

2.2.1 Identification of Zebrafish Dopamine Receptor Genes

Segments of zebrafish dopamine receptor genes were identified using mammalian and fish polypeptide sequences as probes in low-stringency SSAHA (**S**equence **S**earch and **A**lignment by **H**ashing **A**lgorithm) (Ning Z, 2001) searches of the zebrafish genomic reads generated by the Sanger Center (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated). Assembly of overlapping sequence reads led to identification of portions of exons representing a minimum of three D2-like, one D3-like, and three D4-like genes. Subsequent releases of preliminary genomic assemblies and tools for performing BLAST searches (http://www.ensembl.org/Danio_rerio/blastview) facilitated identification of additional exons and assignment of exons to genes.

2.2.2 Cloning of Zebrafish Dopamine Receptor Genes

Polymerase chain reaction (PCR) primers that overlapped conserved initiation and termination codons were designed based on genomic sequence reads. The corresponding full-length cDNAs were generated by RT-PCR. First strand cDNA synthesis was performed using SuperScript reverse transcriptase (Life Technologies) according to the manufacturer's protocols. Random hexamers were used to prime cDNA synthesis and total RNA from adult zebrafish was used as template. REDTaq DNA Polymerase (Sigma) was used for subsequent amplification. For each cDNA, PCR amplification was performed using nested primers. Primers and annealing temperatures are listed in Table 2.1. PCR was carried out using a TD-7500 Thermal Cycler (Hybaid). An initial 4 min denaturation step at 94°C was followed by 34 cycles at 94°C for 30 sec, 45-52°C for 30 sec, and 72°C for 90 sec. A final elongation step was carried out at 72°C for 10 min. All cDNAs were sequenced using an ABI 377 automated DNA sequencer. Sequences were verified by sequencing at least three independent clones and comparison to genomic sequences.

Table 2.1. PCR Primers for the Amplification of Zebrafish D2-like Dopamine Receptors

Clone	Forward Primer	Reverse Primer	Ta (°C)
drd2a			
PCR 1	$^{-34}$ggttctagggtctcagcttg^{-15}	^{1772}caccccatacacagtaatgttatg^{1749}	49
PCR 2	$^{-3}$ctgatggaagtcttcacagcg^{18}	^{1772}caccccatacacagtaatgttatg^{1749}	50
drd2b			
PCR 1	$^{-15}$cagaggatcttcatcatgcct^{6}	^{1320}gtgtgttcaacagtgcaggatcttgat^{1294}	50
PCR 2	$^{-7}$cttcatcatgcctgtcctgaac^{15}	^{1320}gtgtgttcaacagtgcaggatcttgat^{1294}	52
drd2c			
PCR 1	$^{-44}$gaattatgtctctcagtttcaggcttc^{-18}	^{1363}atcctcagcagtgcaatatc^{1344}	50
PCR 2	$^{-11}$ggccacagctcatggatttc^{9}	^{1363}atcctcagcagtgcaatatc^{1344}	52
drd3			
PCR 1	$^{-75}$gttacactgcatgttgtcaag^{-55}	^{1379}atatagcccatggtttagca^{1360}	45
PCR 2	$^{-35}$aaaatctgtccaccctctcc^{-16}	^{1362}gcagctcaggattttaatga^{1343}	47
drd4a			
PCR1	^{1}atggtagaggcagacatgcca^{21}	^{1157}ttagcatgctcaggctagcag^{1137}	54
PCR2	^{1}atggtagaggcagacatgcca^{21}	^{1143}ctagcagcagccaggcagcgt^{1123}	52
drd4b			
PCR1	$^{-5}$gcatcatggtcaatgtgacgcc^{17}	^{1262}caacctcaggaacgacagcagag^{1240}	54
PCR2	^{1}atggtcaatgtgacgcccagt^{21}	^{1257}tcaggaacgacagcagagaag^{1237}	52
drd4c			
PCR1	$^{-90}$gcagagatggaccacagtggaca^{-68}	^{1184}aatcagtacagtcttcagcatc^{1163}	52
PCR2	$^{-15}$ggatcaagaaggacaatgtctgc^{8}	^{1160}tatggtggatgtcagcagcagc^{1139}	52

Nucleotide +1 is the A of the ATG codon for the initiating methionine

2.3 Results

In order to identify zebrafish dopamine receptor genes, we mined the zebrafish genome sequence database. Searches of raw genomic reads (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated) and subsequent genome assemblies generated by the zebrafish genome sequencing project (http://www.ensembl.org/Danio_rerio/blastview) led to identification of portions of seven genes with sequence similarity to mammalian D2, D3, and D4 dopamine receptor genes. We utilized RT-PCR to amplify full-length cDNAs corresponding to each of these genes. There are three zebrafish D2 receptor genes (*drd2a*, *drd2b*, and *drd2c*). The D2a dopamine receptor cDNA (GenBank Accession number AY183456) contains a complete1347 bp ORF. The D2b dopamine receptor cDNA(GenBank Accession number AY333791) contains a complete1314 bp ORF, while the D2c dopamine receptor cDNA (GenBank Accession number AY333792) contains a complete ORF 1359 bp in length. We identified a single D3 dopamine receptor gene (*drd3*). The D3 dopamine receptor cDNA (GenBank Accession number AY183455) contains a complete ORF that is 1365 bp in length. We identified three zebrafish D4 receptor genes (*drd4a*, *drd4b*, and *drd4c*). The D4a DR cDNA (GenBank accession no. AY750152) contains a complete 1128 base-pair (bp) open reading frame (ORF). The D4b cDNA (GenBank accession no. AY750153) contains a complete 1257 bp ORF, while the D4c cDNA (GenBank accession no. AY750154) contains a complete ORF 1149 bp in length.

BLAST analysis indicates that each of the cDNAs encodes a polypeptide with a high degree of sequence similarity to mammalian dopamine receptors. Sequence comparisons indicate that the zebrafish polypeptides show 52-72% amino acid sequence identity to mammalian D2, D3, and D4 receptors (Tables 2.2 and 2.3). One zebrafish clone (*drd3*) shows highest identity (64-67%) to the mammalian D3 dopamine receptor, while the remaining zebrafish clones (*drd2a, drd2b, and drd2c*) share highest identity (65-72%) with the mammalian D2 dopamine receptor (Table 2.2). Pairwise sequence comparisons indicate that the subfamily of zebrafish D2 receptors share 65-74% amino acid sequence identity (Table 2.2). The *drd2a* and *drd2c* subtypes exhibit 74% amino acid sequence identity, whereas *drd2b* is 65% identical to *drd2a* and 67% identical to *drd2c* (Table 2.2*)*. In contrast, *drd3* exhibits 55-56% identity to zebrafish D2 receptors, similar to the 54-62% identity between mammalian D2 and D3 dopamine receptors. Sequence comparisons indicate that the zebrafish D4 receptor polypeptides show 56-61% amino acid identity to mammalian D4Rs (Table 2.3). Pairwise sequence comparisons indicate that *drd4a* and *drd4c* share the highest amino acid identity of 76%, whereas the *drd4b* gene is 60% identical to the *drd4a* gene and 59% identical to the *drd4c* gene (Table 2.3). Pairs of D4R genes similar to *drd4a* and *drd4c* occur in *Fugu* and medaka. The divergent *drd4b* gene also has a counterpart in medaka. Continued mining of the zebrafish genomic and EST databases has failed to uncover additional D2, D3, or D4 dopamine receptor genes. Taken together, these results indicate that zebrafish are likely to possess three separate D2 genes, a single D3 gene and three separate D4 genes.

Table 2.2. Pairwise Comparisons between Zebrafish and Mammalian D2 and D3 Receptors

Zebrafish	D3	D2a	D2b	D2c
Human D3	67	58	58	59
Rodent D3	64	55	53	52
Human D2	58	71	66	71
Rodent D2	58	72	65	72

Numbers represent percent amino acid identity

Table 2.3. Pairwise Comparisons between Zebrafish and Mammalian D4 Receptors

Zebrafish	D4a	D4b	D4c
Human D4	59	57	58
Rodent D4	59	56	61

Numbers represent percent amino acid identity

Sequence alignments of the human and predicted zebrafish dopamine D2, D3, and D4 receptor polypeptides are shown in Figures 2.1, 2.2 and 2.3, respectively. By aligning the zebrafish and human dopamine receptor polypeptides, we identified seven putative transmembrane (TM) domains that are highly conserved with the TM segments of the human D2-like receptors. In addition to the TM segments, several other regions are highly conserved between mammalian and zebrafish dopamine receptors. For D2 receptors these regions include the first and second intracellular loops, three short segments within the third intracellular loop, and the C-terminal tail (Fig. 2.1). D3 receptors exhibit fewer highly conserved regions which include the first (but not the second) intracellular loop and the C-terminus (Fig. 2.2). For D4 receptors, the majority of the conserved regions are the TM segments (Fig. 2.3). In addition, the intron/exon organization of the zebrafish D2, D3, and D4 dopamine receptor genes is virtually identical with respect to their mammalian counterparts (Figs. 2.1, 2.2, and 2.3), suggesting that the zebrafish and mammalian genes arose from a common ancestral gene. Overall, the third intracellular loop is highly divergent between zebrafish and mammals, as well as between zebrafish dopamine receptor subtypes. It is possible that this sequence divergence may reflect functional differences between the various zebrafish dopamine receptors.

Figure 2.1. Comparison of zebrafish and mammalian D2 dopamine receptors. Human, rat, and zebrafish (ZF; drd2a, drd2b, drd2c) D2 receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence. Positions of predicted intracellular loops (IL) are shown above the sequence. Arrowheads indicate the location of introns and are flanked by the corresponding exon numbers.

```
                                                           TM1
Human D2   1 ~~~~~~MDPLNLSWYDDDLERQNWSRPFNGSDGKADRPHYNYYATLLTLLIAVIVFGNVL
Rat D2     1 ~~~~~~MDPLNLSWYDDDLERQNWSRPFNGSEGKADRPHYNYYAMLLTLLIFIIVFGNVL
ZF D2a     1 ~MEVFTAYAFNESFFENA......SRDFNATEQGG.RHQYNYYAMLLTLLIFVIVFGNVL
ZF D2c     1 ~MDFLTEYPYNDTYYDNG......TGALNCTGCEA.KHQYNYYAMLLTLLIFVIVFGNVL
ZF D2b     1 MPVLNVTEELTITASPSSAVFLSLHQSNCSVSPSPSSPPYNFYAVLLVLLIFCVVFGNVL

                  IL1              TM2
Human D2  55 VCMAVSREKALQTTTNYLIVSLAVADLLVATLVMPWVVYLEVVGEWKFSRIHCDIFVTLD
Rat D2    55 VCMAVSREKALQTTTNYLIVSLAVADLLVATLVMPWVVYLEVVGEWKFSRIHCDIFVTLD
ZF D2a    53 VCMAVSREKALQTTTNYLIVSLAVADLLVATLVMPWVVYLEVVGEWRFSTIHCDIFVTLD
ZF D2c    53 VCMAVSREKALQTTTNYLIVSLAVADLLVATLVMPWVVYLEVVGEWRFSKIHCDIFVTLD
ZF D2b    61 VCVAVSREKALQTTTNYLIVSLAVSDLLLATLVMPWGVYLEVVGEWRFSRIHCDVLLTLD
                                                  1↑2

              TM3                          IL2           TM4
Human D2 115 VMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVTVMISIVWVLSFTISCPLLFGL
Rat D2   115 VMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVTVMIAIVWVLSFTISCPLLFGL
ZF D2a   113 VMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVTVMISVVWILSFAISCPLLFGL
ZF D2c   113 VMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVTVMISVVWVLSFAISCPLLFGL
ZF D2b   121 VMMCTASILNLCAISIDRYTAVAMPLLYNTRYSSRRRVALMIAVVWFLSFAISCPLLFGL
                                 2↑3

                                  TM5                         IL3
Human D2 175 NNA...DQNECIIANPAFVVYSSIVSFYVPFIVTLLVYIKIYIVLRRRRKRVNT..KRSS
Rat D2   175 NNT...DQNECIIANPAFVVYSSIVSFYVPFIVTLLVYIKIYIVLRKRRKRVNT..KRSS
ZF D2a   173 NNTVTHDDALCVIANPAFVVYSSIVSFYVPFIITLLVYVQIYVVLRKRRKRVNT..KRTC
ZF D2c   173 NNTATRDGSVCEIANPAFVVYSSIMSFYVPFIITLLVYVQIYVVLRKRRKRVNT..KRSC
ZF D2b   181 NNTASQEGRDCSFADPAFVVYSSVASFYVPFIVTLLVYVQICVVLRRGRRTAPTRRRAA
              3↑4

Human D2 230 RAFRAHLRAPLKGNCTHPEDMKLCTVIMKSNGSFPVNRRRV....EAARRAQELEMEMLS
Rat D2   230 RAFRANLKTPLKGNCTHPEDMKLCTVIMKSNGSFPVNRRRM....DAARRAQELEMEMLS
ZF D2a   231 PVTDMDMSSTIK.KCTHPDDVKLCTVIVKSSGNCPVNNKNVIFIKEVVNNGDDIQMDEIT
ZF D2c   231 QKTDADAQPPLKEKCTHPEDVKLCTVIIKTNGGVPAKNKKAQLIKEVLHQGGDVGMEMVA
ZF D2b   241 NTEPADAQRSCKNKCTHPEDVKLCTLILKPPAAAP.QRKKVTLVKEAV..VHPLAVEPVC
                        4↑5                      5↑6

Human D2 286 STSPPERTRYSPIPPSHHQLTLPDPSHHGLHSTPDSPAKP............EKNGHAK.
Rat D2   286 STSPPERTRYSPIPPSHHQLTLPDPSHHGLHSNPDSPAKP............EKNGHAK.
ZF D2a   290 NRNPSRQRKQDQSGGSQQNSRLVNSNLRETDISPPSPEAGVKP.........ERNGNTSS
ZF D2c   291 GTSPPEKKK............LASSLVVDLLATPPSPNHGSPSHAEYQSNGDEKNGHAKD
ZF D2b   298 SLSA............DREQTQTQPSGRAKLSLSVAPCAGQSGPGPRRDTLQEKTHTEKH

                                                              TM6
Human D2 333 .DHPKIAKIFEIQTMPNGKTRTSL.KTMSRRKLSQQKEKKATQMLAIVLGVFIICWLPFF
Rat D2   333 IVNPRIAKFFEIQTMPNGKTRTSL.KTMSRRKLSQQKEKKATQMLAIVLGVFIICWLPFF
ZF D2a   341 I..TKGAKAFEIQVSPTGKTQTSV.KTLNKRKISQQKEKKATQMLAIVLGVFIICWLPFF
ZF D2c   339 VQSPKETKPVETQALPNGKTRTTVTKTMSKRKMSQHKEKKATQMLAIVLGVFIICWLPFF
ZF D2b   346 .....................AAAKERVRGRLSQQKERKATQMLAIVLGVFIICWLPFF
                                                         6↑7

                             TM7
Human D2 391 ITHILNIHCD.CNIPPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFLKILHC
Rat D2   392 ITHILNIHCD.CNIPPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFMKILHC
ZF D2a   398 ITHIVNTYCQ...VPPELYTAFTWLGYVNSAVNPIIYTTFNIEFRKAFIKILHC
ZF D2c   399 ITHILKTHCTSCVVPLEMYNAFTWLGYVNSAVNPIIYTTFNVEFRKAFIKILHC
ZF D2b   384 LTHVLKAHCGSCCISPSLYSAVTWLGYLNSAVNPVIYTTFNIEFRKAFIKILHC
```

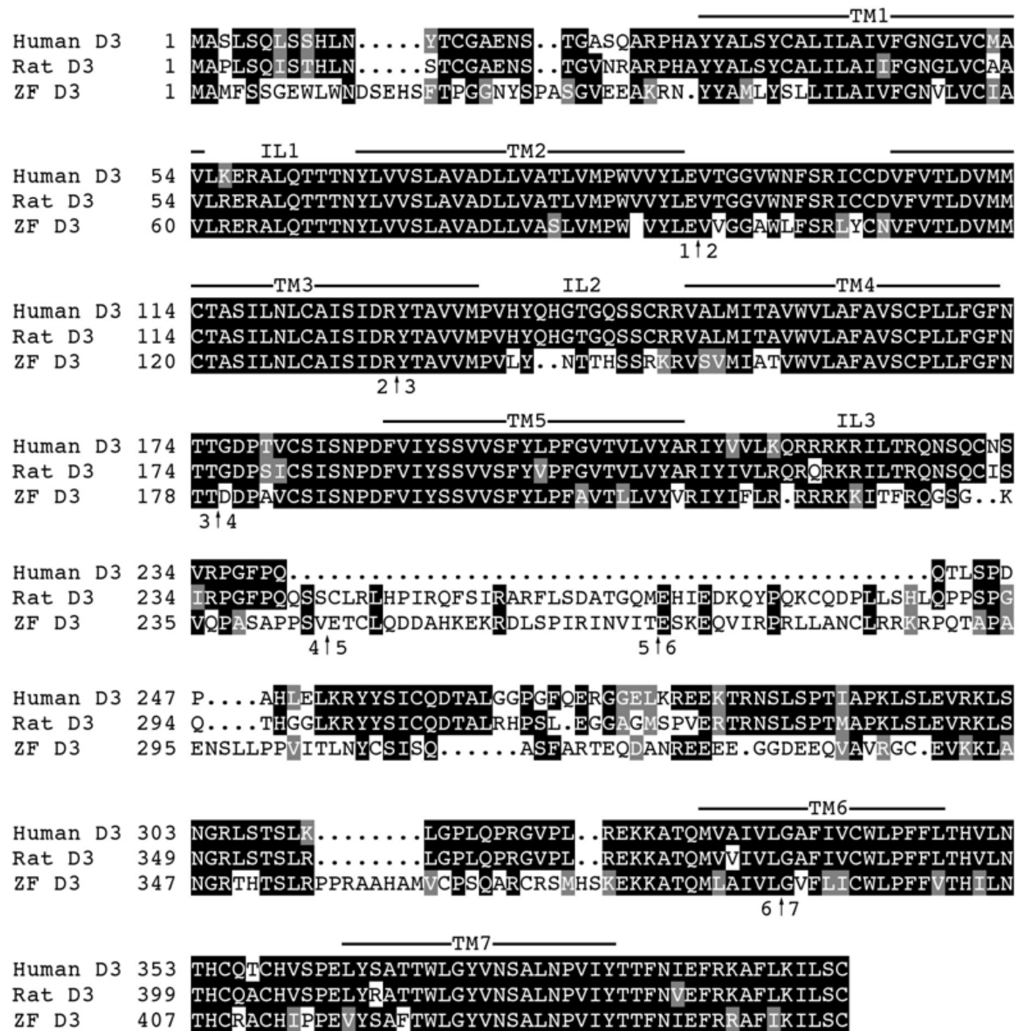

Figure 2.2. Comparison of zebrafish and mammalian D3 dopamine receptors. Human, rat, and zebrafish (ZF; drd3) D2 receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence. Positions of predicted intracellular loops (IL) are shown above the sequence. Arrowheads indicate the location of introns and are flanked by the corresponding exon numbers.

