Modeling and Mapping Addiction in the Zebrafish, Danio rerio

Bradley Serpa

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Modeling and Mapping Addiction in the Zebrafish, 
*Danio rerio*

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My thesis is dedicated to mama Frances Forbus
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ABSTRACT.

Driven by the communication of dopamine, the vertebrate reward system has been evolutionarily conserved to maintain survival and optimize fitness. The neural circuits governing this system integrate sensory stimuli to produce appropriate, self-preserving responses that underlie experience-based learning. In the most primitive vertebrates, dopamine release in neuronal circuits drives homeostatic behaviors, such as seeking nutrients, finding a mate, or avoiding danger. From agnathans to mammals, dopaminergic synthesis and signaling genes and molecules, along with neuronal pathways and reward system-based behaviors, remain highly conserved. Dopamine signaling proteins include two classes of metabotropic G-Protein Receptor Coupled Dopamine Receptors, D1-like (DRD1) and D2-like (DRD2). DRD1 stimulate the neuron by upregulating adenylate cyclase activity, while DRD2 inhibits neurons by blocking or down-regulating adenylate cyclase. Though greatly conserved, the reward system can be hijacked by chemicals that trigger the release of dopamine. Drugs of abuse, like amphetamines, for instance, increase dopamine availability to trigger reward circuits, leading to addiction behaviors. The mechanisms by which amphetamines stimulate dopamine release among reward neurons and the addiction behaviors expressed have not yet been modeled and correlated in zebrafish, a viable translational model for studying drug addiction. In the present study, addictive behaviors in zebrafish were elicited after fish were exposed to amphetamines through a condition place preference paradigm. After the conditioning period, amphetamine-treated fish spent significantly more time in an experimental tank compartment that was paired with amphetamine exposure (p = 0.0031). Likewise, THC (p = 0.0393) and the anesthetic, MS222 (p = 0.0290) significantly affected time spent in the non-preferred tank compartment after conditioning. Amphetamine-treated fish also displayed unique and heightened anxiety and vigilance behaviors. These behaviors and the influence of amphetamines on conditioned learning are likely stimulated by the increased expression of DRD1 receptors measured in dopaminergic brain areas in the fish compared to controls. These data support the hypothesis that drugs of abuse like amphetamines trigger the communication of dopamine among reward circuit neurons.
Chapter 1: Introduction

Addiction continually creates substantial economic, health and societal costs, but available treatments remain inadequate for most individuals \(^1\). By analogy with other medical disorders, an improved understanding of the biological basis of addiction will lead to more effective treatments and eventually to cures and preventative measures. According to the National Institute on Drug Abuse (NIDA), addiction is defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences. This implies that the ‘addicted-state’ is not triggered instantaneously upon exposure to drugs of abuse. Rather, repeated exposure produces neuroadaptations (neuroplastic changes) that contribute to pathological drug-related behaviors \(^2\). These include long-term alterations in gene expression, protein regulation, anatomy, and synaptic function that collectively influence the neural circuits that govern reward, motivation, and higher order cognitive control to produce maladaptive behaviors \(^1,3\).

Although the brain circuitry underlying addiction is complex, it is clear that the mesolimbic dopamine system, consisting of the ventral tegmental area (VTA) and nucleus accumbens (NAc), are crucial substrates for the neural adaptations that underlie addiction \(^4,5,6\). Despite the chemical diversity and individual molecular targets, all addictive drugs directly or indirectly increase dopamine (DA) concentration in projections to the VTA as well as within the VTA itself \(^6\). The drug-induced plasticity observed can be explained by ‘the learning rule’ proposed by Canadian psychologist Donald Hebb, stating that when two neurons are in close
enough proximity for one to excite the other, the influence of repeated stimulation of one cell by the other will promote growth or change to increase the efficiency of communication between neurons [7].

The phenomenon described by Hebb is commonly referred to as long-term potentiation (LTP). Two phases of LTP have been identified: early-LTP (E-LTP; short term memory) and late-LTP (L-LTP; long term memory). Current thinking posits that the efficacy of a synapse can be influenced either by altering the amount of neurotransmitter released by the pre-synaptic neuron or by changes in receptor density on the post synaptic neuron [1,4,8]. Since all addictive drugs influence pre-synaptic release of DA, an explanation for the synaptic dysfunction in these circuits could potentially be explained by changes in the post-synaptic neuron [9]. In this context, the neurotransmitter receptors poised to influence synaptic transmission are likely to play critical roles in the effects of abused drugs and as novel therapeutic targets.

Under normal conditions, the mesolimbic reward system controls an individual’s response to natural rewards, such as food, sex, and social interactions, and is therefore an important determinant of motivation and incentive drive. In simplistic terms, activation of this pathway tells the individual to repeat what it just did to get that reward [10,11]. Additionally, synapses communicate with areas of the brain associated with memory to pay particular attention to all features of that rewarding experience, so it can be repeated in the future. Communication among reward nuclei, Ventral Tegmental Area (VTA), Nucleus Accumbens (NAc), and prefrontal cortex (PC), is mediated through dopaminergic (DAergic) neurons that are governed by balanced signaling of GABAergic (inhibitory) and glutamatergic (excitatory) inputs [12,13].
Within the VTA, there are two general populations of GABAergic neurons: interneurons, which provide local inhibition of DA neurons; and projection neurons, which provide long-range inhibition of multiple brain areas including the NAc\textsuperscript{14-17}. Once stimulated, GABAergic spiny neurons release the inhibitory neurotransmitter γ -aminobutyric acid (GABA), which interacts with GABA receptors present on presynaptic DA neurons\textsuperscript{18}. Binding of GABA to ionotropic GABA receptors causes a conformational change to the membrane receptor protein, resulting in an influx of Cl\textsuperscript{-} ions and hyper polarization of presynaptic DA neuron\textsuperscript{19}. In this case, the hyper polarization event creates a more negative cell membrane potential, thus inhibiting neuronal firing and DA release\textsuperscript{20,21}. Without GABA signaling modulating DAergic neurons, DA is released into the synapse, binds to postsynaptic DA receptors (DAr), and through various intracellular signaling mechanisms alters normal gene expression that potentially influences neuronal structure and communication between neighboring neurons\textsuperscript{22,23}. In general, inhibition is critical for regulating neuronal excitability, and allows flexibility in circuit connectivity.

The rewarding effect of addictive drugs is also mediated by mesolimbic DA. Following exposure to drugs of abuse, DA is elevated throughout the mesolimbic system\textsuperscript{24}. Drugs of abuse modulate release of DA through different mechanisms such as blocking GABA vesicle release, competitive binding of GABA receptor, inhibiting DA reuptake, and influencing vesicle release of DA\textsuperscript{25}. In particular, the psychoactive drugs amphetamine (AMPH) and Δ\textsuperscript{9}-tetrahydrocannabinol (THC) exert their rewarding and reinforcing effects by elevating extracellular DA and prolonging DAr signaling via direct or indirect interaction with DAergic neurons, respectively (Figure 1)\textsuperscript{24,25}. AMPH has been characterized as a DA releaser that
elevates DA by four major mechanisms: 1) It is a substrate for the DA transporter (DAT) that competitively inhibits DA uptake, 2) It facilitates the movement of DA out of vesicles and into the cytoplasm, 3) It promotes DAT-mediated reverse-transport of DA into the synaptic cleft independently of action-potential-induce vesicular release, and 4) It inhibits GABA receptor-evoked hyperpolarization via competitive binding to GABA receptors on the presynaptic DAergic neuron [26-30].

Different from AMPH, THC promotes DA release indirectly through binding of cannabinoid receptors (CB1, CB2) present on GABAergic neurons, leading to a cascade of molecular reactions that reduces GABA vesicle release, thus allowing for DA release from the presynaptic DAergic neurons [25]. This persistent increase in DA results in pathological incentive motivation to drug-associated cues and drug addiction. In this way, drug addiction is an example of adverse behavioral consequences arising from a malfunction in the mesolimbic system [14].

The differential mechanisms by which drugs of abuse alter reward system signaling critically depends on the interaction of these drugs with neurotransmitter receptors within the mesolimbic circuit [5,14]. The main type of receptor is DAr, belonging to the class of G protein-coupled receptors (GPCRs), whose actions modulate the communication of DA that underlies LTP processes leading to addiction [1,5,6].
Figure 1: The mesocorticolimbic dopamine system as target of addictive drugs

Sagittal slice of neural tissue illustrating the ventral tegmental area (VTA), the nucleus accumbens (NAc) and the prefrontal cortex (PFC). The projecting neurons are mostly dopaminergic, and under inhibitory control of GABA neurons. Schematic illustrates three cellular mechanisms by which addictive drugs increase mesolimbic DA levels. Nicotine can directly depolarize DA neurons, while opioids, gamma-hydroxybutyric acid (GHB), benzodiazepines and cannabinoids act indirectly by inhibition of GABA neurons. Cocaine, amphetamines and ecstasy interact with DA transporter on axon terminals and dendrites of DA neurons. Cocaine acts as an inhibitor of DA transporter, while amphetamines and ecstasy promote non-vesicular release of DA. Both mechanisms result in an increase of DA in the VTA, NAc, and PFC \[24,25\].