Figure 2.3. Comparison of zebrafish and mammalian D4 dopamine receptors. Human, rat, and zebrafish (ZF; drd4a, drd4b, drd4c) D2 receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence. Positions of predicted intracellular loops (IL) are shown above the sequence. Arrowheads indicate the location of introns and are flanked by the corresponding exon numbers.

```
                                                 ----------TM1----------
Human D4    1 MGNRSTADADGLLAGRGPAAGASAGASAGLAGQGAAALVGGVLLIGAVLAGNSLVCVSVA
Rat D4      1 MGNSSATEDGGLLAGRGP...ESLGTGAGLGGAGAAALVGGVLLIGLVLAGNSLVCVSVA
ZF D4a      1 ~~~~~~~~~~~~~~~MPANL..TIST..HTTNYNFPALIFGILLIIIIICGNVLVCLSVY
ZF D4c      1 ~~~~~~~~~~~~~~~MSANISSTLQP..ALFTYNVPALVFGILLIIVIICGNVLVCLSVY
ZF D4b      1 ~~~~~~~~~~~~~~~~MVNVTPSIDPTAAHEGYNYLALICGVPLILIIILGNVLVCLSVL

                IL1     ---------TM2---------                  ----------
Human D4   61 TERALQTPTNSFIVSLAAADLLLALLVLPLFVYSEVQGGAWLLSPRLCDALMAMDVMLCT
Rat D4     58 SERTLQTPTNYFIVSLAAADLLLAVLVLPLFVYSEVQGGVWLLSPRLCDTLMAMDVMLCT
ZF D4a     42 TEKALKTTTNYFIVSLAVADLLLAVLVLPLFVYAEFQDGVWSLNMTVCDGLMTMDVMLCT
ZF D4c     44 KEKALKTTTNYFIVSLAVADLMLAVLVLPLFVYAEFQGGVWSLNVSVCDGLMTMDVMLCT
ZF D4b     45 TERSLKTATNYFIVSLAVADLLLAILVLPLYVYSEFLGGIWTLSMYICDALMTMDVMLCT
                                                   1↑2

              TM3------           IL2          ---------TM4---------
Human D4  121 ASIFNLCAISVDRFVAVAVPLRYNRQGGSRRQLLLIGATWLLSAAVAAPVLCGLNDVRGR
Rat D4    118 ASIFNLCAISVDRFVAVTVPLRYNQQGQC..QLLLIAATWLLSAAVASPVVCGLNDVPGR
ZF D4a    102 ASIFNLCAISIDRFIAVSIPLNYNRKHVDQRQIVLLSATWILALAVASPVMFGINNVPNR
ZF D4c    104 ASIFNLCAISVDRFIAVSIPLNYNRKHVDYRQIILLSATWLLALAVASPVIFGINNVPQR
ZF D4b    105 ASILNLCAISVDRYIAVVVPLKYNRNQFSVRQLALITATWVLSLGVASPVIFGLNQVPNR
                          2↑3

                        ---------TM5---------              IL3
Human D4  181 DPAVCRLEDRDYVVYSSVCSFFLPCPLMLLLYWATFRGLQRWEVAR.RAKLHGRAPRRPS
Rat D4    176 DPAVCCLENRDYVVYSSVCSFFLPCPLMLLLYWATFRGLRRWEAAR.HTKLHSRAPRRPS
ZF D4a    162 DHSECKLEDNNYVIYSSVCSFFVPCPIMLLLYCGMFRGLRNWEEAR.KAKLRSNMEACRK
ZF D4c    164 DPSECKLEDNNYVVYSSVCSFFIPCPIMLMLYFGMFHGLRKWEETR.KAKLRNSIQACRS
ZF D4b    165 NPHVCKLEDNQFVVYSSVCSFFVPCPVMLFLYYWMFRGLKRWSSGRNRSHLRRPPRGRRS

Human D4  240 GPGPPSPTPPAPR.........LPQDPCGPDCAPPAPGLPRGPCGP....DCAPAAPSLP
Rat D4    235 GPGPPVSDP..............TQGPFFPDCPPPLPSLRTSPS......DSSRPESELS
ZF D4a    221 LQEA..A..................ASLQPLSGLPPPLPPV.........IEKDITLEEL.
ZF D4c    223 LQHAAVA..................AALPPLGALPAPLPRV.........IERDLAQSSL.
ZF D4b    225 LSLRLGAALQKEKGRAREKVVYLMPAGLSPTSLTATPTTPISSTSPVTLTTDDLAEGQMP

Human D4  287 QDPCGPDCAPPAPGLPPDPCGSNCAPPDAVRAAALP....PQTPPQTRRRRRAKITGRER
Rat D4    275 QRPCSPGC...................LLADAALP....QPPEPSSRRRRGAKITGRER
ZF D4a    252 ........DQ.DHYPDPDSP.......DPVSMLAYPGGGYNQDQNQNRQRKRAKINGRER
ZF D4c    256 ........EELDDFTQPVCPFPPEYKSSTIQTVAYSDIQYGQ...KTQRKKRAKINGRER
ZF D4b    285 GAESDPMTTQMDSVSDAENP...ERATEDDSGRENGVGKNHRPHTGRRHSKSNRVSGRER

                  ---------TM6---------         ---------TM7---------
Human D4  343 KAMRVLPVVVGAFLLCWTPFFVVHITQALCPACSVPPRLVSAVTWLGYVNSALNPVIYTV
Rat D4    311 KAMRVLPVVVGAFLVCWTPFFVVHITRALCPACFVSPRLVSAVTWLGYVNSALNPIIYTI
ZF D4a    296 KAMRVLPVVVGAFLFCWTPFFVVHTMRALCEDCEIPGSVTSIVTWLGYVNSAINPIIYTV
ZF D4c    305 KAMRVLPVVVGVFLFCWTPFFVVHTTRALCESCHISSDLMSTVTWLGYVNSALNPIIYTV
ZF D4b    342 KAMKVLPVVVGVFLACWTPFFVVHVTKALCESCDIGPTLISVVTWLGYVNSAVNPIIYTA
                        3↑4

Human D4  403 FNAEFRNVFRKALRACC
Rat D4    371 FNAEFRSVFRKTLRLRC
ZF D4a    356 FNTEFGKFFRKFLPTLPGCC
ZF D4c    365 FNTEFRKFFRGFVP..RCCC
ZF D4b    402 FNVEFRNVFHKLLCCRS
```

2.4 Discussion

We have isolated full-length cDNAs encoding three D2, one D3 and three D4 dopamine receptors from zebrafish. To our knowledge, this is the first description of dopamine receptor genes in this teleost. Initial BLAST searches of the EST database using human dopamine receptor nucleotide sequences failed to identify any zebrafish dopamine receptor cDNAs. However, by mining the zebrafish genomic sequence database, we identified segments of a single D3 receptor gene, three D2 receptor genes, and three distinct D4-like dopamine receptor genes. Full-length cDNAs encoding the D2, D3 and D4 receptors were subsequently generated by RT-PCR. Sequence comparisons provide strong support for the idea that three of the cloned zebrafish cDNAs encode polypeptides that are orthologs of the mammalian D2 dopamine receptor, while one cDNA encodes an ortholog of the mammalian D3 receptor, and the remaining three cDNAs encodes orthologs of the mammalian D4 receptor. Comparison of genomic sequences indicates that the intron/exon organization of the zebrafish and mammalian D2-like dopamine receptor genes is also highly conserved. Searches of the current zebrafish EST and genomic databases identified an EST (Accession number BI984545) encoding a partial D3 dopamine receptor (amino acids 261-424), but failed to reveal the presence of any additional D2, D3 or D4 receptor genes. Taken together, our data are most consistent with the presence of three D2, one D3 and three D4 dopamine receptor genes in zebrafish.

In mammals two isoforms of the D2 dopamine receptor (D2L and D2S) are generated from the D2 gene by alternative splicing. Compared to the D2S form, the D2L isoform contains an additional 29 amino acid encoded by exon five of the D2 gene. In the D2S isoform, exon five is spliced out whereas this exon is retained in the D2L isoform (Giros et al., 1989; Monsma et al., 1989). In zebrafish, all of the D2 receptor cDNAs that we sequenced contain the fifth exon. PCR analysis of *drd2a* and *drd2b* mRNAs have failed to identify any alternatively spliced transcripts of these genes. In primates, the fifth exon of the D3 receptor gene is nonfunctional and is removed from spliced transcripts, whereas the fifth exon is retained in both the rodent and zebrafish D3 receptors. The functional significance of the fifth exon in D2 and D3 receptors is not well understood. In mammals, the D2S receptor exhibits a predominant presynaptic localization whereas the D2L receptor is postsynaptically situated (Khan et al., 1998). The fifth exon may contribute to dopamine receptor trafficking. A yeast two-hybrid screen revealed an interaction between heart-like fatty acid binding protein and the fifth exon of D2L isoform (Takeuchi and Fukunaga, 2003). This interaction appears to be responsible for the differential subcellular distribution of the D2L and D2S isoforms in NG108-15 cells (Takeuchi and Fukunaga, 2003). In zebrafish, however, the fact that all three D2 receptors appear to contain the fifth exon suggests that the fifth exon is not likely to play a role in trafficking of D2 receptors to pre- or postsynaptic membrane compartments.

Chapter Three

Phylogenetic Analysis and Gene Mapping of D2-like Dopamine Receptors in Zebrafish

3.1 Introduction

We have identified and cloned three D2, one D3 and three D4 dopamine receptor genes in zebrafish. It is believed the zebrafish genome has undergone an additional genome duplication event compared to mammals (Amores et al., 1998; Postlethwait et al., 1998). The presence of multiple D2 and D4 dopamine receptor genes in zebrafish supports this hypothesis. According to the duplication-degeneration-complementation model, the explanation for the retention of duplicated genes is the partitioning of function of the ancestral gene (Force et al., 1999). In addition, the retention of duplicated genes likely suggests that these genes have evolved to obtain specialized, rather than redundant functions. The most common fate for a duplicated gene pair, however, is that one will be lost from the genome as long as the other duplicate gene retains the original function of the ancestral gene (Gloriam et al., 2005).

To further characterize the evolution of D2-like dopamine receptors in zebrafish, we conducted phylogenetic analysis. Additionally, phylogenetic analysis facilitated the confirmation of the identities of the cloned receptors. Each zebrafish D2-like gene was localized to chromosomes via radiation hybrid mapping which allowed for comparative mapping studies to identify conserved synteny between zebrafish and humans.

3.2 Experimental Procedures

3.2.1 Phylogenetic Analysis

D2 and D3 dopamine receptor amino acid sequences were aligned using the PILEUP program (Devreux J, 1984). 253 positions at which alignments were unambiguous were used for phylogenetic analysis, while positions at which alignments were ambiguous due to amino acid insertions or deletions were excluded. The sequences retained for analysis aligned to amino acids 38-175, 181-216, 364-366, and 369-444 of the rat D3 polypeptide. Phylogenetic analysis was performed using the Phylip suite of programs (version 3.573c) described by Felsenstein (1981). Maximum parsimony trees were calculated using PROTPARS. Evolutionary distance trees were constructed by using the algorithm of Fitch and Margoliash (1967). For each method, tree reliability was estimated by analysis of 100 half jackknife subreplicates. Trees were rooted using the D4 dopamine receptor sequence from carp *Cyprinus carpio* (GenBank Accession number CAA74977.1), since previous phylogenetic analysis has clearly identified D4 receptors as the sister clade to D2 and D3 receptors (Le Crom et al., 2004).

D4 dopamine receptor amino acid sequences were also aligned using the PILEUP program (Devreux J, 1984). A total of 208 positions at which alignments were unambiguous were used for phylogenetic analysis. The sequences retained for analysis aligned to amino acids 61-97, 100-175, 185-222, 401-417, and 421-459 of the human D4 polypeptide (Swissprot D4_human, D4.7 variant, 467 amino acids). Phylogenetic

analysis was performed as described above. Trees were rooted using the human and zebrafish D2 and D3 receptors.

3.2.2 Chromosomal Mapping of Zebrafish Dopamine Receptor Genes

Zebrafish dopamine receptor genes were mapped using the Goodfellow T51 radiation hybrid (RH) panel (Kwok et al., 1998). The T51 panel was purchased from Research Genetics and made available to us by Dr. Keith Cheng (Department of Pathology, Penn State College of Medicine). PCR products specific for each zebrafish dopamine receptor gene were amplified using primers corresponding to unique sequences within each gene. A complete list of primers used to determine linkage assignments is detailed in Table 3.1. PCR reactions were performed in duplicate on the RH panel using conditions optimized for each primer pair. PCR reaction products were fractionated on 2% agarose gels, and each sample scored for presence or absence of the zebrafish-specific amplicon. Linkage assignments were computed using the Zon RH mapper resource (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/).

3.3 Results

3.3.1 Phylogenetic Analysis

To examine the evolutionary relationships between zebrafish and mammalian dopamine receptor genes, we conducted a phylogenetic analysis of D2 and D3 dopamine receptors using maximum parsimony (MP) (Felsenstein, 1981) and distance matrix (DM) (Fitch and Margoliash, 1967) methods. The results, shown in Figure 3.1, include the zebrafish, representative mammalian, and all available full-length nonmammalian vertebrate D2 and D3 receptors. Using a D4 receptor sequence from carp to root the tree, a clear demarcation was found between D2 and D3 receptors. This conclusion was strongly supported by bootstrap analysis, with the D3 cluster appearing in 77% (MP) and 79% (DM) of all trees, while the D2 cluster was found in 83% (MP) and 98% (DM) of all trees. The grouping of two mammalian and two fish sequences within the D3 cluster indicates that the mammalian and fish D3 receptor genes are orthologs.

Within the D2 cluster, both MP and DM analyses identified two subclusters. The larger subcluster, containing the receptors encoded by the zebrafish *drd2a* and *drd2c* genes as well as all tetrapod D2 receptors, was supported by 91% (MP) and 100% (DM) of all trees. Although the relationships within this cluster were only weakly supported by the bootstrap analysis, the consensus phylogenetic trees exactly reproduced the expected evolutionary relationships between classes of vertebrates. This analysis strongly suggests that the zebrafish *drd2a* and *drd2c* genes are orthologs of the mammalian D2 receptor

gene. The smaller subcluster contains a pair of highly similar teleost dopamine receptors, the receptor encoded by the zebrafish *drd2b* gene and the carp D2 receptor. This phylogenetic analysis does not allow us to conclude whether the *drd2b* gene is a divergent ortholog of the mammalian D2 receptor gene or if it is the product of an early gene duplication whose counterpart has been lost in the mammalian lineage.

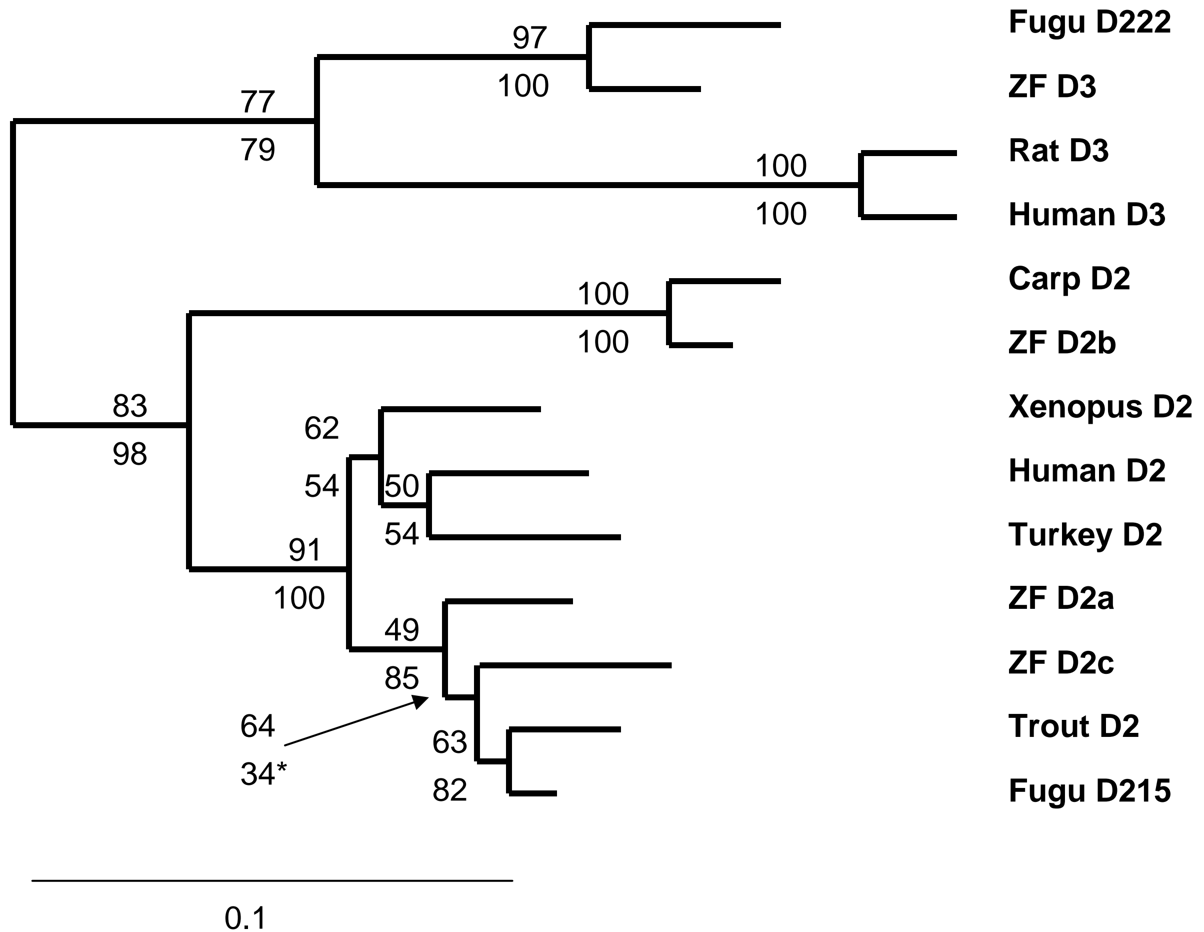

Figure 3.1. Phylogenetic analysis of vertebrate D2 and D3 receptors. Tree was rooted using the carp D4b sequence (CAA74977.1). Scale of evolutionary distance (below) was estimated from the method of Fitch and Margoliash (1967) by using all aligned positions. The maximum parsimony consensus tree determined by bootstrap analysis had identical topology, whereas the zebrafish (ZF) D2a and D2c branches were interchanged in the Fitch/Margoliash consensus tree. Numbers to the left of each node indicate percent support from bootstrap analysis (maximum parsimony above, Fitch/Margoliash below). Clustering of ZF D2a with trout D2 and *Fugu* D215 was supported by 59% of the trees in the Fitch/Margoliash analysis, whereas the topology shown here was supported by 34% of trees. Sources of sequences (accession number or SwissProt identifier): Zebrafish D2a (AY183456), D2b (AY333791), D2c (AY333792), and D3 (AY183455); human D2 (D2DR_HUMAN); human D3 (AAB08750.1); rat D3 (D3DR_RAT); turkey D2 (D2DR_MELGA); *Xenopus* D2 (D2D1_XENLA); carp D2 (CAA74976.1); trout D2 (CAC79663.1); *Fugu* D215 (D2DR_FUGRU); *Fugu* D222 (D56849).

To confirm the evolutionary relationships of zebrafish D4R genes, phylogenetic analysis was conducted using maximum parsimony (MP; Felsenstein, 1981) and distance matrix (DM; Fitch and Margoliash, 1967) methods (Figure 3.2). Clustering of all D4R sequences was strongly supported (100% of trees generated using both methods). Separation of zebrafish *drd4b* and a related medaka sequence from other fish D4 sequences is strongly supported by bootstrap analysis. However, the position of the human D4R sequence within the tree (supported by 84% of DM and 52% of MP trees) is clearly anomalous, suggesting that the mammalian D4R gene is evolving more rapidly than the D4R genes of lower species. Similar to the case with the zebrafish *drd2b* gene phylogenetic analysis cannot distinguish whether the divergent *drd4b* gene arose within a common ancestor of fish and tetrapods, or whether it arose within the fish lineage.

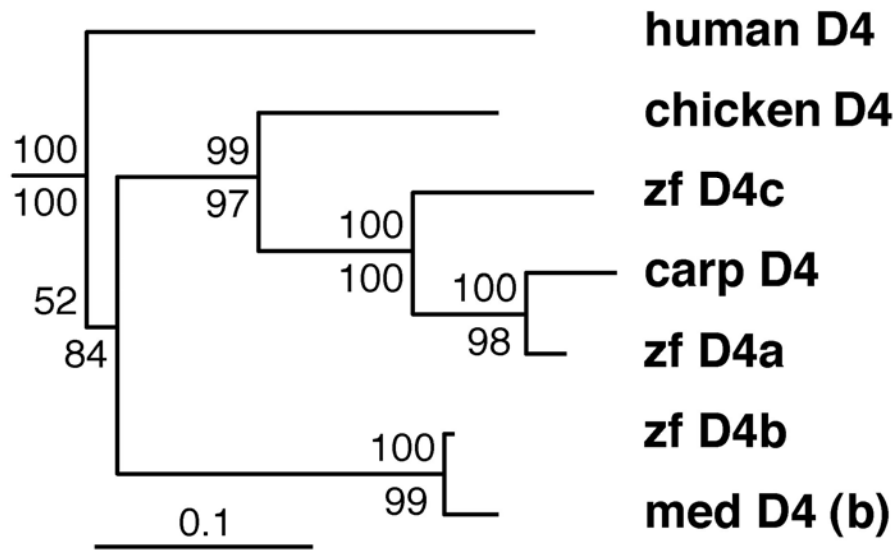

Figure 3.2. Phylogenetic analysis of vertebrate D4 receptors. Tree was rooted using D2 and D3 sequences. Branch lengths were estimated by the method of Fitch and Margoliash (1967) using all aligned positions. Evolutionary distance scale is below tree. The maximum parsimony and Fitch/Margoliash consensus trees obtained by bootstrap analysis showed identical topologies. Numbers to the left of each node indicate percent support from bootstrap analysis (Fitch/Margoliash above, maximum parsimony below). Both methods strongly support clustering of all D4 sequences (100%). Sources of sequences (accession number or SwissProt identifier): Zebrafish D4a (AY750152), D4b (AY750153), and D4c (AY750154); human D4 (D4DR_HUMAN); chicken D4 (XP_420947); carp D4 (CAA74977). Medaka (med) D4 (b) sequence was deduced from scaffold1619 (http://dolphin.lab.nig.ac.jp/medaka/, assembly of 6/2005).

3.3.2 Chromosomal Mapping of Zebrafish Dopamine Receptor Genes

We determined the chromosomal positions of zebrafish D2, D3, and D4 receptor genes using the T51 radiation hybrid panel (Kwok et al., 1998). Gene map positions were calculated with the Zon RH mapper resource (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/). Dopamine receptor genes were found to be dispersed throughout the zebrafish genome. A summary of the map positions of individual dopamine receptor genes and their corresponding LOD scores is presented in Table 3.1. The zebrafish *drd2a* receptor gene mapped to chromosome 15 at a distance 12 cR from EST fa11a11.s1. The *drd2b* gene mapped to chromosome 16 and was located 4 cR from EST fj12d09.x1. The *drd2c* gene localized to chromosome 5 at a distance 6 cR from the marker zK021A24.t7F. The zebrafish *drd3* gene mapped to chromosome 24 at a position 7 cR from the EST fb08c12.u1. The zebrafish *drd4a* gene mapped to chromosome 25 at a distance 7 cR from the marker chunp06816. The *drd4b* gene mapped to chromosome 4 and was located 6 cR from marker zc105f7.za, while the *drd4c* gene localized to chromosome 7 at a distance 11 cR from marker chunp30522.

Table 3.1. Map Positions of Zebrafish and Orthologous Human and Mouse Dopamine Receptor Genes

	Accession			Zebrafish	Human Ortholog		Mouse Ortholog	
Gene	No.	Forward Primer	Reverse Primer	Chromosome (LOD)	Name	Location	Name	Chromosome (cM)
drd2a	AY183456	gacggaactcacttcaatggag	gccattgcttgaagttgtacag	15 (14)				
drd2b	AY333791	ctcatgtgctgaaggctcactgc	cctcctggactgaactagtgcag	16 (16)	DRD2	11q23	Drd2	9 (28)
drd2c	AY333792	catcctcgctagtcgtcgacttgttg	gcaacagagagaatgaagctgagg	5 (18)				
drd3	AY183455	gttacactgcatgttgtcaag	acacgatagccaggatgagca	24 (17)	DRD3	3q13.3	Drd3	16 (23.3)
drd4a	AY750152	agattgtcctgctctccgcca	cagctcctccaaggtaatgtc	25 (16)				
drd4b	AY750153	gagctctggtcgtaaccgctcaca	acaggcagcaccttcattgccttg	4 (18)	DRD4	11p15.5	Drd4	7 (70.1)
drd4c	AY750154	cttactgtctgcaacctggct	tcttcaagactagactgggcc	7 (17)				

Human and Mouse map positions were obtained from LocusLink (http://www.ncbi.nlm.nih.gov:80/LocusLink/)

3.4 Discussion

We have utilized gene mapping and phylogenetic analysis to further study the evolution of zebrafish dopamine receptor genes. Our mapping data shows that the zebrafish *drd3* gene is located on chromosome 24, while the human D3 dopamine receptor *DRD3* gene maps to chromosome 3 (Le Coniat et al., 1991). Zebrafish chromosome 24 contains orthologs of additional human genes located on human chromosome 3 (Table 3.2). Comparative gene mapping thus provides strong additional evidence that the zebrafish *drd3* gene and the mammalian D3 dopamine receptor gene are in fact orthologous. The zebrafish *drd2a* and *drd2c* genes map to chromosome 15 and chromosome 5, respectively, while the human D2 dopamine receptor gene (*DRD2*) has been localized to chromosome 11q23 (Eubanks et al., 1992). Both zebrafish chromosome 15 and chromosome 5 contain multiple orthologs of human genes located on chromosome 11 (Table 3.2). The presence of *lim1* and *lim6* (zebrafish duplicates of the single human *LHX1* gene) on zebrafish chromosome 15 and chromosome 5 (Postlethwait et al., 2000) is consistent with the view that these are duplicate chromosome segments. Taken together, these data support the conclusion that *drd2a* and *drd2c* are duplicate genes strictly orthologous to the single mammalian D2 receptor gene. Our mapping experiments placed *drd2b*, the third zebrafish D2 gene, on chromosome 16. Although we have been unable to identify additional genes that are syntenic between zebrafish chromosome 16 and human chromosome 11 (Table 3.2), improvements in the density of the zebrafish chromosome 16 map may eventually reveal whether or not these are related

Table 3.2. Markers Syntenic with Dopamine Receptor Genes in Zebrafish and Human

	Zebrafish gene		Human Ortholog	
Name	Accession No.	Map Position	Name	Map Position
drd2a	AY183456	15	DRD2	11q23
calmb	AA495026	15	CALM	11q14
cryab	AF159089	15	CRYAB	11q
ctsc	AI436938	15	CTSC	11q14.1-q14.3
hsp47	U31079	15	CBP2	11q13.5
hsp70	AF006006	15	HSPA10	11
kiaa0102	AA494919	15	KIAA0102	11q13.3
mre11b	AI667279	15	MRE11A	11q21
tyr	AJ250302	15	TYR	11q14-q21
drd2b	AY333791	16	DRD2	11q23
drd2c	AY333792	5	DRD2	11q23
apoa	Y13653	5	APOA1	11q23-q24
atdc	AI721600	5	ATDC	11q22-q23
htatip	AI477057	5	HTATIP	11q12.1
wnt11	AF067429	5	WNT11	11q13.5
drd3	AY183455	24	DRD3	3q13.3
cldn11	AF359429	24	CLDN11	3q26.2-q26.3
ek1	U89295	24	EPHB3	3q21-qter
zic1	AF127981	24	ZIC1	3q24
drd4a	AY750152	25	DRD4	11p15.5
cat	AJ007505	25	CAT	11P13
cdkn1b	NM212792	25	CDKN1C	11p15.5
irf7	NM200677	25	IRF7	11p15.5
ldha	NM131246	25	LDHA	11p15.4
mdkb	NM131716	25	MDK	11p11.2
myod	AF318503	25	MYOD1	11p15.4
nap1l4	CF673258	25	NAP1L4	11p15.5
pax6a	AJ507427	25	PAX6	11p13
rag1	U71093	25	RAG1	11p13
rag2	U71094	25	RAG2	11p13
wt1	NM131046	25	WT1	11p13
drd4b	AY750153	4	DRD4	11p15.5
drd4c	AY750154	7	DRD4	11p15.5
cd81	NM131518	7	CD81	11p15.5
cmt4b2	AL923957	7	CMT4B2	11p15.3
copb1	AY294010	7	COPB	11p15.2
cugbp1	AB032726	7	CUGBP1	11p11
f2	BC055596	7	F2	11p11-q12
gas2	AL922142	7	GAS2	11p14.3-p15.2
htatip2	AF329850	7	HTATIP2	11p15.1
lmo1	AF398514	7	LMO1	11p15
mtch2	AF176010	7	MTCH2	11p11.2
nucb2a	BC046077	7	NUCB2	11p15.1-p14
pax6b	AJ507427	7	PAX6	11p13
psmc3	AI437207	7	PSMC3	11p12-p13
slc17a6	AL627163	7	SLC17A6	11p14.3

chromosomal segments. Our gene mapping data indicate that the zebrafish *drd4a* and *drd4c* genes map to chromosomes 25 and 7, respectively, whereas the human D4R gene (*DRD4*) has been localized to chromosome 11p15.5 (Gelernter et al., 1992). Chromosomes 25 and 7 contain multiple orthologs of human genes located on chromosome 11 (Woods et al., 2000). The fact that these zebrafish chromosomes share significant synteny with human chromosome 11 is consistent with the view that these are duplicate chromosome segments and that *drd4a* and *drd4c* are strictly orthologous to the single mammalian D4R gene. Our mapping assignments placed *drd4b*, the third zebrafish D4R gene, on chromosome 4. Although we have been unable to identify additional genes that are syntenic between zebrafish chromosome 4 and human chromosome 11, improvements in the density of the zebrafish map may eventually reveal whether or not these are related chromosome segments. Taken together, our results are consistent with the view that zebrafish posses three paralogous D4R genes.

Analysis of the zebrafish genome indicates that zebrafish have undergone an additional round of genome duplication compared to mammals (Amores et al., 1998; Postlethwait et al., 1998). The most common fate for a set of duplicated genes is that one member of the pair will degenerate to a pseudogene or be lost from the genome (Prince and Pickett, 2002). Our identification of a single zebrafish ortholog of the mammalian D3 receptor gene suggests that the second D3 ortholog was lost during zebrafish evolution. In contrast, we have identified multiple zebrafish D2 and D4 receptor genes. The presence of three zebrafish D2 and three zebrafish D4 dopamine receptor genes suggests there must have been a gene duplication event in addition to the genome-wide duplication.

Because our mapping data place all identified zebrafish dopamine receptor genes on different chromosomes, it is not possible to identify a pair of D2 or D4 genes that arose by local tandem duplication subsequent to the proposed genome-wide duplication event. The sequence similarity of *drd2a* and *drd2c* in addition to the sequence similarity of *drd4a* and *drd4c*, together with their phylogenetic clustering, strongly suggests that these genes are duplicate pairs that were generated from a common ancestral D2 and D4 receptor gene as part of the genome-wide duplication in the teleost lineage. The origin of the *drd2b* and *drd4b* genes is less obvious, however. Phylogenetic analysis suggests that *drd2b* and *drd4b* are the products of a gene duplication event that occurred within the common ancestor of teleosts and mammals. For *drd2b*, the lack of synteny between zebrafish chromosome 16 and human chromosome 11 is consistent with this view as well as the lack of synteny between zebrafish chromosome 4 and human chromosome 11 for *drd4b*. In this scenario, the orthologous gene must have been lost in the mammalian lineage, and a duplicate of the *drd2b* or *drd4b* gene must also have been lost during evolution of the zebrafish lineage. An alternative possibility is that *drd2b* and *drd4b* arose subsequent to the teleost genome-wide duplication event, a scenario that does not require loss of D2 and D4 genes subsequent to gene duplication or species divergence. Although this model is not strongly supported by our phylogenetic analysis, it has been shown that phylogenetic analysis may fail to cluster rapidly evolving branches correctly, the so-called *long-branch attraction* artefact (Felsenstein, 1978). The only described ortholog of the *drd2b* gene is the D2 receptor sequence from carp (Hirano et al., 1998). By analyzing the genome sequence of the pufferfish, *Fugu rubripes*, we identified a putative *drd2b* ortholog (Accession number CAAB01005629) in this species as well

(Canfield et al., unpublished). This *Fugu* sequence exhibits 63% amino acid identity to the pufferfish D2 receptor D215 described previously (Macrae and Brenner, 1995). This newly identified *Fugu* gene shares 81% amino acid sequence identity with the zebrafish *drd2b* gene and clusters with zebrafish *drd2b* and carp D2 in phylogenetic analysis (data not shown). The currently available data do not allow us to derive any firm conclusions regarding the evolutionary origin of the *drd2b* or *drd4b* genes. Analysis of additional vertebrate genomes will probably be necessary to resolve this issue.

Chapter Four

Embryonic Expression Patterns of D2-like Dopamine Receptor Genes

4.1 Introduction

To better understand dopamine receptor function, we have initiated studies designed to characterize the expression of D2-like dopamine receptor genes in zebrafish. There have been several studies aimed at characterizing the organization and function of dopaminergic transmission in zebrafish, but there has been no information on the characterization of dopamine receptors themselves.

Clusters of dopaminergic neurons have been identified in the developing embryo (Guo et al., 1999; Holzschuh et al., 2001; Rink and Wullimann, 2002b) and adult (Ma, 1994a; Ma, 1994b; Ma, 1997; Ma, 2003). Immunocytochemical evidence suggests that zebrafish have a conserved dopaminergic projection pathway that is homologous to the human mesostriatal pathway (Rink and Wullimann, 2002a). Two other ascending dopaminergic pathways have been described that may represent the zebrafish homologs of the mesolimbic and mesocortical pathways found in mammals (Rink and Wullimann, 2002b). The role of dopaminergic signaling in the zebrafish visual system has been investigated using behavioral and electrophysiological methods (Li and Dowling, 2000; Maaswinkel and Li, 2003), and mutants have been identified in the regulatory pathways controlling the development of catecholaminergic neurons (Guo et al., 1999; Guo et al., 2000). In addition, zebrafish have a large repertoire of behaviors some of which (e.g. locomotor activity, conditioned place preference, addictive behavior, and visual

thresholds) can be associated with dopaminergic signaling (Darland and Dowling, 2001; Gerlai et al., 2000; Neuhauss, 2003).

Here we report the spatial and temporal dopamine receptor gene expression in the developing zebrafish. We used whole mount *in situ* hybridization in order to determine the embryonic expression patterns of the zebrafish D2, D3, and D4 genes.

4.2 Experimental Procedure

Whole mount *in situ* hybridization was performed as previously described (Thisse et al., 1994); http://zfin.org/cgi-bin/webdriver?MIval=aa-pubview2.apg&OID=ZDB-PUB-010810-1). The following probes were used to analyze dopamine receptor gene expression.

D2a (*drd2a*; Accession number AY183456), nucleotides 1-1775.

D2b (*drd2b*; Accession number AY333791), nucleotides 337-1294.

D2c (*drd2c*; Accession number AY333792), nucleotides 6-1004.

D3 (*drd3*; Accession number AY183455), nucleotides 36-1397.

D4a (*drd4a*; Accession number AY750152), nucleotides 1-1145.

D4b (*drd4b*; Accession number AY750153), nucleotides 1-1257.

D4c (*drd4c*; Accession number AY750154), nucleotides 1-1117.

4.3 Results

We used whole mount *in situ* hybridization to examine the spatial and temporal expression of the D2-like dopamine receptor genes in developing zebrafish embryos. The expression profiles of the zebrafish D2, D3, and D4 receptor genes are shown in Figures 4.1-4.7.

Expression of the *drd2a* gene was first detected at mid-somitogenesis (15 hpf) exclusively in the epiphysis (Fig. 4.1A). Expression of the *drd2a* gene was similarly restricted to the epiphysis in 24 hpf embryos (Fig 4.1B). In 36 hpf embryos (Fig. 4.1C), *drd2a* transcripts were diffusely expressed throughout the CNS with specific staining present in nuclei of the midbrain (tegmentum), hindbrain (rhombomere 6), and spinal cord. At 48 hpf, expression of the *drd2a* gene was observed in specific tegmental (Fig. 4.1D,F), hindbrain (Fig. 4.1D), and spinal cord nuclei (Fig. 4.1D). Staining was also detected in specific nuclei of the diencephalon (Fig. 4.1D-F). At 5 dpf, basal expression of the *drd2a* gene was detected in virtually all brain regions with specific staining present in the diencephalon, tectum, and hindbrain (Fig. 4.1G,H).

Expression of the *drd2b* gene was first detected at 24 hpf and was prominent in specific nuclei of the diencephalon, tegmentum, hindbrain, and spinal cord (Fig. 4.2A). In hindbrain, *drd2b* transcripts were present in rhombomeres 3, 4, 5, and 6 (Fig. 4.2B). At

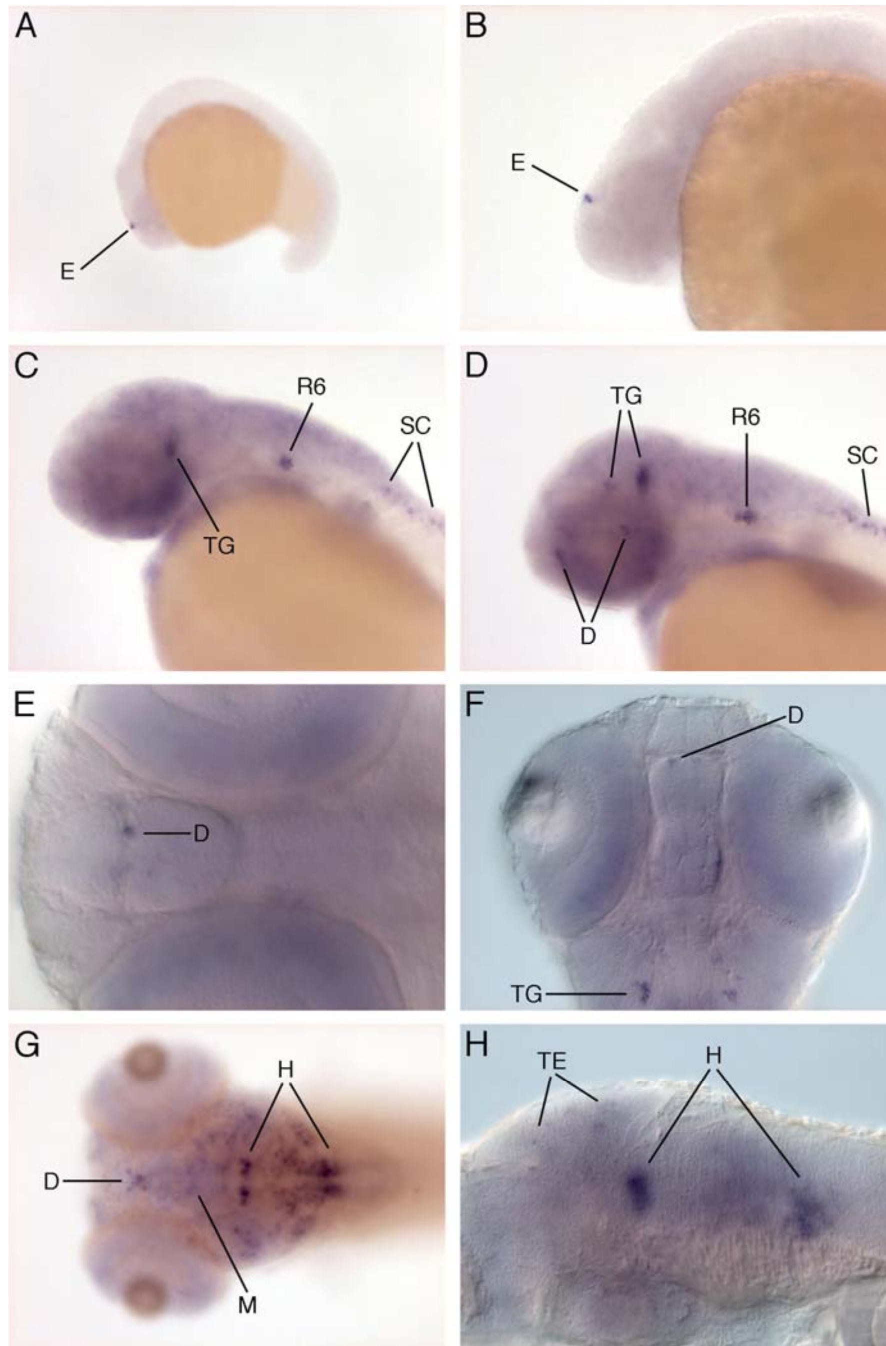

Figure 4.1. Expression of the dopamine receptor *drd2a* gene. A–D: Lateral views of embryos at mid-somitogenesis (15-somite stage, A), 24 hours postfertilization (hpf, B), 36 hpf (C), and 48 hpf (D). **E,F:** Differential interference contrast microscopy (DIC) images at 48 hpf. **G:** At 5 dpf (dorsal view). **H:** DIC image of a 5 dpf embryo. D, diencephalon; E, epiphysis; H, hindbrain; M, midbrain; R, rhombomere; SC, spinal cord; TE, tectum; TG, tegmentum.

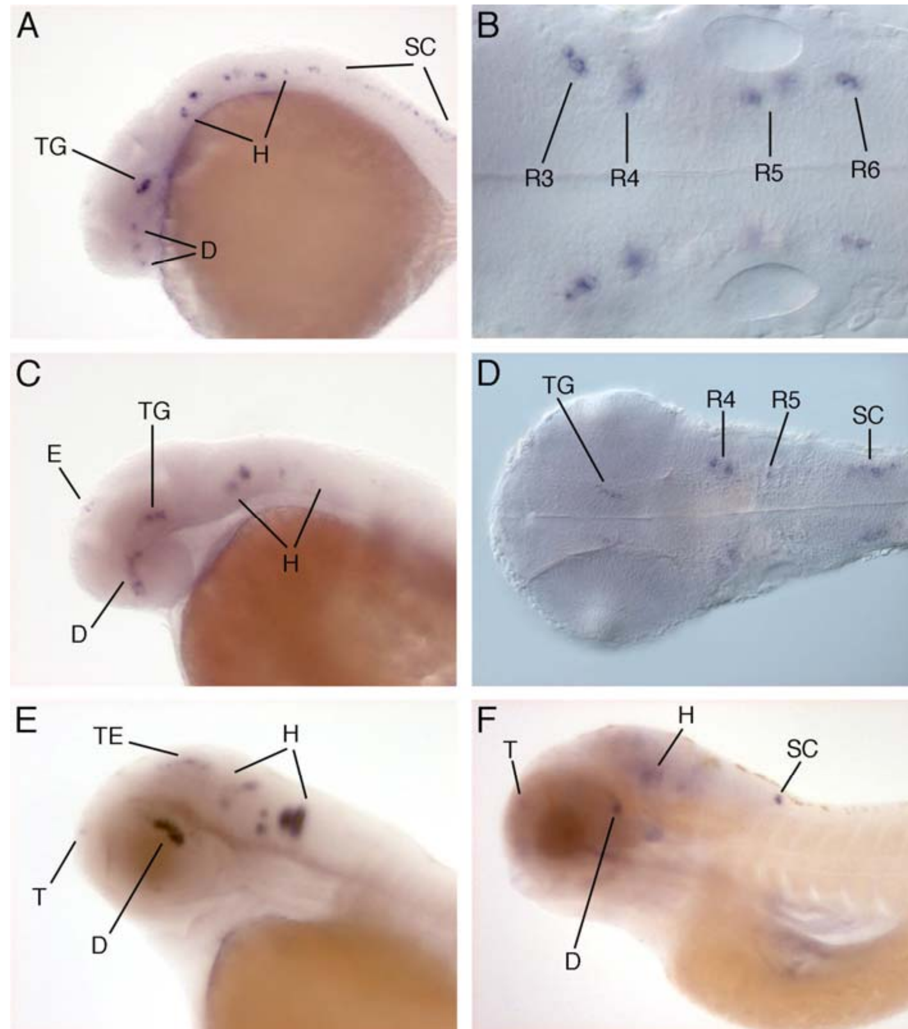

Figure 4.2. Expression of the dopamine receptor *drd2b* gene. A: A 24 hours postfertilization (hpf) embryo (lateral view). **B:** Differential interference contrast microscopy (DIC) image of a 24 hpf embryo. **C:** At 36 hpf (lateral view). **D:** DIC image of a 36 hpf embryo. **E:** At 48 hpf (lateral view). **F:** 5 dpf (lateral view). D, diencephalon; E, epiphysis; H, hindbrain; R, rhombomere; SC, spinal cord; T, telencephalon; TE, tectum; TG, tegmentum. The unstained otic vesicle (OV) is shown for orientation.

36 hpf, *drd2b* transcripts were present in distinct nuclei within the epiphysis, diencephalon, tegmentum, and hindbrain (Fig. 4.2C). Dorsal views of 36 hpf embryos revealed *drd2b* staining in tegmentum, rhombomeres 4 and 5, and spinal cord (Fig. 4.2 D). Expression of the *drd2b* gene at 48 hpf was restricted to specific nuclei of the telencephalon, diencephalon, tectum, and hindbrain (Fig. 4.2E). At 5dpf, *drd2b* gene expression was detectable within specific nuclei of telencephalon, diencephalon, hindbrain, and spinal cord (Fig. 4.2F).

Transcripts of the *drd2c* gene were first detected at mid-somitogenesis and were present exclusively in notochord (Fig. 4.3A). At 24 hpf, *drd2c* expression persisted in the notochord (Fig. 4.3B). Diffuse expression was also detected throughout the CNS with specific staining present in hindbrain and spinal cord nuclei (Fig. 4.3C). At 36 hpf, diffuse expression of *drd2c* was observed throughout the brain and spinal cord with specific expression seen in distinct nuclei of the telencephalon, diencephalon, and rhombomeres 1, 2, 4, and 5 (Fig. 4.3, D-F). By 48 hpf, strong expression of the *drd2c* gene was detected throughout the brain with weaker expression in spinal cord. Specific staining was observed in nuclei of the diencephalon, tegmentum, and hindbrain (Fig. 4.3G). At 5 dpf, expression of *drd2c* was present throughout the CNS with prominent staining observed in the ganglion and intermediate cell layers of the retina (Fig. 4.3H).