Dopamine Receptors

G protein-coupled receptors (GPCR) constitute the largest family of membrane proteins and mediate most general cellular responses to hormones and neurotransmitters, as well as being responsible for key physiological and metabolic functions \[1,5,31\]. At the most basic level, all GPCRs are characterized by the presence of seven membrane-spanning \(\alpha\)-helical segments separated by alternating intracellular and extracellular loop regions that couple to heterotrimeric G proteins \[31,32\]. GPCRs in mammals are classified into five main families according to the GRAFS classification: glutamate, rhodopsin, adhesion, frizzled, and secretin \[33\].
The rhodopsin family is the largest, serving as molecular targets for the neurotransmitters serotonin, DA, norepinephrine, epinephrine, histamine, and acetylcholine \[^{[31,32]}\]. Upon ligand-activation of GPCR, the heterotrimeric G protein dissociates into α and βγ subunits. The activated G proteins can then transduce and amplify GPCR signals via secondary messengers to elicit a variety of cellular responses \[^{[1,31-33]}\] (Figure 2). The cAMP-dependent pathway, also known as the adenylyl cyclase (AC) pathway, is an example of a secondary messenger system involved in signal transduction. GPCRs are often classified by the G protein α subunits with which they prefer to interact and are represented as either G\(_s\) (stimulatory) or G\(_i\) (inhibitory) \[^{[34]}\]. In a cAMP-dependent pathway, the activated G\(_s\)α subunit binds to and activates an enzyme called adenylyl cyclase, which in turn catalyzes the conversion of ATP into cyclic adenosine monophosphate (cAMP). Increases in concentration of cAMP may lead to the activation of protein kinase A (PKA), also known as cAMP-dependent kinase \[^{[30,34]}\]. As a result, PKA phosphorylates additional downstream target proteins, some of which are capable of being transported to the nucleus to bind to sequence specific sites and initiate synapse modifying transcriptional events \[^{[35]}\]. In the case of G\(_i\)α, the opposite is observed. G\(_i\)α mainly inhibits the cAMP dependent pathway by preventing adenylyl cyclase activity \[^{[34,35]}\]. Thus, a decreased production of cAMP from ATP, resulting in reduced intracellular concentrations of active PKA. As mentioned previously, drugs of abuse, in essence, hijack the brain’s reward system and mediate the abnormal release of DA and its exclusive activation GPCR dopamine receptors.

In the brain, DA transmits synaptic information by binding to specific cell surface GPCRs. As members of the GPCR superfamily, DAr have a canonical seven transmembrane structure and can signal through both G protein-dependent and –independent mechanisms \[^{[31,32]}\]. To
date, five subtypes of DAr have been identified and cloned. Based on their structural and pharmacological properties, a general subdivision into two groups of receptors has been made: D1-like (D1, D5) and D2-like (D2, D3, D4) \[^{[1,5,36-41]}\]. Within the mesolimbic reward pathway, the two receptor classes exert opposing intracellular effects via their associated heterotrimeric GTP-binding proteins (G proteins). D1-like receptors couple to G\(_s\) family of G proteins to stimulate cAMP production by AC and are found exclusively postsynaptically on DA-receptive cells, whereas D2-like receptors inhibit AC via G\(_i\) signaling and are present both on pre- and postsynaptic neuron \[^{[1,5,40]}\]. D1-like and D2-like receptors are further distinguished on the basis of differential protein structure and topology of genetic sequences, distinct effector mechanisms, and distribution patterns within the CNS \[^{[42]}\].

![Figure 2: Signaling mechanisms and neuronal effects of dopamine receptors](image)

**Figure 2: Signaling mechanisms and neuronal effects of dopamine receptors**

DA receptors are GPCRs and are subdivided into D1-like (D1 and D5) and D2-like (D2, D3, and D4). D1-like receptors are coupled to G\(_s\), which activates AC, leading to the production of cAMP and activation of PKA, which phosphorylates several downstream substrates. D1-like receptors increase exciteability and firing to promote LTP of striatal medium spiny neurons. D2-like receptors are coupled to G\(_i\), which inhibit the AC-cAMP-PKA transduction pathway. Adapted from Savica, R., & Benarroch, E. E. (2014)
Gene Organization

The D1- and D2-classes of DAR are different at the level of genetic structure, primarily in the presence of introns in their coding sequences \[^{[36]}\]. The D1 and D5 receptor genes do not contain introns in their coding regions, but the genes that encode the D2-like receptors have several introns, with six introns found in the gene that encodes the D2 receptor, five in the gene for D3 receptor, and three in the gene for D4 receptor \[^{[36,43,44]}\]. The existence of introns present in the genetic organization of D2-like receptors provides the basis for the generation of receptor splice variants \[^{[40]}\]. For example, the D2 receptor exists in two main variants, the long isoform (D2L) and the short isoform (D2S), generated by alternative splicing of an 87-base-pair exon between introns 4 and 5 \[^{[36]}\]. These two isoforms differ in the presence or absence of 29 amino acids in the third intracellular loop. These D2 receptor variants exhibit distinct anatomical, signaling, and pharmacological properties. D2S has been shown to be mostly expressed presynaptically and to be involved in autoreceptor functions such as control of dopamine release and regulation of extrasynaptic dopamine levels, whereas D2L is predominantly a postsynaptic isoform \[^{[36,45,46]}\]. The D1 receptors having no intron allows for all members of the gene family to be isolated rather easily from genomic DNA. In mammals, two receptor subtypes were identified (D1\textsubscript{A}/D1, D1\textsubscript{B}/D5), but other subtypes exist in nonmammalian vertebrates; D1\textsubscript{c} (Xenopus), D1\textsubscript{D} (chicken), and D1\textsubscript{x} (teleost). These additional subtypes correspond to gene duplication events that occurred early in the vertebrate lineage \[^{[47,48]}\].
Sequence and Topology

The individual members of the subfamilies of D1- and D2-like receptors share a high level of homology of their transmembrane domains and have distinct pharmacological properties $^{[43,44]}$. D1 and D5 receptors share an 80% homology in their transmembrane domain, whereas D2 receptors share a 75% homology with D3 and a 53% homology with the D4 transmembrane domains $^{[43]}$. The N-terminal domain of all receptor subtypes have a similar number of amino acid residues and carries a variable number of consensus N-glycosylation sites $^{[49]}$. Electron density studies identified D1-like receptors to have short third intracellular loops and long carboxyl terminal tails, whereas the D2-like receptors have long third intracellular loops and short carboxyl terminal tails $^{[36,43,49]}$ (Figure 3). The difference observed in the third intracellular loop is found to be responsible for G protein coupling and signal transmission.

Figure 3: Topology of DA receptors belonging to the family of D1- and D2-like receptors $^{[36]}$. 
CNS Distribution

DAR have broad expression patterns in the brain and in the periphery. The D1 and D2 receptors are present in all of the known target areas of DA in the CNS of vertebrates and their expression territories exhibit considerable overlap. However, neurons do not express D1 and D2 receptors simultaneously (or at best, only at very different levels), revealing that the transcriptional regulation of the two dopamine receptor classes is essentially non-redundant [42]. A summary of this tissue distribution is given in Table 1. The D1 receptors are expressed at high density in the nigrostriatal, mesolimbic, and mesocortical areas, such as the caudate-putamen (striatum), NAc, substantia nigra, olfactory bulb, and frontal cortex. In these areas of the reward system, binding of D1 receptors modulates feelings of reward and pleasure, motivational aspects of movement, experiential and reinforced learning, olfactory stimulus integration, and long-term memory. In the NAc, the outer layer or shell contains high populations of both D1 and D2 receptors and is most heavily influenced by drugs of abuse. D1 receptors in the shell of the NAc mediate reinforced learning and cognition associated with pleasurable experiences. D5 receptors are expressed at low levels in multiple brain regions, including pyramidal neurons of the prefrontal cortex, substantia nigra, hypothalamus, hippocampus, and the dentate gyrus, likely aiding in some of the reward functions, but also playing a part in episodic memory, reasoning, moderation of emotion, and satiety. The highest level of D2 receptors are found in the substantia nigra, olfactory bulb, caudate and putamen, VTA, and NAc. Activation of D2 receptors mediate similar mechanisms of reward and learning as D1 receptors, however D2 receptors in the NAc mediate cognition associated with aversive stimuli. The D3 receptors have a more limited pattern of distribution, the highest level of
expression being observed in the limbic areas, such as the shell of the NAc and the olfactory tubercle, likely pairing motivation/drive with reward, reward with emotion, and operant learning. The D4 receptor has the lowest level of expression in the brain, with documented expression in the frontal cortex, amygdala, hippocampus, hypothalmus, thalmus, and substantia nigra, uniquely contributing to novelty seeking behaviors (curiosity, exploration), working memory, and acquisition of fears [38,39,43,48,49].

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Location</th>
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<tbody>
<tr>
<td>D1</td>
<td>Nigrostriatal, mesolimbic, and mesocortical areas, such as the caudate-putamen (striatum), Nac, substantia nigra, olfactory bulb, and frontal cortex</td>
</tr>
<tr>
<td>D2</td>
<td>Substantia nigra, olfactory bulb, caudate putamen, VTA, Nac</td>
</tr>
<tr>
<td>D3</td>
<td>Olfactory bulb, NAc</td>
</tr>
<tr>
<td>D4</td>
<td>Substantia nigra, hippocampus, amygdala, thalmus, hypothalmus, frontal cortex</td>
</tr>
<tr>
<td>D5</td>
<td>Pyramidal neurons of prefrontal cortex, substantia nigra, hypothalmus, hippocampus, and dentate gyrus</td>
</tr>
</tbody>
</table>

*Table 1: Summary of dopamine receptor locations*

**Dopamine Receptor Function**

The D1- and D2 like receptor classes differ functionally in the intracellular signaling pathways they modulate. The D1-like receptors are coupled to heterotrimeric $G_{as}$-proteins, with activation leading to increase AC activity, and increased cyclic AMP (cAMP) production. This pathway induces the activation of PKA, resulting in the phosphorylation of variable substrates and the induction of immediate early gene expression, as well as the modulation of
numerous ion channels. In contrast, D2-like receptors are coupled to Gαi-proteins and negatively regulate the production of cAMP, resulting in decreased PKA activity, activation of ionotropic K+ channels, and the modulation of other ion channels [30-33].

The signaling pathways and gene expression changes associated with various DA receptors have been suggested to play a critical role in drug-induced neuro-adaptations in the brain (Figure 4). The mechanisms by which drugs of abuse (e.g. amphetamine and THC) are able to induce addiction vary considerably; nonetheless, similarities exist [25,51]. Stimulation of DAergic neuron by drugs of abuse induces the phosphorylation of cAMP-response element binding protein (CREB) and the transient expression of immediate early genes (IEGs) including c-Fos, Jun, and ΔFosB [52]. The transcription factor ΔFosB has been found to be a remarkable molecular biological mediator for addiction [53]. ΔFosB is a member of the Fos family transcription factors, which dimerize with a member of the Jun family to form activator protein-1 (AP-1) transcription factor complexes. AP-1 complexes then bind to AP-1 consensus sequences (TGAC/GTCA) present in the promoter regions of many neural genes. In contrast to all other Fos family proteins and IEGs, ΔFosB presents an unusually high degree of stability and therefore has a conspicuously longer half-life. To date, several downstream target genes for ΔFosB have been identified to be involved in the molecular pathways concerning addictive behavior, memory and learning [5,41,50-55].
Figure 4: Regulation of gene expression by dopamine

Stimulation of dopamine receptors in striatal neurons activates secondary messenger cascades that induce gene expression. Some genes are responsible for homeostatic adaptations that reduce sensitivity to subsequent stimulation; while others are involved in altering the strength of specific synaptic connections\[25\].

Zebrasfish: A versatile addiction model?

Organism survival often depends upon learning the conditions necessary to acquire naturally rewarding and reinforcing stimuli that serve homeostatic, survival, and reproductive purposes. Animals rapidly learn these behavioral responses to obtain natural rewards and can identify the environmental cues that predict them. Additionally, learning occurs following the consumption of rewarding psychoactive substances, such as amphetamine and THC [56,57]. Rapid conditioning occurs when drug use is paired with a place, thing, or emotional state, primarily due to the nature of reward circuitry within the limbic system [58]. Exposure to cued stimuli may
induce cravings for the drug in individuals that are dependent on a substance, and even in those who have been abstinent from drug use for a period of time [59], These classical Pavlovian and operant learning processes are believed to mediate the transition from casual, voluntary drug use, to more habitual and compulsive behaviors [60]. Rats and mice have been the pioneer organism in addiction research, due to the anatomical, biological and genomic homology between rodents and humans. However, use of rodent models are burdened by challenging husbandry, difficult in utero manipulation, and are unamenable to high throughput screening [61]. The zebrafish (*Danio rerio*) provides an alternative model to overcome these limitations [59].

Since being introduced for biomedical research purposes by Streisinger et al. in 1981, the zebrafish model has taken the place of more complex vertebrates in several disciplines such as genetics, developmental biology, and pharmacology. Zebrafish possess sufficient anatomical complexity with physiological and genetic (80-85%) homology to humans, thus allowing effective modeling of human diseases and neurobehavioral disorders [59,62]. Although there are neuroanatomical differences between zebrafish and humans, comparable features of the CNS allow for results to be generalized to mammals. For instance, in zebrafish the majority of DAergic neurons are located across the diencephalon with ascending projections to the telencephalon, while in mammals the midbrain is the main center for DAergic neurons (substantia nigra and VTA) [63,64]. However, previous studies have suggested that zebrafish DA neurons in the posterior tuberculum (periventricular nucleus of posterior tuberculum; PVN) can be a functional equivalent of the main DAergic pathways in the mammalian brain, representing an evolutionarily conserved pathway [47,63,65,66]. Furthermore, the zebrafish CNS uses many neurotransmitters found in mammals that are responsible for higher order cognitive function,
including GABA, glutamate, DA, norepinephrine, serotonin, histamine, and acetylcholine \[67\].

Although the zebrafish CNS is more simplistic, it is capable of mediating complex behaviors such as associative learning and most notably, addiction \[68\]. The study of addiction using animal models, mainly rodents, uses two experimental protocols: the conditioned place preference (CPP) paradigm and different protocols of drug self-administration. In contrast with self-administration behaviors, CPP has been found to differentially assess drug reward and engage distinct neuro-pharmacological circuitry \[69\]. Similar to many rodent behavioral paradigms, CPP has been adopted in zebrafish neurobehavioral research \[69\text{–}72\].

*Conditioned place preference*

In general, this procedure is governed by basic Pavlovian principles, in that the incentive salience of the treatment serves as the unconditioned stimulus (UCS). When paired with neutral environment stimuli, the UCS acquires secondary motivational properties that induce the approach behavior in the absence of the UCS, serving as a conditioned stimuli (CS) \[73\]. The UCS can be virtually any substance that is experienced as rewarding, such as drugs or appetitive stimuli, and tactile, spatial, and visual contextual cues can be used as CS. The CPP apparatus varies by design, but typically consists of a conditioning box consisting of two or three distinct compartments \[61,71,72\]. The procedure generally consists of three testing phases. During phase 1 the animal is permitted to explore all compartments of the conditioning apparatus, and the time spent in each compartment is quantified and used as a baseline place preference. In phase 2, animals are restricted to each compartment for a period of time in which they receive either experimental or control treatment. In phase 3, the animal is once again allowed access to all
compartments and final place preference is measured. Preference for the drug-paired compartment is indicative of the rewarding properties of the drug. Based on previous research studies, it has become widely accepted that increased dopamine transmission is necessary for the establishment of CPP behavior [71-73].

Research Question

In this work, I address the following question: how does the release of DA mediated by psychoactive drugs AMPH and THC influence the expression of D1- and D2-like receptors in the addiction phenotype?

In this study we identify the mechanisms by which two drugs of abuse, AMPH and THC, stimulate the mesolimbic reward pathway. We hypothesized that AMPH and THC both activate the zebrafish reward pathway but result in differential expression of D1-like vs D2-like receptors in various brain regions, more specifically in dopaminergic projections from the midbrain paraventricular organ to the hypothalamus. These essential brain areas receive and integrate sensory stimuli to drive behaviors related to survival, including feeding, mating, migration, and avoidance. In the following chapters, we detail neuroadaptations influenced by the differential expression of D1-like vs D2-like receptors in the zebrafish (Danio rerio) addiction model. Given our model organism, measuring addiction based on drug self-administration is not feasible, however additional behavioral paradigms, such as CPP, allow for translational modeling of addiction. Specifically, in Chapter 2, we used HPLC to confirm the presence of amphetamines in fish brains after drug treatment, then we detailed the various methods we used to address our central research question. Chapter 3 illustrates the interactions between
AMPH and THC with conditioned learning behaviors. We also identify unique, drug-specific behaviors elicited by fish following treatment and compare those with expected behaviors. In this results chapter, we also determine whether chronic exposure to AMPH differentially regulates the expression pattern of dopamine receptors within the zebrafish reward system.

We used immunofluorescent staining to measure expression of TH, DRD1, and DRD2 in controls and AMPH-treated fish. Finally, in Chapter 4 we discuss the impact of our findings and recognize the integrative importance of my study in advancing our understanding of the zebrafish as a translational addiction model, and in understanding the evolutionary significance of the dopaminergic reward circuit that drives our survival.
Chapter 2: Materials and Methods

2.1. Animals and Maintenance

Adult zebrafish of randomly bred genetically heterogeneous ‘wild-type’ strain were obtained from a local distributor (Optimum Aquarium, Kennesaw, GA 30144). All fish were acclimated to the laboratory environment for a minimum of 10 days, housed in 10-liter (L) aquaria at a density of ~2 fish per 1L, and then individually and adjacently housed within 3 L tanks at least 48 hours prior to behavioral testing. Zebrafish used in these studies were ~6 months old and maintained in a circulating system equipped with biological, chemical and mechanical filtration, aeration, and sterilization by UV light (Pentair Aquatic Habitats). Mounted LED lights provided illumination during a 14 h/10 h light/dark cycle. Tank water consisted of reverse osmosis deionized H₂O with supplemented dissolved sea salts (Instant Ocean) and was maintained at ~26-28 °C. Fish were fed twice daily Zeigler zebrafish diet (Pentair Aquatic Ecosystems). All animals were drug and experimentally naïve prior to testing. Behavior was recorded by USB webcam (saved as WMV files for subsequent analysis) mounted to an overhead shelter, which also provided equal light distribution. All protocols for animal use, housing and care were approved and carried out according to the Institutional Animal Care and Use Committee of Kennesaw State University.