The expression profile of the zebrafish D3 dopamine receptor gene (*drd3*) is shown in Fig. 4.4. Expression of the *drd3* gene was first detected at mid-somitogenesis and was

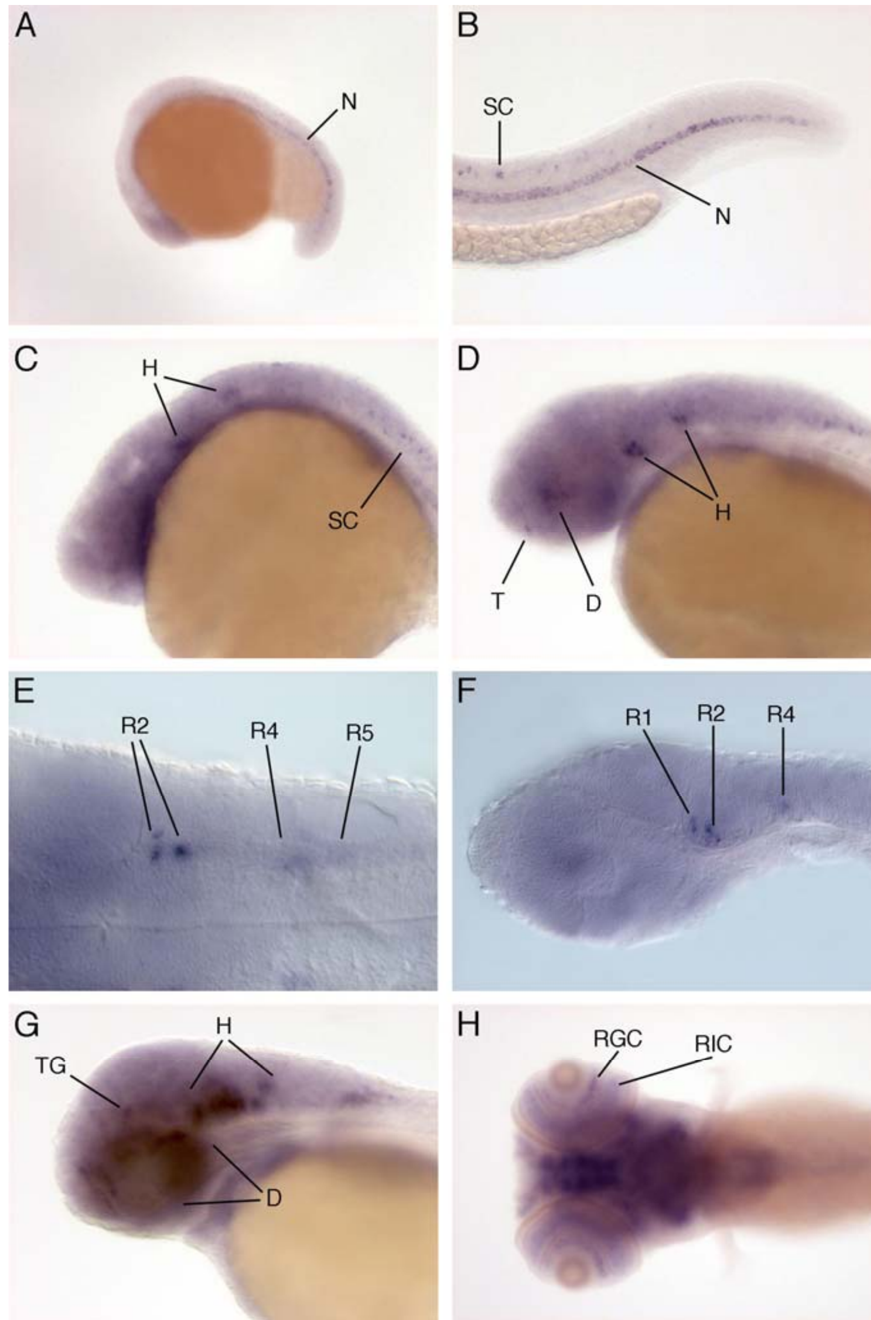

Figure 4.3. Expression of the dopamine receptor *drd2c* gene. A: At mid-somitogenesis (15-somite stage, lateral view). **B:** At 24 hours postfertilization (hpf; tail, lateral view). **C:** At 24 hpf (head, lateral view). **D:** At 36 hpf (lateral view). **E,F:** Differential interference contrast microscopy (DIC) images of a 36 hpf embryo. **G:** At 48 hpf (lateral view). **H:** At 5 dpf (dorsal view). D, diencephalon; H, hindbrain; N, notochord; R, rhombomere; RGC, retinal ganglion cell layer; RIC, retinal intermediate cell layer; SC, spinal cord; T, telencephalon; TG, tegmentum.

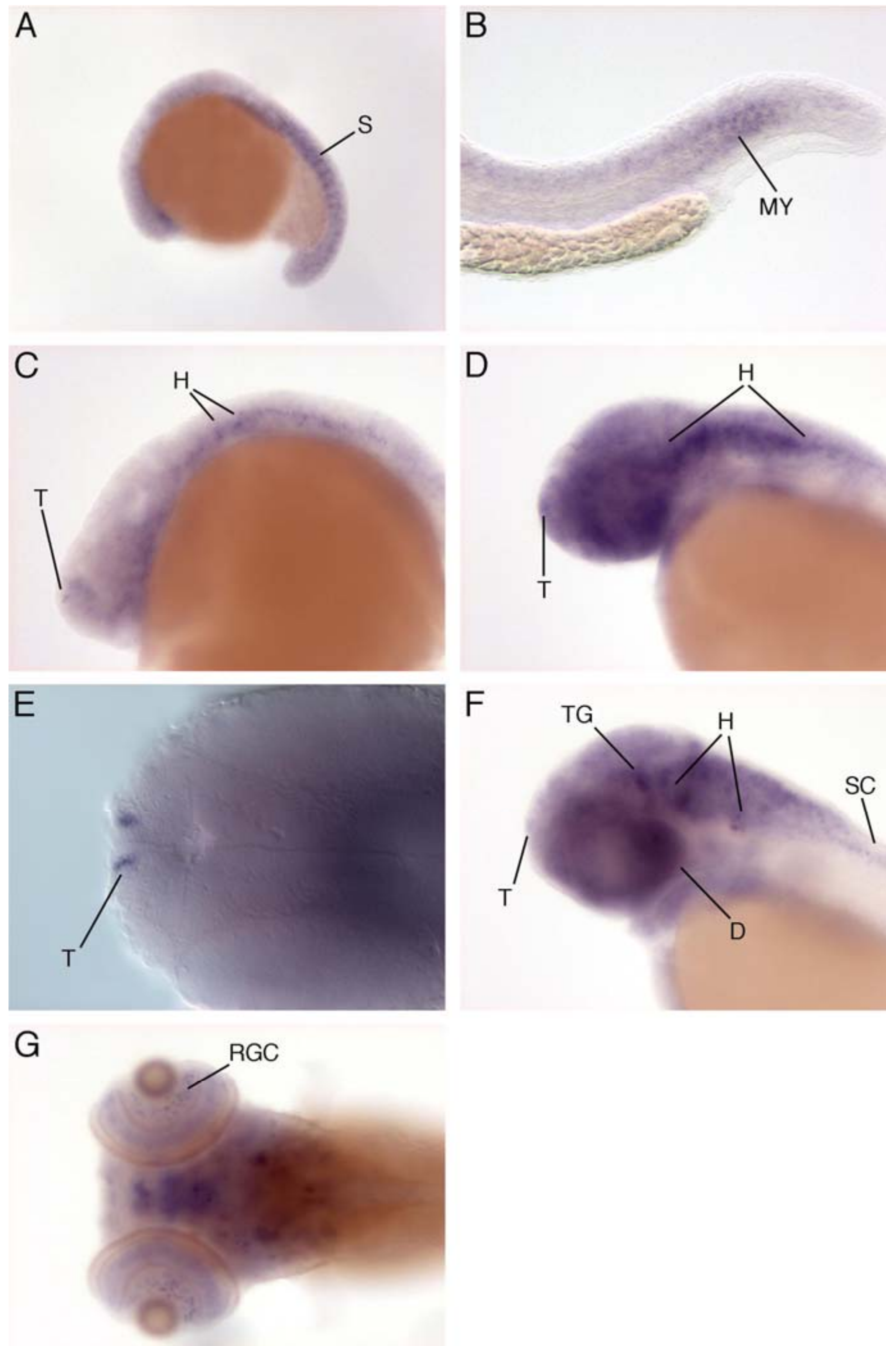

Figure 4.4. Expression of the dopamine receptor D3 gene *drd3*. A–D: Lateral views of embryos at mid-somitogenesis (A), 24 hours postfertilization (hpf; tail, B), 24 hpf (head, C), and 36 hpf (D). **E:** Differential interference contrast microscopy (DIC) image at 36 hpf. **F:** At 48 hpf (lateral view). **G:** At 5 dpf (dorsal view). D, diencephalon; H, hindbrain; MY, myotomes; RGC, retinal ganglion cell layer; S, somites; SC, spinal cord; T, telencephalon; TG, tegmentum.

localized exclusively to somites (Fig. 4.4A). At 24 hpf, *drd3* transcripts persisted in myotomes (Fig. 4.4B). Expression of the *drd3* gene was also detected in brain and spinal cord (Fig. 4.4C), with specific staining observed in distinct telencephalic and hindbrain nuclei (Fig. 4.4C). At 36 hpf, *drd3* expression was detected in telencephalic and hindbrain nuclei (Fig 4.4D, E). Transcripts of the *drd3* gene at 48 hpf were present in telencephalon, diencephalon, tegmentum, hindbrain, and spinal cord (Fig. 4.4F). By 5 dpf, *drd3* transcripts were present throughout the brain with distinct staining observed in the retinal ganglion cell layer (Fig. 4.4G).

Expression of the *drd4a* gene was first detected at 24 hpf in distinct nuclei of the epiphysis and spinal cord (Fig. 4.5A, B). At 36 hpf, transcripts of the *drd4a* gene showed staining in nuclei of the telencephalon, epiphysis, diencephalon, and hindbrain (Fig. 4.5C). In 48 hpf embryos, basal expression of the *drd4a* gene was detected in the brain region with stronger staining in the retina (Fig. 4.5D). By 5 dpf, transcripts of the *drd4a* gene were observed in the photoreceptor cell layer of the retina with basal expression persisting throughout the brain region (Fig. 4.5E, F).

Expression of the *drd4b* gene was first detected at 24 hpf. At this time point, *drd4b* transcripts were restricted to the telencephalon and diencephalon (Fig. 4.6A). At 36 hpf, *drd4b* mRNA was prominently expressed in specific nuclei of the telencephalon, diencephalon, tegmentum, adenohypophysis, and otic vesicle (Fig. 4.6B), while at 48 hpf there was continued expression of the *drd4b* gene in these same brain regions (Fig. 4.6C).

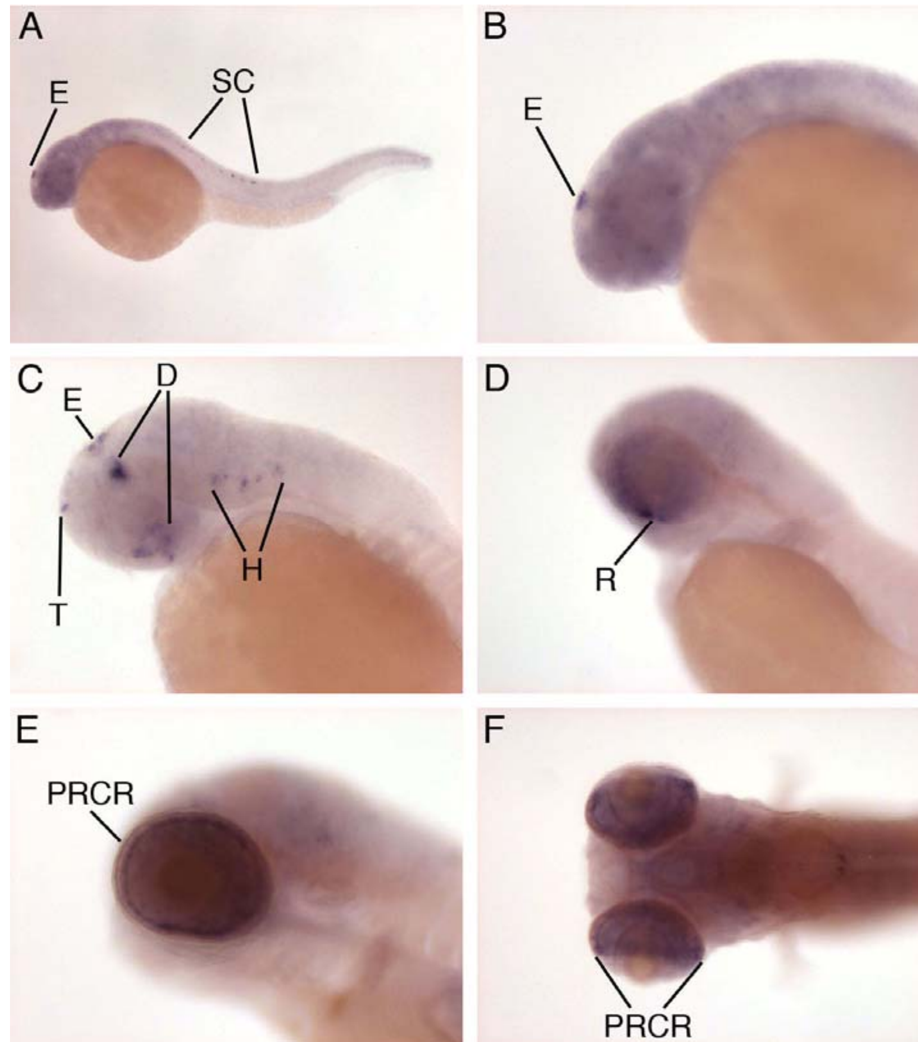

Figure 4.5. Expression of the dopamine receptor D4 gene *drd4a*. A-E: Lateral views of embryos at 24 hours postfertilization (hpf, A), 24 hpf (head, B), 36 hpf (C), 48 hpf (D), 5 dpf (E). **F:** 5 dpf (dorsal view). D, diencephalon; E, epiphysis; H, hindbrain; PRCR, photoreceptor cell layer of the retina; R, retina; SC, spinal cord; T, telencephalon.

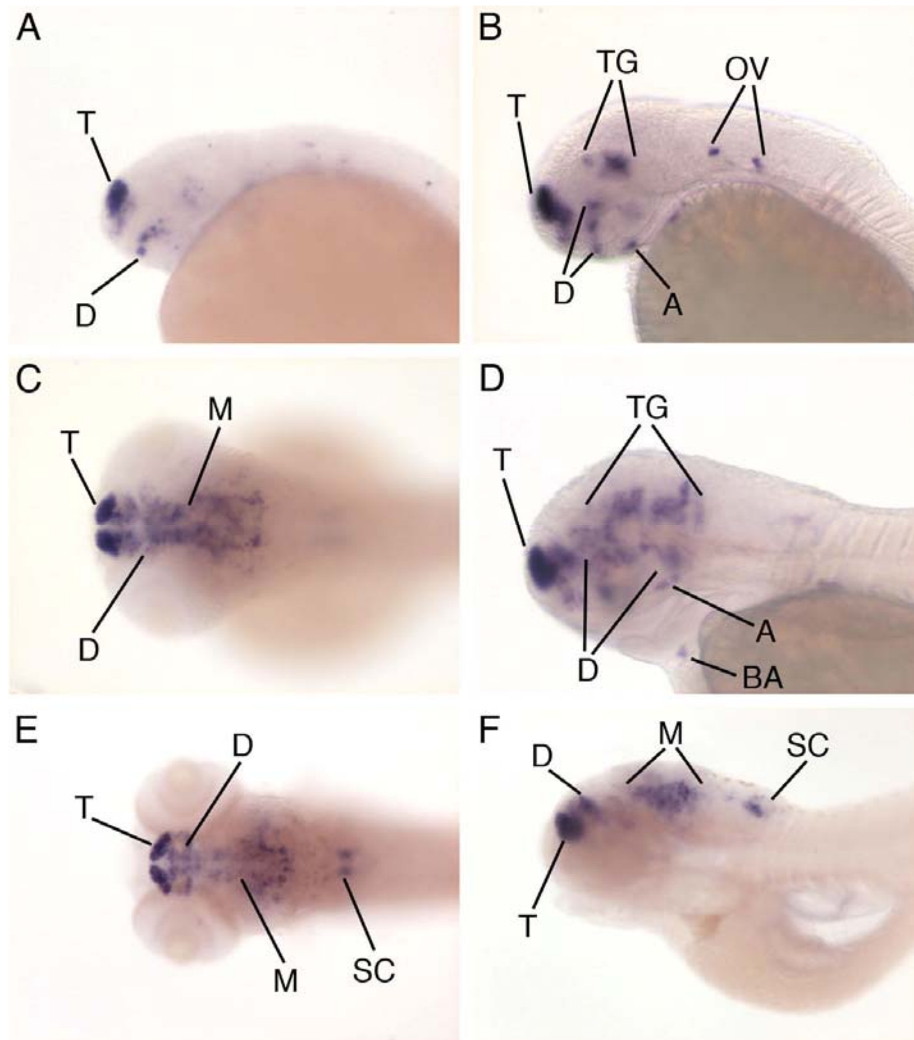

Figure 4.6. Expression of the dopamine receptor D4b gene *drd4b*. A: 24 hpf hours postfertilization (hpf) embryo (lateral view). **B:** 36 hpf embryo (lateral view). **C:** 48 hpf embryo (dorsal view). **D:** 48 hpf embryo (lateral view). **E:** 5 dpf (dorsal view). **F:** 5 dpf (lateral view). A, adenohypophysis; BA, branchial arch; D, diencephalon; M, midbrain; OV, otic vesicle; SC, spinal cord; T, telencephalon; TG, tegmentum.

Lateral views of 48 hpf embryos also revealed the presence of *drd4b* transcripts in the branchial arch (Fig. 4.6D). In 5 dpf embryos, expression of the *drd4b* gene continued to be detected in the telencephalon, diencephalon, midbrain, and in nuclei of the spinal cord (Fig. 4.6E, F).

Expression of the *drd4c* gene was first observed at mid-somitogenesis (15 hpf) at which time *drd4c* mRNA was detected in the spinal cord and endoderm (Fig. 4.7A). At 24 hpf, *drd4c* transcripts were present in specific nuclei of the diencephalon and spinal cord (Fig. 4.7B), while in 36 hpf embryos, *drd4c* transcripts were found in the branchial arch, cranial ganglia, and spinal cord (Fig. 4.7C). At 48 hpf, *drd4c* mRNA was present in the branchial arch with basal levels of *drd4c* expression throughout the brain (Fig. 4.7D). By 5 dpf, *drd4c* transcripts were restricted to the photoreceptor cell layer of the retina (Fig. 4.7E, F).

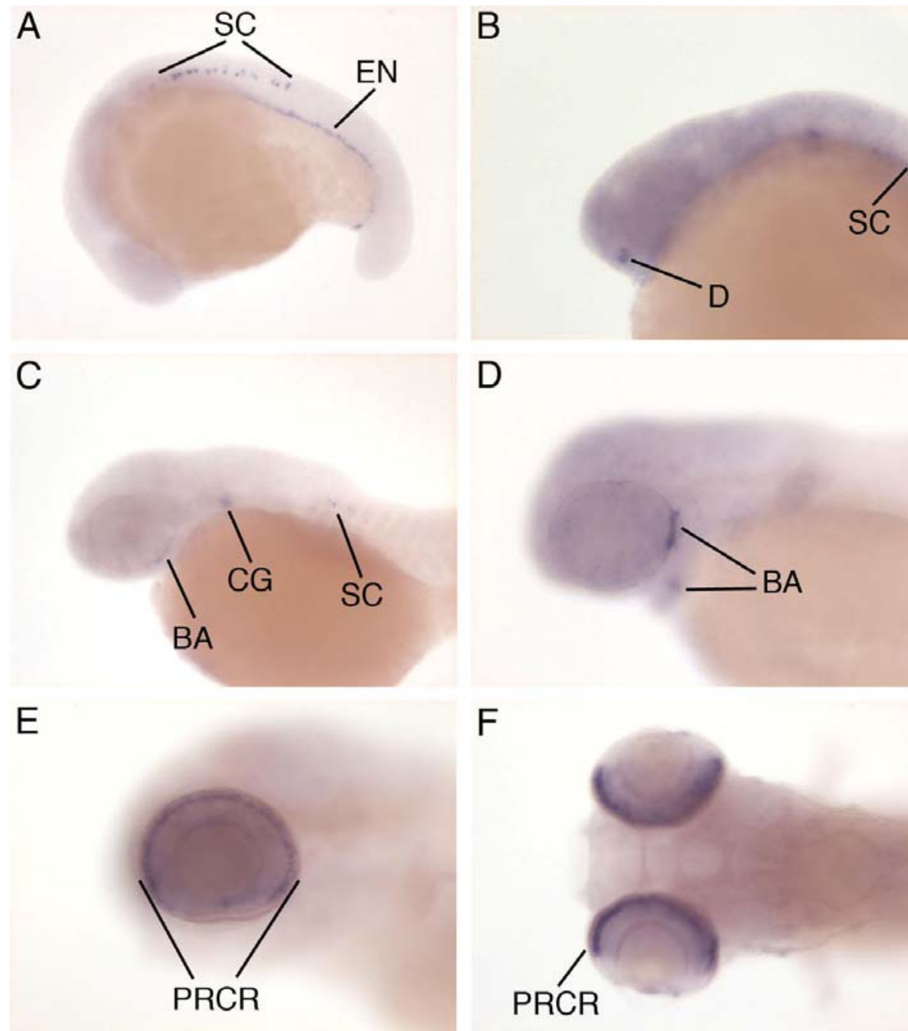

Figure 4.7. Expression of the dopamine receptor *drd4c* gene. Lateral views of embryos at **A:** mid-somitogenesis (15 somite stage), **B:** 24 hours post fertilization (hpf), **C:** 36 hpf, and **D:** 48 hpf. **E:** 5 dpf (dorsal view). **F:** 5 dpf (lateral view). BA, branchial arch; CG, cranial ganglia; D, diencephalon; EN, endoderm; PRCR, photoreceptor cell layer of the retina; SC, spinal cord.

4.4 Discussion

The mammalian dopaminergic system plays a central role in controlling motor coordination, cognition, mood, and reward-associated behavior. The distribution and development of dopaminergic neurons during zebrafish embryogenesis has recently been studied (Guo et al., 1999; Holzschuh et al., 2001; Rink and Wullimann, 2002b). In mammals, dopaminergic neurons develop in the forebrain and midbrain. In contrast, dopaminergic neurons are present predominantly in zebrafish forebrain, and dopaminergic neurons have not been detected in midbrain. However, recent studies have suggested that dopaminergic neurons arising in zebrafish diencephalon may be functionally analogous to the mammalian midbrain substantia nigra neurons (Rink and Wullimann, 2002a).

The data reported here represents the first analysis of the temporal and spatial expression of dopamine receptor genes during zebrafish development. It has been previously been reported that the first dopaminergic neurons are detected in zebrafish embryos at ~22 hpf (Guo et al., 1999). In contrast, we detected expression of the *drd2a*, *drd2c*, *drd3,* and *drd4c* genes by mid-somitogenesis (~15 hpf). Since expression of these genes precedes the appearance of dopamine-producing neurons, it seems reasonable to conclude that the initial expression of D2a (epiphysis), D2c (notochord), D3 (somites), D4c (spinal cord and endoderm) receptors must be localized to postsynaptic membrane sites. By 36 hpf, expression of each of the D2, D3, D4 receptor genes extends throughout the CNS and overlaps the defined dopaminergic pathways and regions associated with dopamine-

containing cell bodies (Guo et al., 1999; Rink and Wullimann, 2002a). The temporal change in D2, D3, and D4 receptor expression most likely reflects a redistribution of receptors from an exclusively postsynaptic localization in early embryos to both presynaptic (autoreceptors) and postsynaptic distribution as embryogenesis proceeds.

Expression of each of the zebrafish D2-like receptor genes has been detected within forebrain, which corresponds to the proposed sites of ascending dopaminergic projection pathways in zebrafish brain. D2, D3, and D4 receptor transcripts (with the exception of *drd2a* and *drd4c*) are detected in the telencephalon and diencephalon. In zebrafish, dopaminergic neurons in diencephalon project to the telencephalon (Rink and Wullimann, 2001; Rink and Wullimann, 2002a). These pathways have been proposed to be homologous to the mammalian mesostriatal, mesolimbic, and mesocortical dopaminergic pathways (Rink and Wullimann, 2001; Rink and Wullimann, 2002a). However, from our analysis of dopamine receptor expression, it is not clear whether the receptors expressed in these projection pathways represent presynaptic autoreceptors, postsynaptic receptors, or both.

Our *in situ* hybridization studies indicate that D2, D3, and D4 dopamine receptor expression in zebrafish parallels for the most part D2-like receptor expression in the major dopaminergic pathways of mammalian brain. We also find significant overlap between zebrafish and mammalian D2-like receptor expression within specific areas of the brain. For example, D2 receptors are expressed in mammalian pineal gland, where they play a role in melatonin synthesis (Ebadi and Govitrapong, 1986). In zebrafish,

drd2a and *drd2b* genes are differentially expressed in epiphysis. *drd2a* is expressed in epiphysis from mid-somitogenesis through 24 hpf, after which its expression was barely detectable. At 36 hpf, expression of *drd2a* was replaced by *drd2b*, raising the possibility that these two D2 receptor subtypes play distinct roles in pineal gland function. In mammals, D2, dopamine receptors are expressed in retinal ganglion cells (Nguyen-Legros et al., 1999). Neurons in the retina project to the optic tectum and convey visual information from the retina to the brain (Isa, 2002). The tectum is involved in complex behaviors such as orienting the eyes, head, and trunk, and deficits in the retinotectal projection pathway have been described in Parkinson's disease and Tourette's syndrome (Isa, 2002). We find prominent expression of the *drd2c* and *drd3* genes in retinal ganglion cells, and *drd2a* and *drd2b* transcripts in the tectum of zebrafish embryos. These results may suggest that D2 and D3 receptors are associated with the processing of visual information and control of visuomotor behaviors in zebrafish. In human tissues, it has been reported that D4R mRNA expression is highest in retina (Matsumoto et al., 1995). We find abundant levels of *drd4a* and *drd4c* transcripts in five-day-old zebrafish embryos. In *DRD4* knockout mice, it has been shown that the D4R plays an important role in regulating photoreceptor metabolism of cAMP and in light adaptation (Nir et al., 2002), whereas in the teleost *Lepomis cyanellus* (green sunfish), the D4R appears to be responsible for mediating cone retino-motor movement (Hillman et al., 1995). Expression of *drd4a* and *drd4c* transcripts in the retinal photoreceptor cell layer of zebrafish embryos suggests that the D4R may regulate retino-motor movement in zebrafish as well as other teleosts. We detected expression of *drd4b* mRNA in the otic vesicle of 36 hpf zebrafish embryos. To our knowledge, this is the first report describing

D4R expression in the ear. The function of D4Rs in the developing zebrafish ear is currently unknown. In mammals D2R and D3R transcripts have been detected in rodent cochlea (Karadaghy et al., 1997), the auditory portion of the inner ear. However, the fact that the zebrafish inner ear does not contain a cochlea suggests that *drd4b* expression may serve a unique role in zebrafish inner ear development or function.

In zebrafish, gene duplication events have led to the evolution of three separate D2 dopamine receptor genes. These genes exhibit distinct yet overlapping expression patterns during zebrafish development. The most striking example of this pattern occurs in the epiphysis, where expression of the *drd2a* gene occurs during early development (15-24 hpf), but is replaced by expression of the *drd2b* gene after 24 hpf. The rhombomeres represent another example in which D2 receptor expression is regulated in a complex temporal and spatial fashion. Rhombomeres are swellings that contribute to the segmental form of the developing hindbrain and the segmental organization of individual cranial motor nerves. In early zebrafish embryos, the *drd2a* gene was expressed exclusively in rhombomere 6. At 24 hpf, *drd2b* was expressed in rhombomeres 3-6. Thereafter, *drd2b* expression was restricted to rhombomeres 4 and 5. In contrast, expression of the *drd2c* gene was detected in rhombomeres 1, 2, 4, and 5 throughout early development. The duplication-degeneration-complementation model has been proposed to account for the retention of duplicated genes in the genome (Force et al., 1999). This model posits that as a result of degenerative changes in duplicated genes, the duplicates together retain the original functions of the single ancestral gene. It will clearly be of interest to determine whether the three zebrafish D2 receptor genes

together perform the function of the single mammalian D2 receptor gene, or whether any of these duplicates have evolved new functional specificities. This question may be best addressed using the reverse and forward genetic approaches which are possible in the zebrafish system.

Chapter Five

Effects of the Antipsychotic Clozapine on Larval Swimming Behavior

5.1 Introduction

Among the D2-like family of DRs, the D4 DR (D4R) has been of particular interest because it is densely expressed in the frontal cortex (Mrzljak et al., 1996), a brain region where dopamine signaling may modulate working memory and the establishment of memory fields (Williams and Goldman-Rakic, 1995). Molecular genetic studies have shown that the human D4R gene is highly polymorphic (Asghari et al., 1994; Lichter et al., 1993; Van Tol et al., 1992). In humans, the D4R gene contains a variable number of tandem repeats (VNTR) of a 48 base pair sequence in exon 3 that codes for a variation in the third intracellular loop of the D4R. The number of repeats varies from 2 to 10 (Lichter et al., 1993; Van Tol et al., 1992). Alleles of the D4R containing specific numbers of repeats have been associated with attention deficit hyperactivity disorder (ADHD) (Barkley, 1998; Swanson et al., 1998), opiate and alcohol abuse (Kotler et al., 1997), and novelty seeking (Benjamin et al., 1996; Ebstein et al., 1996). Consistent with these findings, D4R-deficient mice display reduced behavioral responses to novel stimuli (Dulawa et al., 1999) as well as enhanced sensitivity to drugs of abuse (Rubinstein et al., 1997).

The D4R has also generated interest because it has been found to exhibit a high affinity for the atypical antipsychotic clozapine (Van Tol et al., 1991). Clozapine has therapeutic efficacy in reducing positive and negative symptoms in acutely psychotic and treatment-resistant schizophrenic patients without eliciting extrapyramidal side effects (Lee et al., 1994; Sanyal and Van Tol, 1997). These factors have led to the idea that the D4R may

play an important role in cognitive functions associated with the cortex, and contribute to the etiology or pathogenesis of schizophrenia, a disease characterized by severe alterations in cortical function. However, the neural mechanisms involved in the pathogenesis of schizophrenia, as well as the response to antipsychotic drugs remain largely unknown.

We examined the effects of clozapine exposure on larval zebrafish. Owing to its small size, fecundity, ability to model human diseases, sequenced genome, and vertebrate status, zebrafish make an attractive model organism for both gene and drug discovery (Zon and Peterson, 2005). Zebrafish have also been shown to exhibit a large repertoire of behaviors associated with dopaminergic signaling including locomotion, conditioned place preference, addictive behavior, and visual thresholds (Darland and Dowling, 2001; Gerlai et al., 2000; Neuhauss, 2003).

We examined the effect of clozapine, a D4R-selective antagonist, on the swimming behavior of zebrafish larvae. Exposure of embryos to clozapine produced hypoactivity, an effect that was inhibited by the D4R-selective agonist ABT-724. Our data suggest that clozapine's effect on locomotor behavior may be mediated through D4R's, and that sensitivity to clozapine may be useful for elucidating the genetic basis of response to antipsychotic drugs.

5.2 Experimental Procedures

5.2.1 Drug Treatment

Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo [b,e] [1,4] diazepine; Sigma, St. Louis, MO), ABT-724 trihydrochloride (Tocris), and quinpirole (Sigma) were each dissolved in 0.1% dimethyl sulfoxide (DMSO) at a final concentration of 50 μM. Optimal drug concentrations were determined in pilot locomotor activity assays. For drug protection experiments, larvae were treated with either clozapine (12.5 μM) plus ABT-724 (50 μM or 25 μM), or clozapine (12.5 μM) plus quinpirole (12.5 μM or 6.25 μM) for 60 min, then tested in the locomotor assay. Control fish were exposed to 0.1 % DMSO alone.

5.2.2 Locomotor Assay

Simple observation was used to determine the locomotor activity of zebrafish larvae. Larvae (7 dpf) were preincubated in 2 ml of solution containing drug for 60 min in a clear 12 well tissue culture dish (3 fish per well) at 28.5°C. Each well measured 25mm in diameter x 20mm in depth. A single vertical line was drawn on the bottom of each well dividing the well into 2 equal halves (Fig. 5.1). Locomotor activity was measured for 30 sec by counting the number of times each larva crossed the line. Each dosage group was subjected to 24 independent trials.

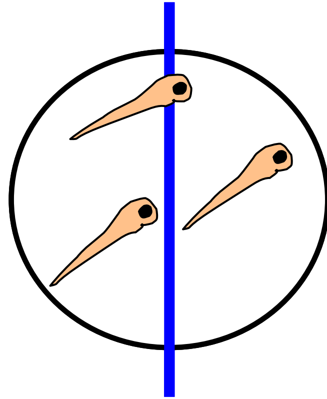

Figure 5.1. Chamber diagram. The viewing chamber was divided into equal halves by a single vertical line (represented in blue). Three larvae were placed in each well. Crossings were counted for 30 sec intervals. The criterion for a crossing was the head of the larvae touching the vertical line.

5.2.3 Statistical Analysis

In order to determine a dose response, we performed a standard repeated measures ANOVA for all outcomes measured at 5 dose levels testing for the significance of the difference in dose levels. We adjusted for the control value and also tested for interaction between well and dose. In some cases this interaction was significant, indicating the sensitivity of the experiment to ambient conditions. SPSS 13.0 for Windows was used for all data analyses.

For drug protection experiments, we performed non-parametrics Wilcoxon Signed Ranks tests. Protection from clozapine-induced hypoactivity was determined by testing the difference between co-incubation of drugs versus clozapine alone. Data for these experiments were displayed as boxplots in which the broad rectangle indicates the inter-quartile range, a thick central line shows the median, and circles depict outliers or extreme observations.

5.3 Results

5.3.1 Effect of Clozapine on Larval Swimming Behavior

Clozapine, a selective D4R antagonist, is an atypical antipsychotic drug used for treatment-resistant schizophrenia (Kane et al., 1988; Taylor and Duncan-McConnell, 2000). Clozapine exhibits a 10 fold higher affinity for the D4R compared to the D2R

(Sanyal and Van Tol, 1997). We examined effects of clozapine on locomotor activity of 7 dpf zebrafish larvae. Larvae were incubated in a 12-well tissue culture dish (3 fish per well). A single vertical line was drawn on the bottom of each well dividing the well into 2 equal halves. Larvae were exposed to clozapine for 60 min, after which locomotor activity was measured for 30 sec by counting the number of times each larva crossed the line. Each experiment was repeated 24 times per dosage group. As shown in Figure 5.2, clozapine affected the locomotor activity of larvae in a dose-dependent fashion. ANOVA analysis indicated that the dose dependent effect of clozapine on swimming behavior was significant ($p= 0.017$). Treatment of larvae with 50 μM clozapine completely blocked locomotor activity, and larvae exhibited motility only upon touch. Treatment of larvae with 12.5 μM clozapine represented the lowest dose of drug that consistently produced hypoactivity as determined by Wilcoxon Signed Ranks Test ($p<0.01$). These results indicate that clozapine produces profound hypoactivity in larval zebrafish. To determine whether the effect of clozapine was due to cytotoxicity, we examined whether larvae were capable of recovery after exposure to clozapine. After a one hr incubation period, larvae were washed three times with charcoal-filtered water, then incubated in charcoal-filtered water. Larvae exposed to 50, 25, or 12.5 μM clozapine showed partial recovery by 2 hours, and full recovery by 3 hours after drug removal.

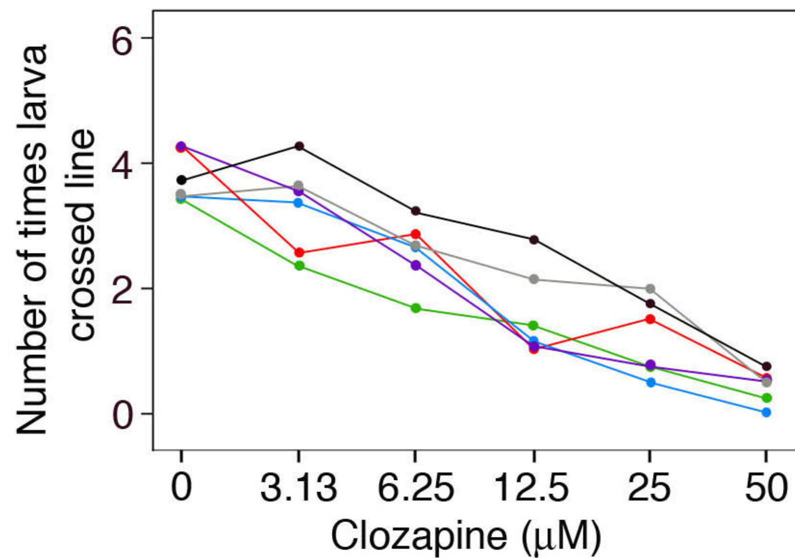

Figure 5.2. Effect of clozapine on larval swimming behavior. 7 dpf zebrafish larvae were incubated in various concentrations of clozapine for 60 minutes. Fish were then tested in the swimming assay as described. Each line represents a different zebrafish larval clutch (studied on separate days), and each point represents the mean of four measurements Clozapine affected the locomotor activity in a dose-dependent fashion ($p=0.017$; ANOVA). Asterisks represent p values ($p<0.01$ compared to control) as determined by Wilcoxon Signed Ranks test.

5.3.2 Drug Inhibition of Clozapine Effects Using a D4R Selective Agonist

To examine whether clozapine might be causing hypoactivity via its effect on D4Rs, we analyzed the swimming behavior of zebrafish larvae after exposure to the selective D4R agonist ABT-724 (Brioni et al., 2004). Treatment of 7 dpf larvae with ABT-724 alone had no significant effect on swimming behavior at any of the doses tested (3.125 μM-50 μM) (Fig. 5.3A; p= 0.744 as determined by ANOVA). We then tested the locomotor activity of larvae that had been coincubated with a hypoactivity-inducing dose of clozapine (12.5 μM) and either 50 μM or 25 μM ABT-724. The results, presented in Figure 5.4A, indicate that ABT-724 protected larvae from the hypoactivity-inducing effects of clozapine in a dose dependent fashion. Larvae treated with clozapine plus 50 μM or 25 μM ABT-724 exhibited a significant increase in locomotor activity compared to larvae treated with clozapine alone ($p < 0.01$ and $p = 0.023$, respectively, as determined by Wilcoxon Signed Ranks test).

We next examined the effect of quinpirole, a selective D2R/D3R agonist (Sovago et al., 2005), on larval swimming behavior. Quinpirole alone at doses ranging from 3.125 μM-50 μM had no significant effect on swimming behavior, except for the 12.5μM dose (Fig. 5.3B). At this dose zebrafish larva were significantly hyperactive ($p < 0.01$). When larvae were coincubated with 12.5 μM clozapine and quinpirole, neither 12.5 μM nor 6.25 μM

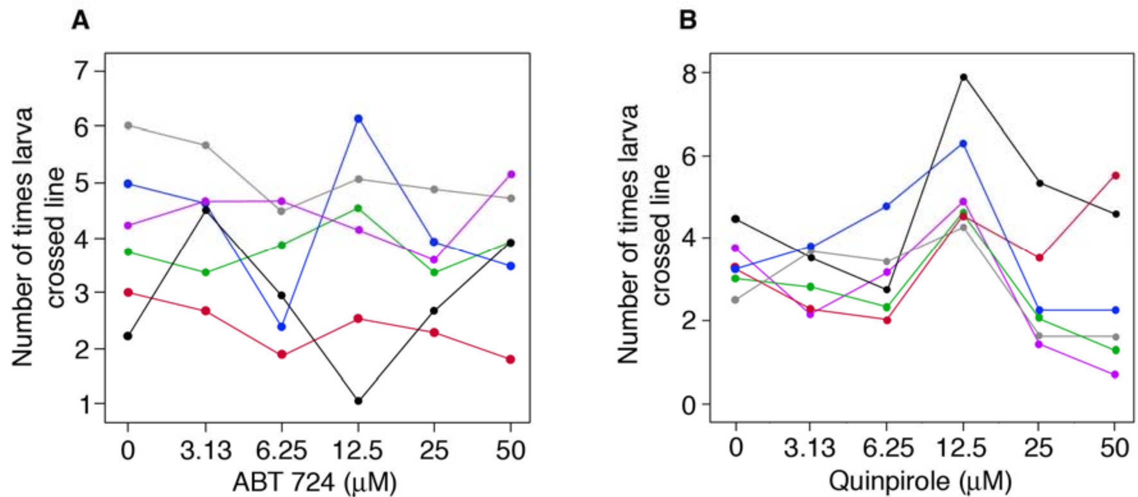

Figure 5.3. Effect of ABT-724 and quinpirole on larval swimming behavior. 7 dpf zebrafish larvae were incubated in either ABT-724 **(A)** or quinpirole **(B)** for 60 minutes. Fish were then tested in the swimming assay as described. Each line represents a different zebrafish larval clutch (studied on separate days), and each point represents the mean of four separate measurements. No significant trend on swimming behavior was measured at any dose of ABT-724 (as determined by ANOVA). Quinpirole had no significant effect on swimming behavior except at a dose of 12.5 μM (ANOVA).

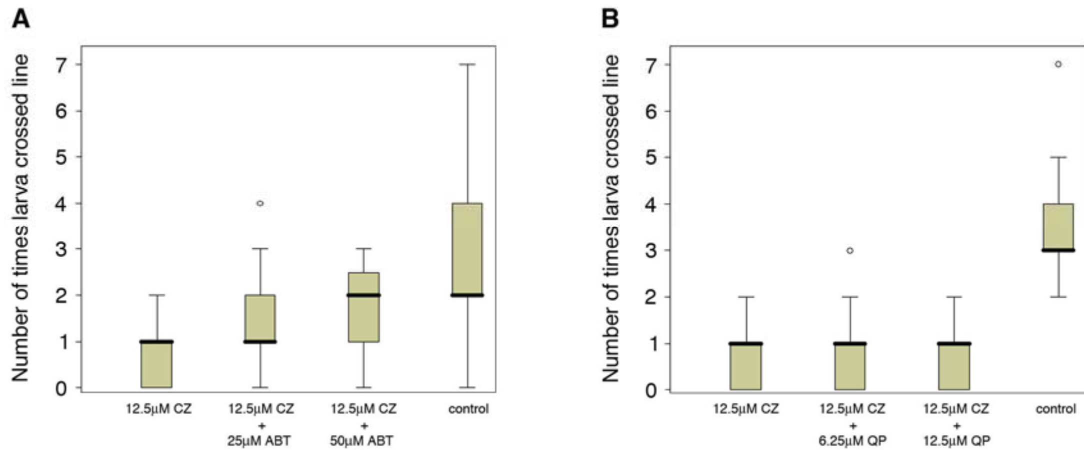

Figure 5.4. Coincubation of clozapine with ABT-724 or quinpirole. 7 dpf zebrafish larvae were incubated with clozapine and either **(A)** ABT-724, or **(B)** quinpirole for 60 minutes. Fish were then tested in the swimming assay as described with 24 independent trials for each dosage group. Boxplots show that ABT-724 reduced clozapine-induced hypoactivity whereas quinpirole had no effect. Data was analyzed by Wilcoxon Signed Ranks tests. Shaded boxes represent the middle 50% of the data set (inter-quartile range). The dark horizontal line within each box represents the median. In cases where the median is not in the middle of the box, the data have a skewed distribution (e.g. panel a; 12.5 µM clozapine). Vertical lines on the top and bottom of shaded boxes are called whiskers and represent the predicted limits of the distribution. Circles indicate outliers. Analysis of the data by Wilcoxon Signed Ranks indicates a significant difference between 12.5 µM clozapine and controls in panels a and b ($p<0.01$).

quinpirole was able to protect larvae from clozapine-induced hypoactivity (Fig. 5.4B, p= 1.0 and 0.71, respectively). Taken together, these studies suggest that the hypoactive effects of clozapine may be mediated, at least in part, by signaling through D4 dopamine receptors.