2.2 Conditioned Place Preference Paradigm

Apparatus. All experiments were conducted between 9:00 and 14:00 h. Our behavioral tank was designed according to Ninkovic and Bally-Cuif [48], with some modifications. The testing tank dimension 30 cm in length, 15 cm in width and 20 cm height. Distinct visual cues divided the experimental tank into two halves: one half colored white and the other was either white with black stripes or white with black dots. Zebrafish did not clearly show a collective preference towards one half
of the experimental tank; therefore, it was considered an unbiased tank. Conditioning tanks were identical in design, with the exception of a central divider. The water level was kept at 17 cm from the bottom of the tank to minimize stress.

*Pretest: Place Preference (PP) Determination.* After an initial introduction into the testing apparatus, each fish was separately accommodated to the new environment for two full days (day 1 and day 2). On the third day, fish were carefully netted from their home tanks and placed directly into the CPP apparatus. Home tanks of animals were placed in close proximity to experimental tank to minimize netting stress and hypoxia. The zebrafish was then permitted to explore the environment for 5 minutes; the initial 5 minutes of exploration is designated for acclimation to the environment. Once acclimated, behavior was recorded for 10 minutes, after which fish were returned to their housing tank. The preferred compartment was defined as the compartment in which a fish spent most of the time on day 3. This value was then expressed as a percentage, which served as the animals’ baseline side preference.

*Conditioning: D-Amphetamine-Induced CPP.* After PP determination, zebrafish were assigned to either Group A (control) or Group B (D-amphetamine). Animals assigned to group B received food containing 40 µg D-amphetamine on days restricted to non-preferred compartment and control food on days restricted to preferred compartment. Zebrafish were fed agarose aliquots in housing tanks 30 minutes prior to being transferred to the experimental tank. On day 4, zebrafish were confined to the non-preferred compartment for 45 min. Restriction to this compartment was achieved using a transparent divider such that visual contact to the preferred compartment was possible. The experimental tank and conditions were otherwise identical to the ones used for PP determination, and each fish was tested alone. After 45 min, the fish was removed from the experimental tank and placed back in its housing tank. On day 5, fish were fed control food then restricted for 45 min into the preferred compartment. Restriction to the non-preferred compartment was repeated on days 6, 8, and
10 and restriction to preferred compartment repeated on days 7 and 9. PP was then measured again on day 11.

**Conditioning: Δ⁹-tetrahydrocannabinol (THC) Induced CPP.** After PP determination (day 3), each fish was weighed (weights varied between 0.2 and 0.8 g per fish) and assigned to one of three treatment groups: control (Group A), vehicle control (Group B), or Δ⁹-tetrahydrocannabinol (Group C). On day 4, zebrafish were sedated (using either 0.01% MS-222 or cold water sedation protocol) then intraperitoneally (IP) injected as followed: Group A injected with saline, Group B injected with ethanol, and Group C injected with Δ⁹-tetrahydrocannabinol. To ensure IP delivery of solution, 3 μg methylene-blue per gram body weight in 110 mM NaCl was added as a tracer. Each zebrafish was immediately confined to the non-preferred compartment for 45 min. After 45 min, the fish was removed from the experimental tank and placed back in the housing tank. On day 5, each fish was injected IP with a saline solution, then restricted for 45 min into the preferred compartment. Restriction to the non-preferred compartment was repeated on day 6 and restriction to preferred compartment repeated on day 7. PP was then measured again on day 8.

**Test: Change Place Preference.** Fish were tested for final preference using identical procedures used to determine baseline preference on day 1. Final preference testing took place at least 16 h following conditioning to ensure that the nervous system was void of any drug. Change in place preference was calculated by subtracting the time spent on the drug-paired environment before conditioning from the time in the drug-paired environment after conditioning.

**2.3 Brain D-Amphetamine Levels**

Extraction and determination of D-amphetamine concentration were carried out according to the high-performance liquid chromatography (HPLC) protocol developed by Bowyer et al. [68]. In this
method o-phthalaldehyde (OPA) in conjunction with the reduced sulfhydryl group, 3-mercaptopropionic acid (3MA), reacts with primary amines to form fluorescent moieties. These derivatives of D-amphetamine are then measured using HPLC with fluorescence detection.

*Instrumentation* – Agilent 1100 series HPLC system comprising an online degasser (G1379), a quaternary pump (G1379), thermostated autosampler(G1329A), and a thermostated column compartment was used. Detection of fluorescent D-amphetamine derivatives was carried out using an Aligent 1100 series diode array detector equipped with a 5μL flow cell. Separations were performed using a 250 x 4.6 mm C18 column (Kinetex 5μ EVO C18 100A), and a guard column containing the same phase, both maintained at 4°C during analysis.

*Reagents and solvents* – D-amphetamine, o-phthalaldehyde, 3-mercaptopropionic acid and sodium tetraborate decahydrate was obtained from Sigma. All solutions were prepared from analytical-grade chemicals (Fischer Scientific), dissolved in Milli-Q water and HPLC-grade solvents.

*Collection of brain samples* – Subjects (n=10) were fed 10 μL aliquot amphetamine spiked food and sacrificed at varying time points post consumption (30, 45, 60 minutes). Brains were quickly removed from the skull and dissected on a chilled glass plate. Samples of brain tissue were also collected from untreated fish (n=4) for blanks and standards. All samples were stored at -80°C until analysis.

*Preparation of OPA reagent* – For fluorescence detection 27mg OPA was dissolved in 500μL ethanol, 5mL of 0.1 borate buffer (pH 9.6) and 40μL of 3-MA. This mixture was kept refrigerated and discarded after 48 hours.

*Sample preparation and extraction* – Brain samples were pooled for each time point, weighed and diluted with 9 volumes of 0.1M borate buffer (pH 10.6). After 20s of ultra-sonication, samples were centrifuged for 10 min at 18000g. 100μL of the supernatant was transferred to clean centrifuge tube and 200μL of ethyl acetate was added. The mixture was vortex-mixed for 2 min and placed on ice. After 10 min on ice an additional 200μL ethyl acetate and 200μL of water were added to the mixture followed by brief vortex-mixing to further facilitate extraction. Centrifugation for 10 min at 18000g was used to separate the organic and aqueous phases. Then 200μL of the organic supernatant was transferred to a glass tube and dried at 45°C under nitrogen. Samples were reconstituted in 80μL KH2PO4 (0.05 M, adjusted to pH 2.6 with phosphoric acid). After adding 20μL of borate buffer (adjusted to pH 11.5 with sodium hydroxide) all samples were derivatized with 20μL OPA reagent. OPA reagent was added to each sample at least 2 min before injection. Derivatization was performed in capped Eppendorf tubes which were kept at 4°C until injection.
**HPLC conditions** – A linear gradient program (Table 2) was run at ambient temperature with mobile phase A consisting of 65% KH₂PO₄ (0.05 M, adjusted to pH 5.5 with potassium hydroxide) and 35% methanol, and mobile phase B with inverse proportions of buffer (35%) and methanol (65%). Fluorescent detector settings were: λ exciting 340 nm, λ emission 440 nm. Linear equation was calibrated with standard amphetamine solutions prepared at three different concentrations (2.5, 5.0 and 10 µM) in microdialysis buffer. Calibration curves were constructed by plotting the peak areas of OPA-3-MA-amphetamine (y, mAU) against the concentration (x, µM).

**Table 2: Chromatographic linear gradient conditions for HPLC determination of OPA-3-MA/amphetamine derivatives**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow-rate (ml/min)</th>
<th>Buffer A (%)</th>
<th>Buffer B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (0)</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0-3</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>18</td>
<td>1.5</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

### 2.4 Locomotor activity assay in a novel environment

To measure zebrafish locomotor activity, individual zebrafish were weighed (weights varied between 0.4 and 1.1 g per fish), injected IP and placed in a transparent tank with dimensions 21.5 cm long, 10.1 cm wide and 12.5 cm high, filled with 1.5 L of system water. A camera, placed 1 m in front of the tank, started recording 10 min after the fish was placed in the chamber. Recording time was 10 min at 25 frames per second (fps). Subsequent video clips were analyzed using behavioral tracking software (AnyMaze). The locomotor activity was scored based on distance traveled in cm (activity) and time spent in the “safe” bottom portion of the tank and in the “risky” top portion of tank (vigilance). Drugs and dosages were as followed: D-amphetamine (40µg/g IP), DRD1 agonist SKF 81297 (0.3µg/g IP), and DRD2 agonist Quinpirole HCl (0.3µg/g IP). Drug exposure was repeated every day for four days, and measurements were collected and behaviors recorded at days one and four of the experimental period.
2.5 Statistical Analysis

*IPP vs. CPP.* For each individual, initial place preference was identified and calculated as the difference in time spent in the preferred versus the non-preferred tank side. Following the conditioning paradigm, the differences between preferred and non-preferred tank were determined, depending on the initial preferred side for each fish. The differences in IPP and CPP for each fish in each treatment group were analyzed with an F-Test to compare for variances within treatments. Once equal variance was determined, we compared IPP time differences to CPP time differences with two-tailed Paired T-Tests using Graph Pad Statistical Software (Prism). For each analysis \( p < 0.05 \), and error was measured using standard error of the mean (SEM). Table 3 below lists the treatment groups and the number of samples per group (n).