5.4 Discussion

Since the pioneering work of Streisinger (Streisinger et al., 1981), zebrafish has gained wide acceptance as a model system for the study of vertebrate development. The zebrafish system has also shown promise for the genetic analysis of behavior, and mutant strains with visual system defects (Neuhauss, 2003), and alterations in drug (Darland and Dowling, 2001) and alcohol (Lockwood et al., 2004) response have been obtained. Here we studied the effect of clozapine, the preferred drug for treatment-resistant schizophrenia (Tandon and Fleischhacker, 2005), on swimming behavior of zebrafish larvae. Clozapine is a selective D4R antagonist, and has an *in vitro* affinity that is approximately 10 times greater for the D4R than that for D2Rs (Sanyal and Van Tol, 1997; Tandon and Fleischhacker, 2005). Clozapine was found to produce hypoactivity in zebrafish larvae, an effect that was prevented by treatment of larvae with ABT-724, D4R-selective agonist. Quinpirole a D2/D3 specific agonist, did not protect larvae from clozapine-induced hypoactivity, a result consistent with the view that clozapine is acting through D4 rather than D2 or D3Rs.

Future pharmacological studies will be needed to characterize the affinity of clozapine for various G-protein-coupled receptors in zebrafish. In addition to the D4R, clozapine is known to interact with subsets of serotonin 5HT receptors (Bergqvist et al., 1999; Hermann et al., 1996; Kuoppamaki et al., 1993; Zhukovskaya and Neumaier, 2000), and our experiments do not allow us to distinguish whether the effects of clozapine on zebrafish behavior are mediated via D4 and/or serotonin receptors. Regardless of its mechanism of action, clozapine produced a profound behavioral response in larval zebrafish. The robustness of this response should make it feasible to carry out a genetic screen for mutants exhibiting an altered response to the hypoactive effects of clozapine. The availability of such mutants may permit identification of genes involved in the biological effects of clozapine and other antipsychotic medications.

Chapter Six

Neuroprotection of MPTP-induced Toxicity in Larval Zebrafish and the Characterization of Adenosine Receptors

6.1 Introduction

Parkinson's disease is characterized by the selective degeneration of dopaminergic neurons in the substantia nigra resulting in a decrease of dopamine in the brain. The symptoms of PD include tremor, rigidity, slow movement, and postural imbalance and affects approximately 1% of the population over the age of 65. Rare cases of familial PD have uncovered mutations in several proteins; however a majority of PD cases are sporadic suggesting that environmental toxins may contribute to the disease (Gasser, 2001).

The neurotoxin MPTP has been shown to cause the selective degeneration of dopaminergic neurons in the mammalian midbrain and can elicit symptoms similar to those seen in PD patients. MPTP-induced neurotoxicity is one of the most common methods of studying PD in animal models ranging from mice to primates (Betarbet et al., 2002; Kordower et al., 2000; Ramirez et al., 2003). Goldfish have also been shown to confer sensitivity to MPTP administration and exhibit significant bradykinesia (Pollard et al., 1992). Recent studies have shown that exposure of zebrafish embryos and larvae to MPTP lead to a significant reduction of dopaminergic neurons in the diencephalon (Bretaud et al., 2004; Lam et al., 2005). In addition, MPTP-induced toxicity can be prevented by treating zebrafish embryos and larvae with the dopamine transporter (DAT) inhibitor, L-deprenyl and by DAT knockdown (McKinley et al., 2005). From these animal studies, it is hypothesized that the hydrophobic nature of MPTP allows it to readily cross the blood brain barrier where it gets metabolized into MPP+ by monoamine

oxidase-B in glial cells. MPP+ is selectively taken up into DA neurons by the DAT (Miller et al., 1999). Once inside the DA neuron, MPP+ exerts its toxic effects by the inhibition of mitochondrial complex I, leading to an increase in reactive oxygen species and ultimately, cell death (Blum et al., 2001; Nicotra and Parvez, 2000).

It has been shown that administration of caffeine (A1/A2a antagonist) or genetic depletion of the A2a receptor is neuroprotective against MPTP in mice (Chen et al., 2001). Additionally, strong epidemiological evidence suggests that caffeine consumption may lower the risk of developing PD (Ascherio et al., 2001; Ross et al., 2000). To determine if the same phenomenon exists in zebrafish, we sought to determine whether caffeine is neuroprotective against MPTP in zebrafish embryos in order to further validate zebrafish as a model system to screen drugs for PD. Toward this end, we exposed embryos to caffeine co-incubated with MPTP and found a significant neuroprotective effect on dopaminergic neurons.

Studies suggest that the mechanism of caffeine neuroprotection is mediated via the A2a adenosine receptors (Chen et al., 2001). In mammals, the adenosine receptor family is made up of four receptor subtypes (A1, A2a, A2b, and A3) and all are putative seven transmembrane G-protein coupled receptors. A1 and A3 receptors couple to G_i proteins to inhibit adenylyl cyclase, while A2a and A2b receptors couple to G_s to increase cAMP. As a necessary step in this analysis, we sought to identify and characterize zebrafish adenosine receptors. We performed searches of the zebrafish genome which yielded several contigs containing A2-like receptors. From these sequences, we amplified full-

length cDNAs encoding two A2a and one A2b adenosine receptor genes. The predicted proteins ranged from 62%-74% identity when compared to the human A2 adenosine receptors. Furthermore, we used whole mount *in situ* analysis to elucidate the embryonic expression of the zebrafish A2 adenosine receptor genes.

6.2 Experimental Procedures

6.2.1 MPTP and Caffeine Treatment

Zebrafish embryos were collected and incubated at 28.5°C in Petri dishes for 24 hours. At 24 hpf, embryos were dechorionated and transferred to a 6-well plate containing either one of four treatment groups; vehicle (charcoal filtered water), 40uM MPTP, 10uM caffeine, and 40uM MPTP/10uM caffeine (n = 15 embryos per treatment group). All treatment groups contained 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigment biosynthesis. The different water treatments were changed daily and at 5 dpf, the zebrafish larvae were collected for *in situ* hybridization analysis for the dopamine transporter.

6.2.2 In Situ Hybridization Analysis of *dat* Expression

Whole mount in situ hybridization was performed as previously described (Thisse et al., 1994; http://zfin.org/cgi-bin/webdriver?MIval aa-pubview2.apg&OID ZDB-PUB-010810-1). The following probe was used to analyze the dopamine transporter; *slc6a3*

(accession no. NM131755), nucleotides 391-1055 (from W. Driever, University of Freiburg).

6.2.3 Cloning of Zebrafish Adenosine Receptors

Segments of zebrafish adenosine receptor genes were identified by using mammalian polypeptide sequences as probes in low stringency SSAHA (Ning Z, 2001) searches of the zebrafish genomic reads generated by the Sanger Center (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated). PCR primers that overlapped conserved initiation and termination codons were designed based on genomic sequence reads. The corresponding full-length cDNAs were generated via RT-PCR. First-strand cDNA synthesis was performed by using SuperScript reverse transcriptase (Life Technologies) according to the manufacturer's protocols. Random hexamers were used to prime cDNA synthesis and total RNA from adult zebrafish was used as template. REDTaq DNA Polymerase (Sigma) was used for subsequent amplification. Primers and annealing temperatures are listed in Table 6.1. PCR was carried out using a TD-7500 Thermal Cycler (Hybaid). An initial 4-min denaturation step at 94°C was followed by 34 cycles at 94°C for 30 sec, 51° for 30 sec, and 72°C for 90 sec. A final elongation step was carried out at 72°C for 10 min.

Table 6.1. PCR Primers for the Amplification of Zebrafish A2 Adenosine Receptors

Clone	Forward primer	Reverse primer	Ta (°C)
adora2a.1	$^{-44}$tacattgaggcgaggcatggtcc^{-22}	^{1373}tcaggaaacctccgtgagttc^{1353}	51
adora2a.2	$^{-19}$cacactggttgatagcaccatg^{+3}	^{1455}gcgcacaccactgattacttc^{1435}	51
adora2b	$^{-28}$catggcatcttaagtgaagtgagc^{-5}	^{1094}acaccggcgtctatagcagag^{1074}	51

Nucleotide +1 is the A of the ATG of the initiating methionine

All generated cDNAs were sequenced using an ABI 377 automated sequencer. Sequences were verified by sequencing three independent clones and comparison to genomic sequences. We identified three A2 adenosine receptor genes (*adora2a.1*, *adora2a.2*, and *adora2b*). The A2a.1 adenosine receptor cDNA (GenBank accession no. AY945800) contains a complete ORF that is 1328 bp in length. The A2a.2 adenosine receptor cDNA (GenBank accession no. AY945801) contains a complete ORF that is 1343 bp in length, while the A2b adenosine receptor cDNA (GenBank accession no. AY945802) contains a complete ORF that is 1055 bp in length.

6.2.4 Phylogenetic Analysis

Dopamine receptor amino acid sequences were aligned using the PILEUP program (Devreux J, 1984). The sequences retained for analysis aligned to amino acids 9-140, 170-210, 222-260, and 275-291 of the human A2a receptor polypeptide. Phylogenetic analysis was performed using the Phylip suite of programs (version 3.573c) described by Felsenstein (1981). Maximum parsimony trees were calculated using PROTPARS. Evolutionary distance trees were constructed by using the algorithm of Fitch and Margoliash (1967). For each method, tree reliability was estimated by analysis of 100 half jackknife subreplicates. Trees were rooted using the human beta2 adrenergic receptor and the human H2 histamine receptor.

6.2.5 Chromosomal Mapping of Zebrafish Adenosine Receptor Genes

Zebrafish adenosine receptor genes were mapped by using the Goodfellow T51 radiation hybrid (RH) panel (Research Genetics) (Kwok et al., 1998). Primers were designed to unique sequences for the adenosine receptor genes to amplify specific PCR products. A list of the primers used and corresponding linkage groups can be found in Table 6.3. PCR conditions were optimized for each primer pair. PCR products were run on 2% agarose gels and scored for the presence or absence of the zebrafish specific amplicon. Linkage group assignments were computed using the RH Zon mapper resource (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/).

6.2.6 In Situ Hybridization Analysis of A2-like Adenosine Receptor Genes

Whole mount in situ hybridization was performed as described above. The following probes were used to analyze adenosine receptor expression: A2a.1 (*adora2a.1*; accession no. AY945800), nucleotides 1-1373; A2a.2 (*adora2a.2*; accession no. AY945801), nucleotides 1-1455; A2b (*adora2b*; accession no. AY945802), nucleotides 1-1094.

6.3 Results

6.3.1 Caffeine Attenuates MPTP-induced Dopaminergic Toxicity in Larval Zebrafish

It has been shown that MPTP will significantly decrease the amount of dopaminergic neurons in larval zebrafish (Bretaud et al., 2004). It has also been shown in mammalian animal models that administering caffeine (A1 and A2a AR antagonist) can protect against MPTP dopaminergic toxicity (Chen et al., 2001). Therefore, we decided to test whether caffeine can also protect against MPTP in larval zebrafish. We exposed zebrafish embryos to MPTP and caffeine from 24 hpf to 5 dpf (Fig. 6.1). The effect on DA neurons was assessed at 5 dpf by *in situ* hybridization of the dopamine transporter gene (*dat*), which specifically labels DA neurons (Holzschuh et al., 2001). As seen in Figure 6.1A, the *dat* gene is expressed predominantly in the ventral diencephalon and pretectal area. The most dramatic effect was observed in embryos treated with 40µM MPTP with a reduction of dopaminergic neurons in the ventral diencephalon and pretectum (Fig. 6.1B). We found that caffeine provided a significant level of protection for dopaminergic neurons exposed to MPTP (Fig. 6.1C). Expression levels of *dat* closely approximated the expression of *dat* observed in untreated zebrafish larvae (Fig. 6.1A, C) suggesting caffeine also maintains its neuroprotective properties in teleosts. Pretectal dopaminergic neurons are absent in zebrafish larvae exposed to MPTP and caffeine, suggesting these clusters of neurons are not protected by caffeine (Fig. 6.1C). Caffeine

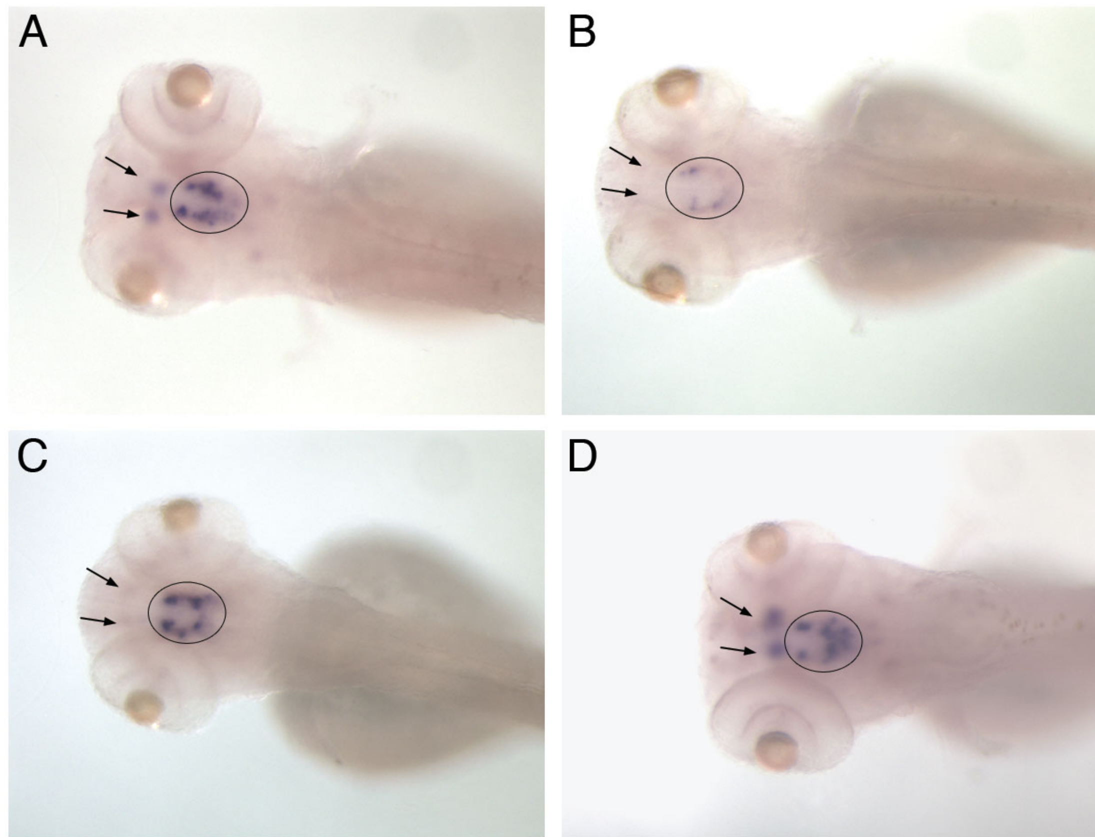

Figure 6.1. Caffeine protects dopaminergic neurons from MPTP-induced toxicity. Whole mount *in situ* hybridization of 5 dpf embryos with *dat* probe, dorsal views. **A**: Untreated controls. **B**: Embryos treated with 40µM MPTP. **C**: Embryos treated with 10 µM caffeine and 40µM MPTP. **D**: Embryos treated with 10µM caffeine. Neurons of the ventral diencephalon are circled. Black arrows point to the bilateral pretectal cluster of neurons.

treatment (10uM) alone appeared to have no effect in the reduction of DA neurons (Fig. 6.1D).

6.3.2 Cloning of Zebrafish A2 Adenosine Receptor Genes

Because the neuroprotective properties of caffeine are believed to be mediated via the A2a adenosine receptor, we hypothesized that zebrafish possess this receptor subtype. Zebrafish homologues of mammalian adenosine receptors were identified by BLAST searches of the zebrafish genome sequence database (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio_translated and http://www.ensembl.org/Danio_rerio/blastview). We used RT-PCR to amplify full-length cDNAs corresponding to three A2-like receptor genes. Sequence comparisons indicate that the zebrafish A2Rs share a high degree (62-74%) amino acid sequence identity. Two zebrafish clones (*adora2a.1* and *adora2a.2*) show highest identity (71-74%) to the human A2a AR, while the remaining zebrafish clone (*adora2b*) shares highest identity (65%) with the human A2b AR (Table 6.2). Sequence alignments of the human and predicted zebrafish A2a and A2b adenosine receptor polypeptides are shown in Figures 6.2 and 6.3, respectively. By aligning the zebrafish and human A2 receptors, we identified seven putative transmembrane (TM) regions that are highly conserved to the TM domains of the human A2 receptors.

Table 6.2. Pairwise Comparisons between Zebrafish and Human A2 Adenosine Receptors

Zebrafish	A2a.1	A2a.2	A2b
Human A2a	71	74	62
Human A2b	62	63	65

Numbers represent percent amino acid identity

```
Human A2a    1  -MPIMGSSVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVTNYFVVSLAAADIAVGVLA
Mouse A2a    1  ----MGSSVYIMVELAIAVLAILGNVLVCWAVWINSNLQNVTNFFVVSLAAADIAVGVLA
ZF A2a.1     1  MLNNVFDVLYMILELLIALLSVLGNVLVCWAVGLNSNLQSITNFFVVSLAVADIAVGVLA
ZF A2a.2     1  ----MSSLVYIVLELVIAVLAVAGNVLVCWAVCLNSNLQSITNFFVVSLAVADIAVGVLA

Human A2a   60  IPFAITISTGFCAACHGCLFIACFVLVLTQSSIFSLLAIAIDRYIAIRIPLRYNGLVTGT
Mouse A2a   57  IPFAITISTGFCAACHGCLFIACFVLVLTQSSIFSLLAIAIDRYIAIRIPLRYNGLVTGM
ZF A2a.1    61  IPFSIVISTGFCANFYGCLFIACFVLVLTQSSIFSLLAIAIDRYIAIKIPLRYNSLVTGQ
ZF A2a.2    57  IPFAVTISIGFCSNFHGCLFIACFVLVLTQSSVFSLLAIAVDRYIAIKIPLRYNSLVTGR
                                                                1↑2

Human A2a  120  RAKGIIAICWVLSFAIGLTPMLGWNNCGQPKEGKNHSQGCGEGQVACLFEDVVPMNYMVY
Mouse A2a  117  KAKGIIAICWVLSFAIGLTPMLGWNNCSQTDE--NSTKTCGEGRVTCLFEDVVPMNYMVY
ZF A2a.1   121  RARGIIAICWVLSVIIGLTPMLGWHKARLQEG---HNGTCPPGMMECLFEEVVVMDYMVY
ZF A2a.2   117  RAKGIIAVCWILSVVIGLTPMFGWNTSIDAG----TNSSCPQGMTECLFEKVVTMGYMVY

Human A2a  180  FNFFACVLVPLLLMLGVYLRIFLAARRQLKQMESQPLPGE--------RARSTLQKEVHA
Mouse A2a  175  YNFFAFVLLPLLLMLAIYLRIFLAARRQLKQMESQPLPGE--------RTRSTLQKEVHA
ZF A2a.1   178  FNFFACVLVPLLLMLAIYLRIFMAARHQLKCIESKAIPCEL-------KSRSTLQKEVHA
ZF A2a.2   173  FNFFGCILIPLFAMLAIYTWIFTAARRQLRQMEQKLAHLQGHAHKEGSSSRSTLQKEVHA

Human A2a  232  AKSLAIIVGLFALCWLPLHIINCFTFFCPDCSHAPLWLMYLAIVLSHTNSVVNPFIYAYR
Mouse A2a  227  AKSLAIIVGLFALCWLPLHIINCFTFFCSTCQHAPPWLMYLAIILSHSNSVVNPFIYAYR
ZF A2a.1   231  AKSLAIIVGLFAVCWLPLHIINCFTLFCPECERPPALIMYLAIILSHANSVVNPFIYAYR
ZF A2a.2   233  AKSLAIIVGLFAVCWLPLHIINCFTLFCPQCDRPQDWVMYLAIILSHANSVVNPFIYAYR

Human A2a  292  IREFRQTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSD---------GEQVSLRLNGHP
Mouse A2a  287  IREFRQTFRKIIRTHVLRRQEPFRAGGSSAWALAAHSTE---------GEQVSLRLNGHP
ZF A2a.1   291  IREFRHTFRKIVRYHILGRREPLSCNGSTR--TSTRTSV---------ADSLRIKVNGLV
ZF A2a.2   293  IRDFRQTFRRIIRRHFLWHESRLAIGNSNGGMTASSAAVSVIETSCTMSNGYVMDAANPI

Human A2a  343  PGVWANGSAPHP--ERRPNGYALGLVSGG-----------SAQESQGNTGLPDVELLSHE
Mouse A2a  338  LGVWANGSAPHS--GRRPNGYTLGPGGGG-----------STQGSPG-----DVELLTQE
ZF A2a.1   340  RELYAEQSSTTSSCESAEPGHTHRPVSTENSILDNQPIEISNSHRHTALRHPESPLTGNN
ZF A2a.2   353  PGMISCDNFTKELPAKIKPQEEFQDLGYS----------LNGSLDHSFNANSTPIFSSHS

Human A2a  390  LKGVCPEPPGLDDPLAQDGAGVS----------------------
Mouse A2a  380  HQ-EGQEHPGLGDHLAQGRVGTASWSS--EFAPS-----------
ZF A2a.1   400  EGLACRKHAGLDITDGKDLSSPLHIKS--ALYVQTAHCVELTEVS
ZF A2a.2   403  REEVSSIRDHVEITTVKDCSDFTHVQDRCLMPVRTSNSSGLAEVS
```

Figure 6.2. Comparison of zebrafish and mammalian A2a adenosine receptors. Human, mouse, and zebrafish (ZF; A2a.1, A2a.2) A2a receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence.

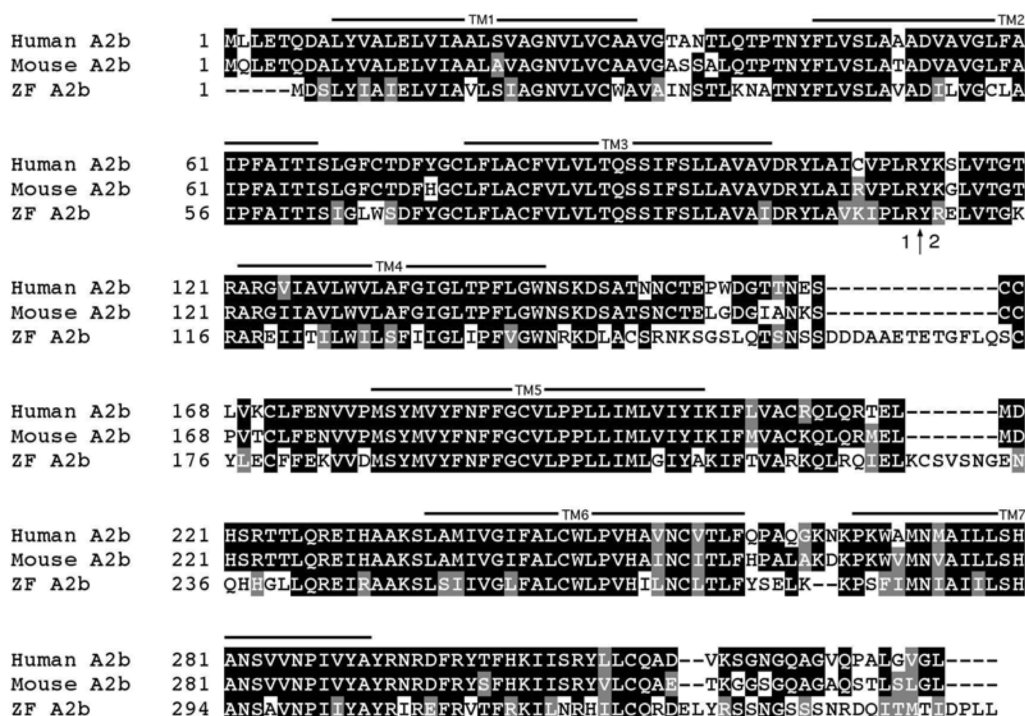

Figure 6.3. Comparison of zebrafish and mammalian A2b adenosine receptors. Human, rat, and zebrafish (ZF; A2b) A2b receptors were aligned using CLUSTALW. Ellipses in sequences allow optimal alignment for amino acid insertions/deletions. Identical amino acids are highlighted in black, and conserved amino acids are highlighted in gray. Amino acids are numbered to the left of each line. The transmembrane (TM) domains are indicated by solid lines above the sequence.

6.3.3 Phylogenetic Analysis of Zebrafish A2a and A2b Receptors

To confirm the evolutionary relationships of zebrafish A2R genes, we conducted a phylogenetic analysis using maximum parsimony (MP; Felsenstein, 1981) and distance matrix (DM; Fitch and Margoliash, 1967) methods (Figure 6.4). Clustering of A2a with other vertebrate A2a sequences was strongly supported (MP,95%; DM, 97%) by trees generated. Clustering of zebrafish A2b with other fish A2b sequences is supported by 91% (MP) and 100% (DM).

6.3.4 Chromosomal Mapping of Zebrafish A2a and A2b Receptor Genes

We determined the chromosomal positions of the zebrafish A2a.1, A2a.2 and A2b receptor genes by using the T51 radiation hybrid panel (Kwok et al., 1998). Gene map positions were calculated with the Zon RH mapper resource (http://zfrhmaps.tch.harvard.edu/ZonRHmapper). A summary of the map positions of the individual A2 receptor genes is presented in Table 6.3. The zebrafish A2a.1 gene mapped to chromosome 8 at a distance of 11cR from marker GA482T7. The zebrafish A2a.2 gene mapped to chromosome 21at a distance of 4cR from marker chunp306. The human A2a gene is located on chromosome 22, however synteny between zebrafish chromosomes 8 and 21 and human chromosome 22 has yet to be established (Woods et

Figure 6.4. Phylogenetic analysis of vertebrate adenosine receptors. Tree was rooted using the human beta2 adrenergic receptor (NP_000015) and the human histamine receptor (NP_071640) peptide sequences. Branch lengths were estimated by the method of Fitch and Margoliash (1967) using all aligned positions. Evolutionary distance scale is below tree. The maximum parsimony and Fitch/Margoliash consensus trees obtained by bootstrap analysis showed identical topologies. Numbers to the left of each node indicate percent support from bootstrap analysis (Fitch/Margoliash below, maximum parsimony above). Both methods strongly support clustering of all A2a and A2b sequences (100%). Sources of sequences (accession number or SwissProt identifier): Zebrafish A2a.1 (AY945800), A2a.2 (AY945801), and A2b (AY945802); chicken A1: P_989647, chicken A2a: XP_425280, chicken A2b: P_990418, chicken A3: NP_989482, mouse A1: NP_001008533, mouse A2a: NP_033760, mouse A2a: NP_031439, mouse A3: NP_033761, Xenopus laevis A2a: AAH84390, human A1: NP_000665, human A2a: NP_000666, human A2b: NP_000667, human A3: NP_000668. The following sources of sequences are DNA accession numbers of species-specific genomes: medaka A2b: BAAF02026954, fugu A2b: CAAB01003799, fugu A2a: CAAB01000409.

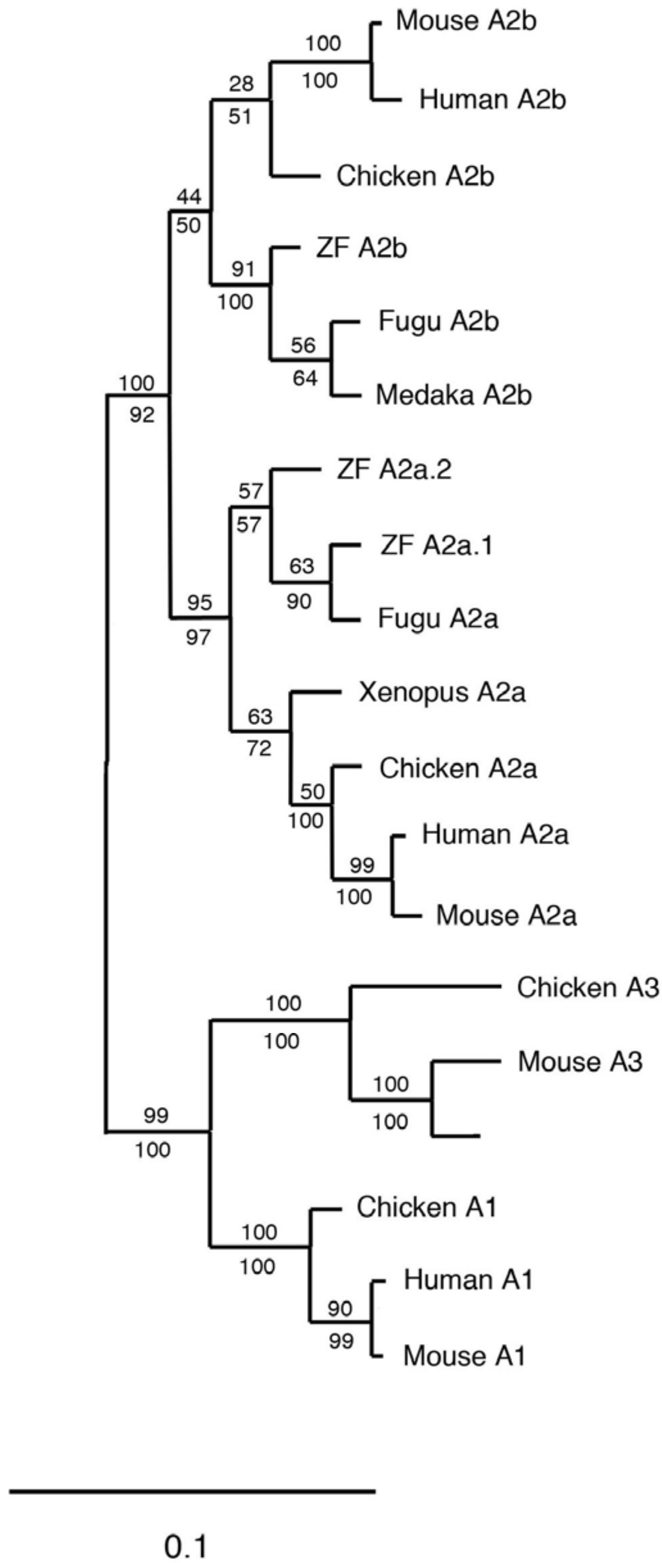
Mouse A2b
100
100
Human A2b
28
51
Chicken A2b
44
50
ZF A2b
91
100
Fugu A2b
56
64
Medaka A2b
100
92
ZF A2a.2
57
57
ZF A2a.1
63
90
Fugu A2a
95
97
Xenopus A2a
63
72
Chicken A2a
50
100
Human A2a
99
100
Mouse A2a
Chicken A3
100
100
Mouse A3
100
100
99
100
Chicken A1
100
100
Human A1
90
99
Mouse A1
0.1

Table 6.3. Map Positions of Zebrafish and Orthologous Human and Mouse Adenosine Receptor Genes

	Accession			Zebrafish	Human Ortholog		Mouse Ortholog	
Gene	No.	Forward Primer	Reverse Primer	Chromosome (LOD)	Name	Location	Name	Chromosome
adora2a.1	AY945800	tacattgaggcgaggcatggtcc	gatggcaatgtatcggtcaatggc	8 (14)	ADORA2A	22q11.23	Adora2a	10
adora2a.2	AY945801	cacactggttgatagcaccatg	ctgccttcaaccaatacacgg	21 (14)				
adora2b	AY945802	catggcatcttaagtgaagtgagc	tcatgcgcggaatgtcgtgca	5 (16)	ADORA2B	17p12-p11.2	Adora2b	11B2

Human and Mouse map positions were obtained from Entrez Gene (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene)

al., 2000). The zebrafish A2b gene localized to chromosome 5 at a position 9cR from marker zc199f23.za. Zebrafish chromosome 5 and human chromosome 17 (location of A2b gene) share synteny, with four genes identified on chromosome 17 also mapping to zebrafish chromosome 5 (Woods et al., 2000).

6.3.5 Expression of Zebrafish A2a and A2b Receptor Genes

We used whole-mount *in situ* hybridization to examine the spatio-temporal expression of the A2 adenosine receptor genes during zebrafish embryogenesis. The expression patterns of the zebrafish A2 receptor genes are shown in Figures 6.5-6.7.

Expression of the *adora2a.1* gene begins at early somitogenesis where transcripts are present in the ventral mesoderm (Fig. 6.5A). At mid-somitogenesis (15 hpf), the *adora2a.1* gene persists in the ventral hematopoietic mesoderm (Fig. 6.5B). In 24 hpf embryos, *adora2a.1* transcripts were detected in the blood of the intermediate cell mass (ICM) of the mesoderm (Fig. 6.5C). By 36 hpf, transcripts of *adora2a.1* were found in the blood, and in distinct nuclei of the diencephalon, tegmentum, and hindbrain (Fig.6.5 D). At 48 hpf, *adora2a.1* transcripts were diffuse throughout the brain with more robust staining in the hindbrain (Fig. 6.5E).

Expression of the *adora2a.2* gene was first detected at gastrulation in the yolk syncytial layer (YSL) and ventral margin (Fig. 6.6A). Expression of the *adora2a.2* gene persists in the YSL through early somitogenesis and also is detected in the tail bud (Fig. 6.5B). At

mid-somitogenesis, transcripts of adora2a.2 continued to be expressed in the YSL and in the tail bud (Fig. 6.6C). In addition, expression of the adora2a.2 gene was also detected in neurons of the spinal cord (Fig. 6.6C). At 24 hpf, *adora2a.2* transcripts were detected in the interrenal tissue and in a few neurons in the spinal cord (Fig. 6.6D). By 48 hpf, transcripts of the *adora2a.2* gene are expressed only in the telencephalon (Fig. 6.6E).

The *adora2b* gene was first detected at early somitogenesis in the YSL (Fig. 6.7A). The expression of *adora2b* persisted in the YSL at mid-somitogenesis and 24 hpf (Fig. 6.7B,C). At 36 hpf, *adora2b* transcripts were expressed in distinct nuclei of the telencephalon and diencephalon (Fig. 6.7D). By 48 hpf, the *adora2b* gene was basally expressed in the brain with more intense labeling in the anterior lateral hindbrain and in the neurons of the spinal cord.

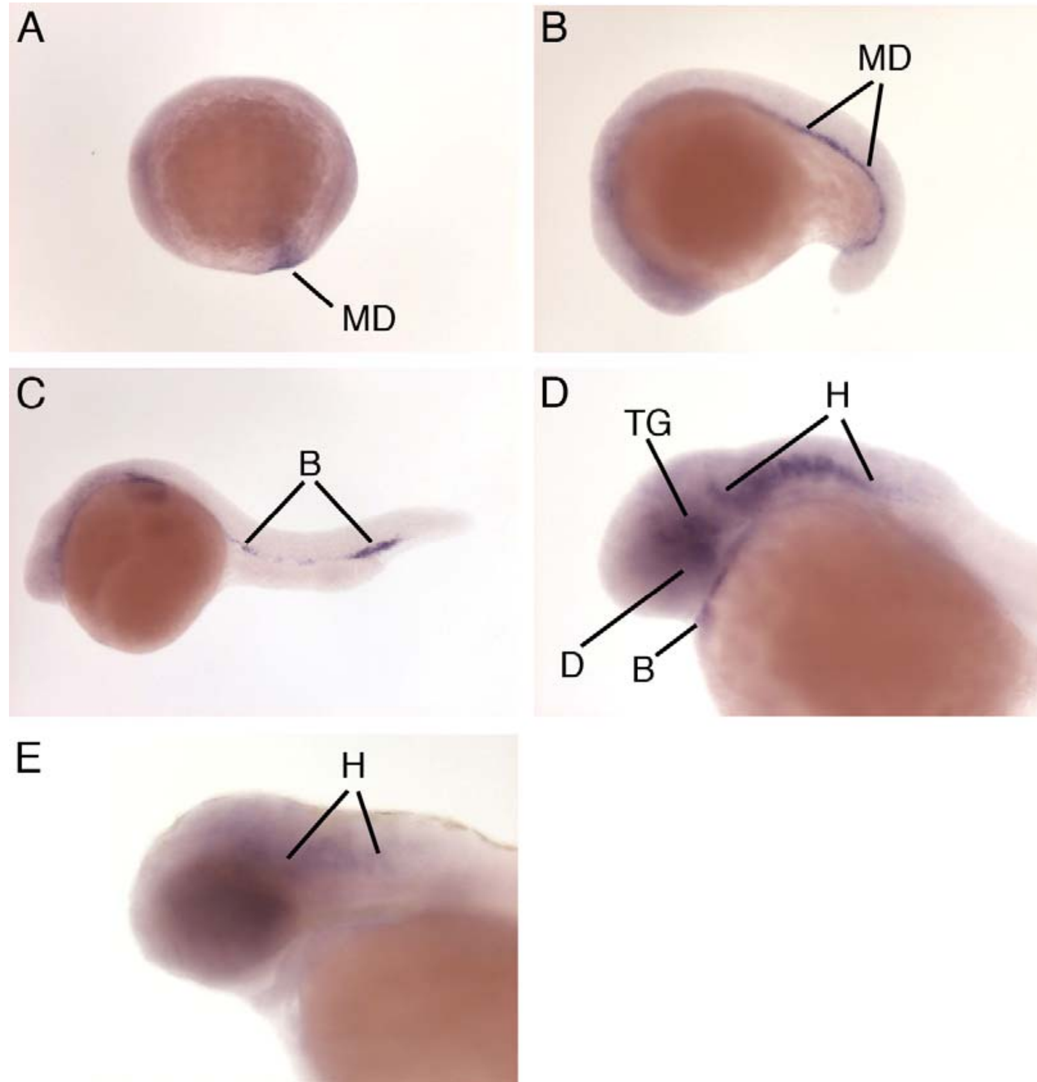

Figure 6.5. Expression of the adenosine receptor A2a.1 gene *adora2a.1*. Lateral views of **A:** early somitogenesis 11 hours postfertilization (hpf) embryo **B:** mid-somitogenesis (15 hpf) **C:** 24 hpf embryo **D:** 36 hpf embryo **E:** 48 hpf. B, blood; H, hindbrain; MD, mesoderm; TG, tegmentum.

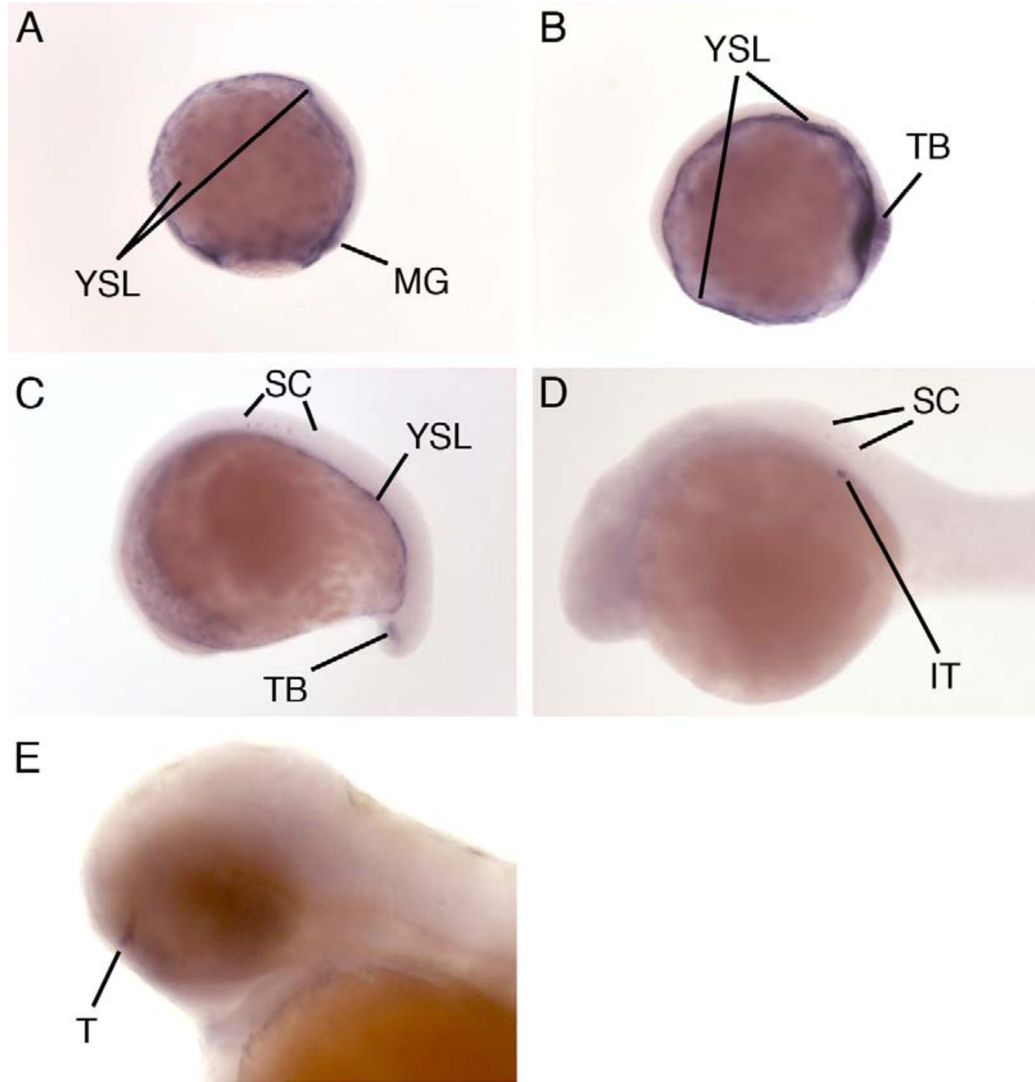

Figure 6.6. Expression of the adenosine receptor A2a.2 gene *adora2a.2*. Lateral views of **A:** gastrulation embryo **B:** early somitogenesis 11 hours postfertilization (hpf) embryo **C:** mid-somitogenesis (15 hpf) **D:** 24 hpf embryo **E:** 48 hpf embryo. IT, interrenal tissue; MG, margin; SC, spinal cord; T, telencephalon; TB, tail bud; TG, tegmentum; YSL, yolk syncytial layer.