*Locomotor Assay.* For these experiments, locomotor activity parameters (distance traveled, time at top or bottom of the tank, and other behaviors) were measured then compared at day 1 of treatment and day 4 of treatment to recognize the effects of these drugs on locomotor behavior and vigilance. Like the IPP vs. CPP experiment, the time the fish spends at the “risky” top region of the tank is subtracted from the time spent at the “safer” bottom region of the tank. These differences are compared between day 1 and day 4 of treatment using T-tests (\( p < 0.05 \), and error was measured using SEM). Total distance traveled for each treatment was also compared at day 1 and day 4 also using T-tests to assess effects of the drugs on activity. Table 3 below lists the treatments and the number of fish per treatment in each behavioral experiment.
Table 3. Behavioral Assay Treatment Groups

<table>
<thead>
<tr>
<th>IPP vs CPP Treatments</th>
<th>n</th>
<th>Locomotor Assay Treatments</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control agarose fed</strong></td>
<td>13</td>
<td><strong>Control cold water anesthetized</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>Amphetamine agarose fed</strong></td>
<td>20</td>
<td><strong>Amphetamine cold water anesthetized</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>Control MS222 anesthetized</strong></td>
<td>24</td>
<td><strong>SKF 81297 DRD1 agonist</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>THC MS222 anesthetized</strong></td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control cold water anesthetized</strong></td>
<td>8</td>
<td><strong>Quinopirole HCl DRD2 agonist</strong></td>
<td>8</td>
</tr>
<tr>
<td><strong>THC cold water anesthetized</strong></td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6 Immunohistochemistry and Microscopy

The fish were anesthetized with 0.1% MS-222. Brains were dissected and fixed in 4% paraformaldehyde at 4°C overnight. Fixed brains were then washed three times for 10 min with 1xPBS and then transferred into a solution of 30% sucrose in PBS (w/v) overnight or until tissue sinks. On the next day, cryoprotected brains were transferred to Tissue-Tek O.C.T, froze using dry-ice chilled isopentane and maintained in -80°C freezer until processing. Transverse sections through mesencephalon region of brain were taken at 20 µm and collected on charged slides.

*Immunofluorescent staining for DRD1, DRD2, and TH.* Tissues were double-stained using an immunohistochemistry procedure previously optimized to show expression of TH, DRD1, or DRD2 throughout the mesencephalon (Ganser et. al., 2013). For TH expression, mouse anti-TH1 monoclonal antibody, (Immunostar) was applied at a concentration of 1:500, then counterstained with super clonal Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen, green) at 1:500 (2ng/µL). DRD1 expression was highlighted with a 1:500 dilution (0.334 ng/µL) of rabbit anti-DRD1 monoclonal antibody (Abcam) and labeled with Alexa Fluor 568 goat anti-rabbit secondary antibody at 1:500 (6 ng/µL)
(Molecular Probes, red). Rabbit anti-DRD2 polyclonal antibody applied at 1:500 (6ng/µL) (Immunostar), counterstained with Alexa Fluor 568 goat anti-rabbit secondary antibody 1:500 (6ng/µL) (Molecular probes, red) labeled expression of DRD2 in zebrafish brain.

Completed slides were observed using a Zeiss LSM 700 laser scanning confocal microscope. 3 – 4 repeated trials were used for each treatment with 6-10 tissue-sections per slide. Pictures were captured of each cross section using identical settings (master gain: 756, laser intensity: 6.0 %, pinhole 45 µm, digital gain: 1.0) with a z-stack of each tissue-section. Average expression was in the form of fluorescence arbitrary units (F. A. U.) for DRD1 (555 channel) stains and were scored by manually circling the region of interest (periventricular nucleus of the hypothalamus (PVN)) on each cross section with open-source software Fiji. A negative control stain was prepared with the absence of the primary antibodies and also analyzed and subtracted from final treatment values to account for background signal.
Chapter 3: Results

Amphetamine Concentrations in the Brain.

Fish were euthanized at various time points following amphetamine exposure, brains were dissected and analyzed using HPLC for amphetamine presence and represented as average concentration obtained within each group (Table 3). Chromatograms in Figure 5 detail the presence of amphetamines (peaks demarcated with an asterisk (*) in treated fish at 30 min (pooled n=3), 45 min (pooled n=4), and 60 minutes (pooled n=3) after exposure. Peaks detected much earlier than amphetamine on the chromatograms, indicate the presence of nonspecific biological amines from reagent and brain tissue. The relative standard deviation (RSD) for retention time of amphetamine derivative within the column was <7.3% and had an average of 5%. Thus, indicating an acceptable level of precision for the identification of amphetamine derivative in analyte sample.

Table 4. Determined amount of d-amphetamine present in brain tissue samples

<table>
<thead>
<tr>
<th>Subject</th>
<th>Number of subjects</th>
<th>*Theoretical μg/ aliquot dose</th>
<th>Average amount d-amphetamine in brain tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>n=4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 min amph fed</td>
<td>n=3</td>
<td>40μg</td>
<td>30.15μg</td>
</tr>
<tr>
<td>45 min amph fed</td>
<td>n=4</td>
<td>40μg</td>
<td>44.8μg</td>
</tr>
<tr>
<td>60 min amph fed</td>
<td>n=3</td>
<td>40μg</td>
<td>42.6μg</td>
</tr>
</tbody>
</table>

* Theoretical dosage is based on the assumption of 30% of drug being absorbed via ingestion
Figure 5. HPLC chromatogram of brain tissue analytes of OPA-3-MA derivatives. (A) OPA-3-MA in microdialysis buffer (n=2), (B) Control brain tissue (n=4), (C) d-amphetamine standard (10 µM), (D) brain tissue 30 min post ingestion of amphetamine food (n=3), (E) brain tissue 45 min post ingestion of amphetamine food (n=4), (F) brain tissue 60 min post ingestion of amphetamine food (n=3). * represents d-amphetamine-OPA-3-MA derivative. Additional peaks represent unknown amounts of other biological amines contained in reagent and brain tissues that peaked much earlier than amphetamine, and as such were not further evaluated. (x, time min), (y, mAU)
Behavior

**IPP vs. CPP.** Experiences with various stimuli condition our responses to increase the likelihood of survival. Learned responses drive our motivation to seek stimuli that are rewarding and improve fitness and to avoid dangerous stimuli. Dopaminergic neuronal circuits underlie conditioned learning and the physiological motivation behaviors for self preservation. Zebrafish learn behaviors through a Pavlovian Condition Place Preference Paradigm (CPP) for which the fish is exposed to an environment split between visually unique patterns. In the present study, fish chose between an all white environment, and an all white environment with black stripes. Fish generally prefer the striped compartment, most likely because the black stripes give the illusion of safe cover. In the present study, however, I allowed the fish to determine initial preference (IPP) and used this IPP to set CPP. For each treatment group, I calculated the difference between time spent in the preferred side and the non-preferred side at IPP then CPP, then compared the differences at these two time points using paired T-Tests.

**Amphetamine.** Controls (n=13) and amphetamine-treated fish (n=20) were subjected to the CPP paradigm, and differences in time spent in preferred versus non-preferred compartments were calculated for fish in each treatment at both IPP and CPP (Figure 6). Control fish showed no difference in place preference between IPP and CPP (p = 0.2549, \( \alpha = 0.05, t = 1.196, df = 12 \)). Amphetamine treatment, however, did demonstrate a significant associative effect on place preference in IPP versus CPP (p = 0.0031, \( \alpha = 0.05, t = 3.388, df = 19 \)), with subjects showing no preference for the preferred environment following treatment. Suggesting that amphetamine influences conditioned learning in zebrafish.
Figure 6. Time Differences in IPP vs CPP

**A.** Average Time in Preferred Side

**Control**

<table>
<thead>
<tr>
<th>Average Time (s)</th>
<th>Control IPP</th>
<th>Control CPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>

**B.** Average Time in Preferred Side

**Amphetamine**

<table>
<thead>
<tr>
<th>Average Time (s)</th>
<th>Amphetamine IPP</th>
<th>Amphetamine CPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>-100</td>
<td>-100</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6. Average differences in time spent between preferred and non-preferred sides of the tank were measured at Initial Place Preference (IPP) and compared to the average differences in time after Conditioned Place Preference (CPP) using T-Tests. A.) Controls showed no significant difference between IPP and CPP (p = 0.2549, α < 0.05, n = 13). B.) Amphetamine exposed fish, however, displayed a significant difference between IPP and CPP (p = 0.0031, α < 0.05, n = 20), indicating addiction-like behaviors with preference change.

**THC and MS222.** When designing the drug delivery methods for this study, I consulted an oft-cited and frequently followed protocol for CPP in zebrafish involving the use of the common aquatic animal anesthetic, tricaine methanesulfonate (MS222) to anesthetize the fish enough that intraperitoneal injections of the drug or with vehicle control and sham injections could be done without stressing the fish enough to confound conditioned learning response. Behavioral monitoring and statistical analyses of control fish anesthetized in MS222 and fish injected with THC after being anesthetized, suggested an interaction between MS222 and conditioned place preference. Figure 7 shows the results following the CPP paradigm for the group of sham and vehicle injected control fish anesthetized with MS222 (C-MS222) and THC injected fish anesthetized with MS222 (THC-MS222).
during the protocol. For controls anesthetized in MS222, time spent in the preferred compartment was significantly different at IPP compared to CPP ($p = 0.0290$, $\alpha = 0.05$, $t = 2.33$, $df = 23$), suggesting MS222 influences place preference. Likewise, for MS222 anesthetized, THC-treated fish, time spent in the preferred compartment was significantly different at IPP compared to CPP ($p = 0.0392$, $\alpha = 0.05$, $t = 2.168$, $df = 27$). The results from this experiment suggest that using MS222 as an anesthetic could be causing conflicting results as compared to other methods; therefore, alternative methods for anesthetizing subjects was evaluated.

Figure 7. Time Differences in IPP vs CPP
THC & MS-222

A. Average Time in Preferred Side Control - MS-222

B. Average Time in Preferred Side THC - MS-222

Figure 7. Average differences in time spent between preferred and non-preferred sides of the tank were measured at Initial Place Preference (IPP) and compared to the average differences in time after Conditioned Place Preference (CPP) with T-tests. A.) Controls anesthetized in tricaine methanesulfonate (MS-222) showed a significant difference between IPP and CPP ($p = 0.0290$, $\alpha < 0.05$, $n = 12$), suggesting that MS-222 may have an effect on place preference in treated fish. THC exposed fish that were anesthetized in MS-222 also showed significant difference between IPP and CPP ($p = 0.0392$, $\alpha < 0.05$, $n = 28$), confounding whether the changes in preference were due to MS-222 or THC.
THC and cold water anesthetization. Because our data indicated MS-222 having independent effects on conditioned learning, we used a chemical free anesthetizing process. After using cold water as an anesthetic, control fish displayed no difference in place preference, even after conditioning (p = 0.7491, α = 0.05, t = 0.3348, df = 7), suggesting that temperature anesthetization does not influence CPP. However, cold water anesthetized fish that were injected with THC during the conditioning protocol displayed a significant change in place preference to the non-preferred compartment after THC was introduced in the non-preferred side during conditioning (p = 0.0393, α = 0.05, t = 2.338, df = 11). Figure 9 details the average difference between time spent on the preferred side of the tank and the non-preferred side at IPP compared to CPP for cold water anesthetized control and THC-injected fish.

**Figure 8. Time Differences in IPP vs CPP**

**THC – Cold Water**

![Graph A](image1.png)

![Graph B](image2.png)

Figure 8. Average differences in time spent between preferred and non preferred sides of the tank were measured at Initial Place Preference (IPP) and compared to the average differences in time after Conditioned Place Preference (CPP) with T-tests. A.) Controls anesthetized in cold water did not display a significant difference between IPP and CPP (p = 0.7491, α < 0.05, n = 8), suggesting that temperature based anesthetization likely does not interact with place preference. THC exposed fish that were also anesthetized in cold water, though, exhibited a significant difference in place preference after THC treatment (p = 0.0393, α < 0.05, n = 12).
**Locomotor Assays.** Controls. Fish (n=8) were injected with one of four treatments over a four-day period, to assay the drugs' effects on distance traveled (locomotor activity) and water level preference (vigilance). In sham-injected controls, there was no difference in distance traveled \( (p = 0.9975, \alpha = 0.05, t = 0.003266, df = 7) \) or water level preference \( (p = 0.9768, \alpha = 0.05, t = 0.03019, df = 7) \) between day 1 and day 4 of treatment (Figure 9).

**Figure 9. Control:** Activity and Vigilance

![Graph](image)

Figure 9. A.) Activity in control fish was measured in distance traveled during the 10-minute observation period on day 1 and compared to distance traveled on day 4. Controls did not exhibit a significant difference in activity \( (p = 0.9975, \alpha < 0.05, t = 0.003266, n=8) \). B) Vigilance, or the time spent in the “safe” bottom level of the tank versus the “risky” top part of the tank, was also not significantly different at day 1 versus 4 \( (p = 0.9768, \alpha = 0.05, t = 0.03019, n=8) \)

**Amphetamine.** Fish were given I.P injection of d-amphetamine (n=8) at 40µg per gram weight during the locomotor assay and showed changes in behavior after several days of exposure (Figure 10). Specifically, amphetamine-treated fish had an increase in activity at day 4 compared to day 1. The distance traveled increased recognizably, but was not significant \( (p = 0.0592, \alpha = 0.05, t = 2.25, df = 7) \), nor was vigilance significant between days 1 and 4 \( (p = 0.8076, \alpha = 0.05, t = 0.253, df = 7) \).
Figure 10. Amphetamines: Activity and Vigilance

A. Distance Traveled
Amphetamine: IPP vs CPP

B. Average Time Difference At Tank Level
Amphetamine: IPP vs CPP

Figure 10. A.) Activity in fish on the amphetamines was measured in distance traveled during the 10-minute observation period on day 1 and compared to distance traveled on day 4. Though exhibiting an obvious increase in activity by day 4, amphetamine fish did not display a significant difference in activity ($p = 0.0592$, $t < 0.05$, $t = 2.25$, $n=8$), suggesting that a larger sample size may show significance in activity by day 4 of treatment. B) Vigilance, or the time spent in the “safe” bottom level of the tank versus the “risky” top part of the tank, was also not significantly different at day 1 versus 4 ($p = 0.8076$, $t = 0.05$, $t = 0.253$, $n=8$).

Additional behaviors exhibited by treated fish were unique to amphetamine. Thigmotaxis, a behavior associated with habituation, or initial fear and vigilance within a new environment, usually decreases in zebrafish over the first few minutes in a new tank. Thigmotactic behavior begins when the fish is placed in a novel environment, and immediately swims to the lower and safer part of the tank along the walls. The fish explores the tank along the walls until it habituates to the new environment, and the perceived stress of threats eases. Thigmotaxis ends when the fish swims away from the walls and explores the middle and more shallow depths of the tank. Both control and amphetamine treated fish exhibited thigmotaxis, though amphetamine treated fish displayed more exaggerated forms of thigmotaxis, anxiety behavior and home base behavior. Anxiety behavior, includes a suite of behaviors,
including exaggerated swimming behavior (darting), diving, scototaxis (natural preference for darker parts of the tank), and episodes of freezing. Anxiety behaviors were consistently displayed in both the CPP behavioral studies, as well as the novel environment/locomotor behavior studies (videos links available in supplemental data). Finally, another type of thigmotactic-related anxiety behavior uniquely observed in amphetamine treated fish was home base establishment behavior. Fish exhibit home base behavior when fear and stress do not completely subside, manifesting in short bursts of exploration and return to an established safe part of the tank. Amphetamine treated fish in both behavioral experiments established home base territories, while never fully habituating to the experimental environments (videos links available in supplemental data).

**SKF 81297 DRD1 Agonist.** In the zebrafish, the areas of the brain similar to mammalian brain areas that express DRD1-like receptors include associative learning areas and midbrain regions that drive motivational behaviors, like the habenula, diencephalon, and midbrain. Compared to DRD2-like receptors that are most concentrated in the striatal projections between the midbrain and hypothalamus, DRD1 receptors are diffusely expressed from forebrain to hindbrain. These regions are associated with reward and pleasure, as well as activating motivation and memory associated with learning. As such, we expect that locomotor activity would increase with DRD1 agonist treatment. Figure 11 illustrates, however, that there was not a significant difference in activity after four days’ treatment ($p = 0.3451$, $t < 0.05$, $t = 1.012$, $df = 7$), but there was a significant decrease in vigilance by the end of treatment ($p = 0.0435$, $t < 0.05$, $t = 2.459$, $df = 7$).
Figure 11. DRD1 Agonist SKF 81297: Activity and Vigilance

A. Distance Traveled
SKF 81297: IPP vs CPP

B. Average Time Difference At Tank Level
SKF 81297: IPP vs CPP

Figure 11. A.) Fish treated with the DRD1 agonist, SKF 81297 did not display a significant difference in activity at day 1 compared to day 4 (p = 0.3451, t < 0.05, t = 1.012, n=8). B) Vigilance, or the time spent in the “safe” bottom level of the tank versus the “risky” top part of the tank, however, was significantly less at day 4 versus 1 (p = 0.0435, t = 0.05, t = 2.459, n=8).

During the locomotor behavior experiments, DRD1 agonist treated fish commonly and uniquely displayed akinetic behaviors indicating brain-wide locomotor deficiencies resulting in hypolocomotion. Though measured activity did not decrease significantly after SKF 81297 exposure, fish spent much less time in the upper parts of the tank, likely indicating deficits in locomotion. In particular, DRD1-agonist treated fish exhibited “droopy tail” specific akinesia. While the fish swims, tail movement briefly ceases and the tail end of the fish dips down and must physically be corrected. In extreme cases, the droopy tail led to inclined swimming (videos links available in supplemental data).