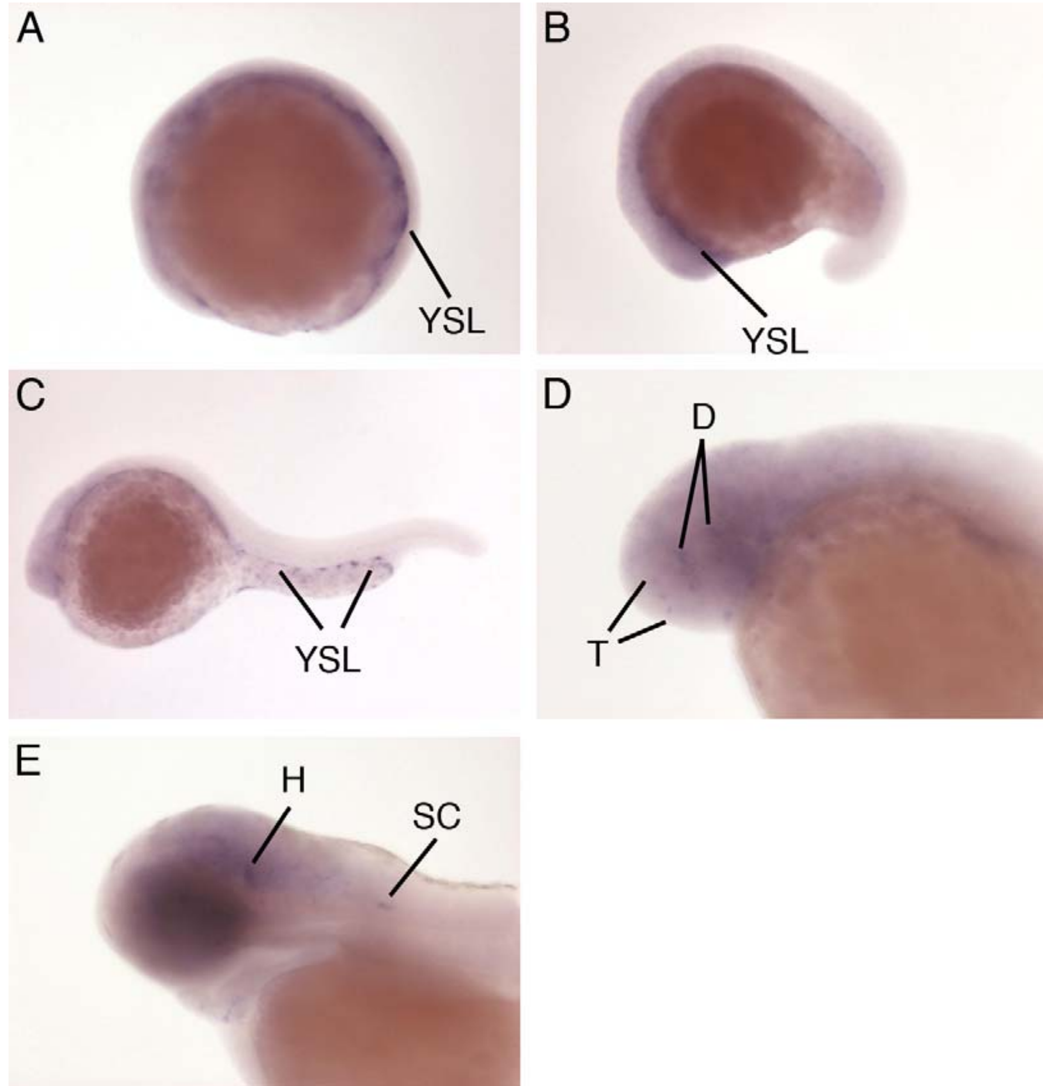

Figure 6.7. Expression of the adenosine receptor A2b gene *adora2b*. Lateral views of **A:** early somitogenesis 11 hours postfertilization (hpf) embryo **B:** mid-somitogenesis (15 hpf) **C:** 24 hpf embryo **D:** 36 hpf embryo **E:** 48 hpf embryo. D, diencephalon; H, hindbrain; SC, spinal cord; T, telencephalon; YSL, yolk syncytial layer.

6.4 Discussion

Dopaminergic neurons in the zebrafish embryo are destroyed by the neurotoxin MPTP. The mechanism of MPTP neurotoxicity appears to be similar to that observed in mammals. A recent study has shown specific compounds (MAO-B and DAT inhibitors) protect zebrafish dopaminergic neurons from MPTP (McKinley et al., 2005). In addition to these drugs, our results have shown caffeine, an A1/A2a adenosine receptor antagonist, protected dopaminergic neuron clusters in the ventral diencephalon of 5 dpf embryos. No protection was observed for the pretectal neuron clusters. It has been shown previously that the pretectal cluster is particularly sensitive to MPTP (McKinley et al., 2005). In adult zebrafish, axons of pretectal neurons project to the optic tectum (Ma, 2003), however little is known about their function.

Previously, caffeine has been shown to possess neuroprotective properties in mouse models of PD. Neuroprotection by caffeine likely involves the antagonism of A2a receptors. It has been observed that A2aR knockout mice have decreased MPTP neurotoxicity, suggesting a role of this receptor in facilitating MPTP toxicity and contributing to neuroprotection via the antagonistic action of caffeine (Chen et al., 2001). The mechanism of neuroprotection of A2aR inactivation and antagonism remains unclear. The zebrafish genome revealed that two A2a and one A2b adenosine receptor genes exist in this species. Therefore, a similar mechanism of neuroprotection via A2aRs may be present in zebrafish.

Sequence comparisons and phylogenetic analysis provide strong support that zebrafish possess two A2a and one A2b adenosine receptor genes. Alignment of the zebrafish and mammalian A2a and A2b receptor polypeptides reveal seven putative TM domains that are highly conserved to segments of the human A2R TM domains. In addition, comparison of the genomic sequences show that the intron/exon organization of the zebrafish and mammalian A2R genes is highly conserved, suggesting the genes arose from a common ancestral gene. Gene duplication events have likely led to the evolution of two A2aR genes in zebrafish. The duplication-degeneration-complementation model has been proposed to account for the retention of duplicate genes in the genome (Force et al., 1999). This model proposes that as a result of degenerative changes in duplicated genes, the duplicates together retain the original functions of the single ancestral gene. The most common fate for a set of duplicated genes is that one member of the pair will degenerate to a pseudogene or be lost from the genome (Prince and Pickett, 2002). Our identification of a single zebrafish ortholog of the mammalian A2b receptor gene suggests that the second A2b ortholog was lost during zebrafish evolution.

The mammalian adenosine signaling system plays a role in a variety of physiological functions in the cardiovascular system, behavior, respiratory system, and neuroprotection. The data presented here represents the first analysis of temporal and spatial expression of A2 AR genes in the developing zebrafish embryo. Our *in situ* hybridization studies indicate that A2a and A2b adenosine receptor expression in zebrafish parallels for the most part A2-like receptor expression in mammals. For example, A2a receptor transcripts are expressed in lymphocytes and platelets, where their functions are largely

unknown. Zebrafish *adora2a.1* transcripts are found in blood of 24 through 36 hpf of development. Mammalian A2aR genes are expressed in the kidney and the activation of these receptors has been reported reduce renal injury in models of ischemic renal failure (Okusa, 2002). At 24 hpf, zebrafish *adora2a.2* transcripts are localized to interrenal tissue which may suggest that this receptor is associated with preventing renal injury in zebrafish. Mammalian A2aR transcripts are found in various mammalian brain regions including the striatum. From 36 hpf through 48 hpf, basal expression of *adora2a.1* transcripts is found in the diencephalon, tegmentum, and hindbrain of the developing zebrafish. *adora2a.2* transcripts are localized to the telencephalon in 48 hpf zebrafish embryos. In zebrafish, dopaminergic neurons are located primarily in the diencephalon. Here, we have localized transcripts of *adora2a.1*, but not *adora2a.2*, to the developing zebrafish diencephalon. It is interesting to note that while the ventral diencephalic neurons were protected by caffeine, the pretectal cluster of dopaminergic neurons seemed to remain susceptible to the toxicity of MPTP. Our *in situ* analysis failed to identify transcripts of *adora2a.1* and *adora2a.2* in the pretectal cluster. It will be interesting to determine whether the neuroprotective effect of caffeine on dopaminergic neurons is mediated through the A2a.1 or A2a.2 adenosine receptor or both. This question may be best addressed using morpholino knockdowns of the A2a receptor subtypes in zebrafish.

In summary, we have shown that caffeine can protect against the neurotoxicity of MPTP in zebrafish embryos. Caffeine is believed to be neuroprotective due to its antagonistic actions on A2a ARs. We have cloned and characterized two A2a and one A2b ARs genes in zebrafish. Thus, zebrafish may be a useful model system for understanding the

mechanism of caffeine/A2a AR neuroprotection and for screening other drugs with neuroprotective properties.

Chapter Seven

Closing Discussion

7.1 Identification of D2-like Dopamine Receptor Genes in Zebrafish

Dopaminergic signaling controls a wide variety of complex functions including locomotion, cognition, and emotion. Dysfunction in dopaminergic signaling contributes to neurological disorders such as SZ and PD. The primary treatments of these diseases involve alterations in neuronal dopaminergic signaling but the underlying mechanisms that lead to disease remain unclear. The primary treatments for SZ are antipyschotics that block the dopamine receptors, while the primary treatment for PD is L-DOPA, a precursor of dopamine. While these drugs treat the primary symptoms of disease, they also produce unwanted side effects. Current research is aimed at identifying novel drugs and their targets for the management of these diseases. The mechanisms that lead to disease states most likely involve complex genetic components that have yet to be identified. Zebrafish, a newly established genetic vertebrate model, has the potential to provide important insights into the mechanisms of dopamine signaling. The specific focus of this dissertation is the cloning and characterization of D2-like dopamine receptor genes in zebrafish.

The presence of dopamine receptors in zebrafish illustrates the importance of dopaminergic signaling throughout evolution. We have identified three D2, one D3 and three D4 dopamine receptor genes in zebrafish. The most plausible explanation for multiple D2 and D4 genes is the supposition that gene duplication events have occurred in bony fishes, including zebrafish. It has been suggested that gene duplication events have played a key role in the development of complexity in vertebrates through the

evolution of new gene functions (Taylor and Raes, 2004). The duplication-degeneration-complementation (DDC) model suggests the retention of duplicated genes allows the partitioning of the ancestral gene function (Force et al., 1999). It will clearly be of interest to determine whether the subtypes of the zebrafish D2 or D4 genes together function as the single mammalian ortholog or whether these subtypes have evolved to acquire novel functions. The unique expression profiles of these receptors suggest that the functions of the D2-like receptor genes may have unique functions as well. For example, the D4b receptor subtype is expressed in the otic vesicle at 36 hpf. The function of D4Rs in the developing zebrafish is unknown but the expression pattern suggests D4b may play a novel role in inner ear development.

7.2 Function of D2-like Dopamine Receptor Genes in Zebrafish

Identification of the molecular components of dopamine signaling in zebrafish suggests this species may be a useful model system to study neurological disease. Zebrafish possess a balance of complexity and simplicity. This species is a small diploid vertebrate with unique features that include robust reproduction, transparency of embryos, and ex utero development. These properties make the zebrafish system amenable for large-scale genetic study. Together with the forward and reverse genetic techniques and the identification of multiple subtypes of dopamine receptor genes, zebrafish have the potential of elucidating the functions of the diverse dopamine receptor subtypes found in mammals.

Forward genetic analysis may be more favorable than gene knockdowns in determining function of the zebrafish dopamine receptor genes. A zebrafish mutant lacking a particular subtype can be studied throughout its lifetime. Furthermore, complex behaviors such as swimming and self-feeding do not occur until five days post fertilization (dpf). Gene knockdown studies using morpholinos are advantageous for studying genes essential for development in which the knockdown causes specific morphological abnormalities. In the case of the dopamine receptor genes, morpholinos may not be the ideal genetic tool for determining gene function if, in fact, the function involves complex behaviors. The disadvantage of the morpholino, which is injected at the one-cell stage, is as the embryo grows the morpholino gets diluted by 2-3 dpf and begins to lose its efficacy. Additionally, non-specific effects of morpholino oligos have been noted which widespread cell death, neural degeneration, and defects in epiboly (Heasman, 2002). In fact, our lab has knocked down two of the DR subtypes in zebrafish. Morpholinos targeted to the ATG start site of *drd2a* and *drd4b* were injected at the one-cell stage. While there were no gross morphological defects, behavior was difficult to interpret in the developing embryo, which may have been due to a lack of behaviors in zebrafish embryos. In addition, there may have been other subtypes compensating for the lack of the receptor that was knocked down. It may be necessary to knock-down the entire subtype receptor family in order to observe a behavioral or morphogical defect. Due to the limitations of morpholinos and the plasticity of the brain during development, it is likely that results of morpholinos targeted to zebrafish dopamine receptor subtypes will be difficult to interpret.

The limitations for the use of dopamine receptor targeted morpholinos may be overcome with target-selected mutagenesis called TILLING (Targeting Induced Local Lesions in Genomes) that is now available in zebrafish. For this method, male zebrafish are mutagenized using N-ethyl-N-nitrosourea (ENU) and these males are subsequently used to produce a population of F1 animals that may harbor random heterozygous mutations in a gene of interest. In order to detect mutations, the method of TILLING is implemented which is based on the enzymatic cleavage of heteroduplex DNA using a plant endonuclease CEL-1 (Oleykowski et al., 1998). The gene of interest is amplified via PCR using genomic DNA of the F1 animals. The PCR product is incubated with CEL-1 and cleavage products are electrophoresed in order to identify the presence of mutations. With the identification and cloning of the D2-like dopamine receptor genes in zebrafish, it will be possible to identify specific receptor subtype mutant zebrafish using these sequences and the method of TILLING. This will allow for a permanent knockout zebrafish in which genetic and, perhaps more importantly, behavioral studies can be conducted throughout the life-span of the animal.

As an alternative to disrupting dopamine receptor gene function, antagonists and agonists of the receptors can be used to analyze their physiological roles. Compounds, including DR antagonists, can be directly administered to the tank water where it can easily be taken up inside the zebrafish embryo and adult. We have shown that zebrafish larvae are sensitive to the D4 antagonist clozapine, an atypical antipsychotic used in treatment-resistant schizophrenic patients. The zebrafish response to clozapine was a rapid and robust sedative-like effect on locomotion. The molecular and cellular mechanisms of

how dopaminergic pathways in the brain regulate locomotion remain largely unknown. The robustness of the response in zebrafish to the exposure of clozapine establishes a rapid assay system in which genetic screens can be used to identify mutants with altered responses to clozapine. In addition to clozapine, other antipsychotics (haloperidol and fluphenazine) were also found to impair locomotion in larval zebrafish (Giacomini et al., 2006). This study further strengthens the conclusion that this animal model may prove to be an ideal system in which to conduct genetic screens for the identification of novel genes involved the dopaminergic control of locomotor activity.

Due to the divergence in amino acid sequence of the zebrafish D2-like dopamine receptors, it is not known whether the binding affinities of these antipsychotics are similar to those in mammals. The presence of multiple D2 and D4 receptor genes in zebrafish may suggest different binding affinities for these drugs. We have attempted to determine the binding profiles of spiperone (D2 antagonist) for the zebrafish D2 and D3 receptors using a ligand binding assay. However, we were unsuccessful in detecting spiperone binding to the zebrafish D2 and D3 receptors. From these results, it is not known whether spiperone does not bind to the zebrafish DRs or whether the methodology was prone to error. In future studies, it will be of great importance to determine the binding affinities of DR antagonists and agonists on zebrafish dopamine receptor subtypes.

In addition to determining binding affinities of various DR drugs, the anatomical distribution of the binding of these drugs in the zebrafish brain may aid in determining

the physiological effects of these drugs on the DA neurotransmitter system. With the use of radiolabelled drugs, it may be possible to determine the brain regions (ascending and descending dopaminergic tracts) in which these drugs bind. In contrast to the localization of mammalian DAergic neurons which are found in the midbrain, the majority of zebrafish DA neurons are located in the basal forebrain (diencephalon). However, anatomical and tracing experiments have shown that these neurons send ascending projections to the telencephalon, which is hypothesized to represent the homologous mammalian mesostriatal pathway. Therefore, the DA neurons in the zebrafish diencephalon may function similarly to the mammalian midbrain DA neurons. We have determined the gene expression profiles of the entire D2-like DR family in embryonic zebrafish. It is likely that results of radiolabelled drug studies will overlap with these expression profiles and the proposed DA projection pathways.

7.3 Zebrafish: An Animal Model for Schizophrenia

It is often misconceived that animal models can reproduce all aspects of complex human diseases such as SZ. Complex neuropsychiatric illnesses cannot be exactly mimicked in available animal model systems. However, animal models are useful and important for understanding the role of neurotransmitter systems and genes involved in the symptoms of SZ and related illnesses. Because it is impossible to model the entire spectrum of symptoms of SZ in an animal, researchers have focused their studies on the endophenotypes of SZ (intermediate phenotypes that are biological markers that may indicate susceptibility to SZ). Many of the endophenotypes studied can and have also

been reported in the animal model, zebrafish. Selected phenotypes associated with SZ include drug-induced alterations in locomotor activity and pre-pulse inhibition (PPI).

It is important for any animal model to have predictive validity when using them in research of a human disease. Predictive validity refers to the idea that both the experimental and clinical observations are similar (Ellenbroek and Cools, 1990). One of the discomforting side effects of antipyschotics is sedation. We have shown larval zebrafish exhibit a dose dependent decrease in locomotor activity after exposure to the atypical antipsychotic clozapine. Locomotor activity can be quantitated by several means. We chose to develop a locomotor assay that uses human observations to count the number of times larval zebrafish cross a line in a given area. While this method is labor intensive and may be subject to human error, the particular assay design and DR antagonist used was successful in observing a significant reduction in locomotion of larval zebrafish. In cases in which certain drugs do not have a robust effect on swimming behavior it will be necessary to use an automated system for measuring locomotion. More sophisticated behavioral analysis such as video tracking may lead to a more precise measure of overall locomotion in zebrafish. Video tracking would allow for the measurement of swimming speed and for the tracing of positions of the fish in the tank.

Another endophenotype of schizophrenia used to model the disease in animals is PPI. PPI is a measure of sensorimotor gating that has been found to be disrupted in SZ patients. In mammals, the startle response is a defensive reaction to a sudden intense stimuli. When a weaker prepulse stimuli is administered prior to the stronger pulse

stimuli, the startle reflex is reduced. It may be possible to conduct a forward screen using zebrafish to find mutants with alterations in PPI. The acoustic/vibrational startle response is present in 72 hpf larvae and can be measured by the swimming trajectory of the fish (Guo, 2004). Initial studies have suggested that this startle response is weakened by a prepulse (Burgess et al, 2004 unpublished). Mutants with enhanced and reduced PPI may be valuable models for studying underlying mechanisms involved in neuropsychiatric disorders.

Cognitive deficits in learning and memory are present in patients with SZ. Learning and memory tests have already been studied in adult zebrafish with the use of a T-maze (Ninkovic et al., 2006). This experimental paradigm can be used to study the working memory in zebrafish and to assess cognitive changes in drug treated or mutagenized fish. Other negative symptoms of SZ include flattening affect and social withdrawal. These symptoms are the most difficult to model in animals. However, social withdrawal which is relatively easy to study in animals such as rodents can also be studied in zebrafish. Zebrafish are also social animals that display schooling behavior. These social groups are called shoals which consist of fish with similar morphologies (Peichel, 2004). It will clearly be of interest to find mutant zebrafish that lack this social interaction and to observe whether treatment with antipsychotic drugs can inhibit social withdrawal.

7.4 Zebrafish and Neuroprotection

Dysfunction in dopaminergic signaling is also found in patients with Parkinson's disease, which is characterized by the selective degeneration of dopaminergic neurons in the substantia nigra. Because the pathogenesis of PD remains poorly understood, researchers model this disorder in animal systems using the neurotoxin MPTP. It has recently been shown that the administration of MPTP to zebrafish embryos and larvae causes a significant reduction in dopaminergic neurons in the diencephalon (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005). While rare cases of familial PD do exist, most cases of the disease are sporadic suggesting these cases may be a result of exposure to environmental toxins. Therefore, there is a current need to identify compounds with neuroprotective properties.

Similar to mammals, it has been shown in zebrafish that MPTP is transported into dopaminergic neurons via the dopamine transporter (McKinley et al., 2005). Our studies have shown that coincubation of caffeine and MPTP significantly prevents the degeneration of dopaminergic neurons in larval zebrafish. Therefore, zebrafish may be useful for screening drugs with neuroprotective properties in addition to being a valuable model system to uncover the molecular mechanisms underlying neurotoxicity.

Epidemiologic and laboratory studies have shown that caffeine, an A1/A2a antagonist, reduces the risk of developing PD (Ascherio and Chen, 2003). The neuroprotective properties of caffeine are believed to be mediated via the A2a adenosine receptors located

in the striatum. A2a knockout mice have been found to be resistant against damaging effects of MPTP on dopaminergic neurons. However, the cellular and molecular mechanisms by which A2a receptors contribute to neuronal death remain unclear. We have identified two A2a receptor subtype genes (*adora2a.1 and adora2a.2*) in zebrafish. The identification of A2a receptors and the neuroprotection of caffeine from the toxin MPTP in larval zebrafish may allow for the identification of a mechanism for how the inactivation of A2a receptors prevents the degeneration of dopaminergic neurons. It is possible the A2a receptor blockade may protect against MPTP toxicity by preventing its uptake via the dopamine transporter. In this case, it may be necessary for the A2a receptor and DAT to be colocalized, which has yet to be shown. However, in the striatum, A2a and D2 receptors are found to colocalize and the two receptor subtypes have also been shown to directly interact to form heteromers (Canals et al., 2003; Hillion et al., 2002). In future studies, it will be interesting to determine if this heterocomplex resides near or interacts with the dopamine transporter. By using the sophisticated genetic techniques available in zebrafish, this vertebrate system has the potential for identifying a neuroprotective mechanism for the inactivation of A2a adenosine receptors.

Despite the complexity of SZ and PD, our studies have been aimed at the development of zebrafish as a model system to study these neuropyschiatric disorders. The work described in this thesis identifies and characterizes the D2-like dopamine receptor gene family in zebrafish thereby providing the framework to investigate the functions of the individual receptor subtypes using the powerful genetic tools available in this vertebrate model. Additionally, the conserved behavioral effect induced by the antipsychotic

clozapine on larval zebrafish should help to identify mutants with altered responses to the drug. Finally, we have presented data that suggests zebrafish can be used as a high-throughput animal model to identify neuroprotective compounds in addition to elucidating the molecular mechanisms involved in PD pathogenesis.

In the past several decades, zebrafish have proven to be an invaluable animal model. With an almost completely sequenced genome and development of behavioral assays in combination with forward genetic screens, it is expected that zebrafish will provide important insights into dopamine-related neurological disorders.

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Curriculum Vitae

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Publications

Blasiole B, Degrave A, Canfield V, Boehmler W, Thisse C, Thisse B, Mohideen MA, Levenson R. Differential expression of Na,K-ATPase alpha and beta subunit genes in the developing zebrafish inner ear. Dev Dyn. 2003 Nov;228(3):386-92.

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