Quinpirole HCl DRD2 Agonist. DRD2 receptors are heavily expressed in dopaminergic projections between the midbrain and hypothalamic areas, suggesting heavy influence on the
communication of other neurotransmitter pathways that influence dopaminergic signaling. Figure 12 shows that fish treated with Quinpirole HCl (n=8) did not show significant differences in activity or vigilance between days 1 and 4. Distance traveled was not significantly different at day 1 compared to day 4 (p = 0.3658, df < 0.05, t = 0.967, df = 7). Likewise, vigilance was not significantly different at day 4 compared to day 1 (p = 0.8231, df < 0.05, t = 0.2321, df = 7).

**Figure 12. DRD2 Agonist Quinpirole HCl: Activity and Vigilance**

A.) Fish treated with the DRD2 agonist, Quinpirole did not display a significant difference in distance traveled at day 1 compared to day 4 (p = 0.3658, df < 0.05, t = 0.967, n=8). B) Vigilance, or the time spent in the “safe” bottom level of the tank versus the “risky” top part of the tank was also not significantly different at day 4 versus 1 (p = 0.8231, df < 0.05, t = 0.2321, n=8).

Though no significant differences in locomotion were measured in DRD2-agonist treated fish, these fish exhibited extreme anxiety behaviors. Like all fish in these behavioral experiments, initial placement in a novel environment elicits thigmotaxis eventually subsiding within two minutes after placement. Quinpirole HCl-exposed fish, however, exhibited hyperbolic anxiety behaviors that did not
subside throughout the experimental period. Fish behavior alternated between episodes of freezing and darting, along with diving to lower parts of the tank (videos links available in supplemental data).

Behavioral assays indicated that drugs of abuse interact with conditioned or associative learning. Though the acute locomotor assays did not yield many significant differences, these behavioral recordings did reveal many unique behaviors that were common to particular drugs and call into question the differential behavioral phenotypes resulting from stimulation of DRD1 versus DRD2 receptors in the reward system.

**Immunofluorescent Mapping of Dopaminergically Active Brain Areas.**

*Control.* Dopamine receptors in the zebrafish are categorized into two main classes. Type 1 receptors (DRD1, DRD5), after binding dopamine, upregulate cyclic AMP mechanisms and stimulate the neuron$^{33}$. Potentiation of DRD1 is necessary for cognitive processes, including learning, memory, and locomotor responses, and also mediate DRD2 expression$^{30,31}$. Conversely, type 2 receptors (DRD2, DRD3, DRD4), down-regulate cyclic AMP activity, thus help govern regulatory processes involving dopaminergic signaling in response to non-novel or non-life threatening stimuli. Genes encoding dopamine receptors (D1 and D2) have been identified and localized primarily in the midbrain and diencephalic regions of the zebrafish brain, specifically the tuberculum (projections from the midbrain to the thalamus and hypothalamus that mirror the striatal projections in mammals that govern motivational locomotion in the nigrostriatal, mesolimbic, and mesocortical reward system projections), paraventricular organ and periventricular thalamic nuclei extensions (orthologous to mammalian ventral tegmental area), and midbrain association cortices for sensory inputs$^{77}$. Dopaminergic signaling in zebrafish brain has been confirmed through immunocytochemical labeling methods with TH primary antibodies to illustrate CNS areas where dopamine is synthesized, and antibodies against DAT, to label dopamine-specific, reuptake
proteins involved in signaling among reward system neurons. Never before, however, have studies successfully identified specific dopamine receptor proteins within the zebrafish reward system. In the present study, the brains of control and amphetamine-treated fish were prepared for immunofluorescence study, specifically to identify areas of TH expression, as well as the presence of DRD1.

Figure 13 shows an illustrated anatomical atlas of a transverse section through a control zebrafish midbrain (Figure 13A-B). Referencing Figure 13B, Figures 13C (20x) details areas in the control zebrafish midbrain where TH is expressed (green). The highest concentration of TH staining was most reliably found in this midbrain area where tegmental (RT) and tubercular nuclei (TPp), along with the paraventricular organ (PVO), and caudal hypothalamic nuclei (HC, Hd) project anteriorly to the hypothalamus (H) and infundibulo-pituitary regions of the diencephalon. Figure 13D (40x) details the regions of the medial midbrain, focusing on the large, TH-labeled somata occupying hypothalamic nuclei and the paraventricular organ. Recall that TH catalyzes the conversion of tyrosine to L-Dopa, the initial rate limiting step in dopamine synthesis, and can indicate brain areas where DA signaling takes place. The presence of TH, however, could also indicate the presence of other catecholaminergic neurotransmitters, like epinephrine (EPI) and norepinephrine (NEPI). Once L-Dopa is converted to DA by aromatic amino acid decarboxylase (AADC), DA itself serves as the precursor molecule to EPI and NEPI. TH, therefore, cannot be used as a specific marker of DA, but rather a general marker of catecholamines.

In the present study, DRD1 serves as the specific indicator of DA signaling in the zebrafish brain. In Figure 13E (20x), DRD1 expression (red) in control fish is sparsely present in several areas that parallel TH staining, the vascular lacunae of the area postrema (Vas) near the tectal ventricular space, the posterior commissure (Cpost), the rostral tegmental nucleus (RT), the paraventricular organ (PVO), and three hypothalamic nuclei: the periventricular nucleus of the posterior tubercle (TPp), the posterior tuberal nucleus (PTN), and the dorsal zone of the periventricular nucleus (Hd). Punctae labeling DRD1 in
controls appear to surround mostly smaller cell bodied neurons, compared to neurons labeled with TH-labeled where green punctae representing TH expression, are found in the cytoplasm of neurons with small somata and large cell-bodied neurons with elongate extensions. In Figures 13F-H, DRD1 labeling (red) brightly stains midbrain sections of zebrafish treated with amphetamine. Figure 13F, in particular, shows highly concentrated punctae representing DRD1 expression surrounding small somata in the Vas. Compared to controls, DRD1 is heavily expressed in the Vas and also concentrated in the TPp, PTN, and PVO, while larger cell bodies and extensions present in the Hd are also more heavily stained in amphetamine-treated fish and nearly absent in controls. Magnified (40x) amphetamine-treated fish show specific labeling of DRD1 on large somata located in the TPp, PTN, and PVO (Figure 13G). Finally Figure 13H shows heavy DRD1 labeling in the mibrain of another amphetamine-exposed fish in the Vas and medial hypothalamic nuclei, but diffuse punctae are also visible in anteriorly projecting tracts in the solitary tract and tegmentum (striatal projections), as well as the caudal thalamic and medial hypothalamic nuclei.
Figure 13. Midbrain Dopamine Signaling in Control and Amphetamine-Treated Fish
Total DRD1 expression was measured by quantifying total fluorescent arbitrary units (F.A.U.) in four specific midbrain areas where DRD1 was visualized in both controls and amphetamine-treated fish. Representative cross sections of the midbrain region at the level of the corpora quadrigemina and the caudal hypothalamus (as in Figure 13A), show the specific areas in Figure 14A, C (control) and Figure 14B, D (amphetamine) where DRD1 expression was measured. These areas are summarized (14E), and histograms (14F) detail the measured differences in DRD1 expression in each midbrain area for controls.
and amphetamine-treated fish. Amphetamine-exposed fish showed greater DRD1 expression in all four areas compared to controls.

Because staining confirms the presence of hypertrophied dopamine-specific signaling in pertinent midbrain areas, we can draw conclusions that justify behavioral anomalies associated with drug exposure and how these particular stimuli can hijack the reward system in organisms whose motivational and physiological drive is mediated by dopamine communication.

**Figure 14. Total DRD1 Expression**

![Figure 14](image)

Figure 14. DRD1 expression was measured in four different midbrain regions to compare expression in controls and amphetamine-exposed fish. Total expression was measured by setting standard gain and intensity for DRD1 (555 red) channel and DAPI (405 blue) channel, based on control sections, and recording signal intensity in fluorescence arbitrary units (F.A.U.). Expression was manually scored by encircling the same areas of interest in each slide and taking the average F.A.U. for controls (n=4) and fish treated with amphetamine (n=8). A) Control midbrain (20x) showing areas where DRD1 expression was measured. Left dorsal zone of the hypothalamic periventricular nucleus (Hd), Left rostral tegmental nucleus (RT), Periventricular nucleus of the posterior tubercle of the hypothalamus (TPp). B) Amphetamine-treated midbrain (20x) showing the same areas where DRD1 expression was measured: Hd, RT, TPp. C) Control dorsal midbrain (20x) showing the right vascular lacunae of the area postrema (Vas) where DRD1 was measured and compared to D) the right Vas area in amphetamine-exposed fish.
E) Histogram shows total DRD1 expression per midbrain area in controls versus amphetamines. F) Expression of DRD1 in total F.A.U. is detailed in this table for each region selected in the midbrain.

Chapter 4: Discussion

Communication across dopaminergic circuits in the brain underlies our basal physiological motivators that drive survival. Whether we seek nourishment, companionship, accomplishment, or avoid situations that risk our well-being, the neurons governing these behaviors and the genes that encode these pathways have not evolved much further than their protochordate roots\textsuperscript{47,78}. Phylogenetic analyses show that the enzymes that catalyze various steps in catecholamine synthesis (TH, AADC) are present in protostomes, urochordate taxa that far predate the divergence of chordates\textsuperscript{47}. Likewise, genes that encode catecholaminergic signaling proteins (vMAT) are present in urochordates, while genes for dopamine-specific signaling (DAT) have not been confirmed in taxa more primitive than teleost fish\textsuperscript{47}. Other dopamine-specific signaling molecules, aside from vesicle packaging proteins and synaptic transporters, are dopamine receptors, which fall into two categories, Type 1, or D1-like, and Type 2, or D2-like\textsuperscript{78}. Like DAT and vMAT, these two DA receptor categories emerged with bony fish (for which there was a genome duplication resulting in eight known receptor subtypes), for which five subtypes are functional in the human reward system\textsuperscript{77}. D1-like receptors (DRD1, DRD5) activate cyclic AMP to stimulate dopamine release and signaling, and D2-like receptors (DRD2, DRD3, and DRD4) that, when bound, down regulate dopaminergic signaling and decrease dopamine release\textsuperscript{77}. Because zebrafish hold these genes and signaling mechanisms in common with humans, they serve as an ideal translational model to study both evolutionary drivers or motivation, learning, memory, and cognition, as well as neuropsychiatric diseases associated with dopaminergic pathways\textsuperscript{79,80,81}.

The goals of the present study aimed to add support to the growing utility of zebrafish as translational models for human disease, even if the diseases are neurological and cognition-based. By subjecting zebrafish to conditioned learning paradigms, and introducing dopamine signal altering drugs,
our study draws parallels to the homologous nature of the neuronal circuits driving associative learning, choice, motivation, and vigilance in zebrafish and humans. Because zebrafish have well-categorized, stereotypic behaviors in response to certain stimuli, we can quantify responses, observe novel behaviors, and inform on or compare behaviors to humans under similar conditions. Specifically, knowing that zebrafish that exhibit seeking and vigilant behaviors in response to experientially learned stimuli, confirm that reward and fear behaviors are heavily conserved as evolutionary motivators of self-preservation and fitness, along with the neural circuits that mediate these responses, to drive these behaviors. Likewise, the use of addictive amphetamines to demonstrate similarities to human amphetamine-induced responses and addictive behaviors, supports the use of zebrafish as translational models of addiction and neuropharmaceutical responses. Our study, however, goes beyond behavioral assays to detail neuroplastic changes in dopamine signaling within the zebrafish reward system in response to drugs of abuse. When I initially planned this study, I knew that primary research measured amphetamine and THC-induced behaviors in zebrafish. Some studies also mapped the expression of genes, synthesis proteins, or signaling proteins in the brain related to drug exposure in zebrafish. None of these studies, however, were able to correlate amphetamine-induced behavioral changes with dopamine receptor-specific signaling in the zebrafish reward circuit. The two most dominant dopamine receptor subtypes, DRD1 and DRD2, are active and present in the zebrafish brain, and the genes that encode these receptor subtypes have been confirmed in dopaminergic signaling pathways through in situ analyses. Though the presence of dopamine synthesis (TH) and signaling molecules (DAT) has been confirmed and mapped in the zebrafish brain through immunohistochemical analyses, studies that imply dopamine specific signaling because of the presence of TH in similar reward type circuits in the zebrafish brain, can only definitively say that the presence of TH indicates general synthesis of catecholamines. Immunolabeling studies utilizing the signaling protein, DAT, however, can specify the presence of dopamine-related signaling, thus the goal
of my study was to confirm the presence of DRD1 in the zebrafish brain and to map its expression in reference to TH expression, and to recognize any differences in DRD1 expression following amphetamine exposure.

CPP and MS222. I based my behavioral experimental setups on three seminal zebrafish CPP papers\textsuperscript{59,62,71}, that detailed analyses of measurable behaviors. All studies recommended IP injection of study agents, and the protocols for humane anesthetization with tricaine methane sulfate (MS222) for brief immobilization and intraperitoneal injection, however the behavioral data measured in anesthetized controls indicated that MS222, itself, affected CPP (Figure 7). MS222, a sodium channel blocker with no particular channel specificity, slows neuronal signaling by temporarily blocking sodium channels that must open to propagate an action potential. Because movement of sodium across the neuronal membrane affects all neuronal signaling, MS222 was likely influencing dopaminergic signaling that affected experiential learning. The interaction of anesthetic with CPP led us to use non-chemical, cold water anesthetization to sedate the fish. Control fish anesthetized with cold water showed no difference in place preference following conditioning, and thus the remainder of our experiments used this immobilization protocol to study the effects of amphetamines on behavior. Though behavioral tests assaying the effects of THC on CPP following cold anesthetization were done, the brains of these fish were not analyzed.

Previous zebrafish CPP studies that used MS222 as an anesthetizing agent may have had underlying changes to conditioned behavior that may not be directly correlated with the study treatment. The synergistic effects of drugs tend to compound neurobehavioral disorders as well, and emerging reports on the comorbidity of prescribed drugs in veterans with PTSD, details how wounded veterans are often prescribed opiate pain killers along with benzodiazepines for their PTSD-related anxiety\textsuperscript{85,86}. The long-term prescription of each of these medications alone significantly increases risky behaviors in veterans\textsuperscript{84}. However, as they are often prescribed together, incidents of self-harm,
addiction to other substances or behaviors, noncompliance with prescriptions, development of OCD and social anxiety, and suicide dramatically increase⁸⁴,⁸⁵. What the zebrafish tells us, in its behavioral responses to MS222 apart from THC, is that other stimuli or agents may not only influence dopaminergic circuits, but may also make the brain more susceptible to altered signaling.

*Amphetamine.* Zebrafish in all of our treatment groups responded to CPP as expected, specifically, amphetamine-exposed fish spent significantly less time on the initial preferred side after conditioning the fish on the nonpreferred side with amphetamine. Though the differences were not significant, in the locomotor assays, the activity, measured in distance traveled, did show an increase nearing significance as expected. Vigilance, measured in time spent on the risky versus safe water levels of the tank, was unexpectedly not significantly different following drug exposure. Amphetamines are broadly acting central nervous system stimulants, meaning they upregulate the activity of all the excitatory neurotransmitters in the brain (DA, 5HT, NEPI, EPI, and ACh). Amphetamines specifically upregulate dopamine transmission by blocking reuptake of DA by inward rectifying presynaptic dopamine transporters, influencing presynaptic pH such that DA does not get packaged into vesicles, and reversing reuptake transporters such that the cytoplasmic presynaptic dopamine that is unbound by vesicles quickly floods the synaptic cleft⁷⁹. Because AMPH is a global excitatory neurotransmitter agonist, we expected upregulation of activity and increased vigilance due to the upregulation of fight or flight neurochemicals. Though some of our locomotor assays did not show significant upregulation of activity, specific behaviors displayed (thigmotaxis, diving, anxiety behaviors) by amphetamine treated fish indicate hypertrophied stress. Thigmotaxis, specifically, a habituation behavior exerted by fish exposed to novel environments was more common and exaggerated in amphetamine-exposed fish versus control, and likewise in DRD1-agonist treated fish.

We expected that amphetamine treated fish would show an increase in DRD1 signaling, because its mechanism of action upregulates dopamine transmission into the synapse. Likewise, because of its
stimulatory effects, we expected an increase in DRD1 activity, because the potentiation of DRD1 receptors upregulates cAMP to stimulate DA signaling. Behaviors observed, like increased activity and anxiety-like behaviors do support the hypothesis that DRD1 signaling would be upregulated in amphetamine exposed fish. Moreover, the influence of amphetamine in place preference strongly supports its altering (upregulation) of dopaminergic signaling related to experiential or conditioned learning. Interestingly, the strong labeling of DRD1 expression in the midbrain also suggests that amphetamine increases dopaminergic signaling, but primarily in midbrain areas projecting to striatal areas governing movement (Tegmentum), thalamic association nuclei (integrating centers for appropriate responses to sensory inputs), and hypothalamic projections that mediate motivation, or seeking behaviors, conditioned or experiential learning, and fear/startle responses. Expression of DRD1 was particularly strong in dorsomedial and medial areas of the midbrain. Figure 13 shows that DRD1 expression was centered primarily in the vascular lacunae of the area postrema (Vas). This particular nucleus contains chemosensory neurons that come in contact with heavily fenestrated capillaries that likely monitor the blood brain barrier for toxins and osmoregulatory homeostatic cues86. Though the Vas is present in Chondrichthyes and repeatedly labeled and identified in zebrafish brain atlases, its specific functions remain a mystery86. Its presence in early vertebrates and its unique specialization in mammals as the chemical trigger zone (CTZ) that mediates vomiting in response to chemical toxins detected in the blood, along with its conserved location, anatomy, and stimulation by catecholaminergic inputs, suggests a similar function among vertebrates in avoidance. Morita and Finger (1987)86 did the only teleostean study of the Vas in which the anatomy, connectivity, and immunocytochemistry confirmed afferent and efferent vagal inputs as well as heavily concentrated TH staining. In mammals, the Vas (CTZ) is also highly vascularized and serves as the body’s safe guard against toxin infiltration. When poisons are detected by chemoreceptors in the Vas, DA floods the area inducing immediate vomiting86. Because of its proximity to and innervation by dopaminergic projections involving
avoidance, the Vas likely serves as a chemical trigger zone of sorts for zebrafish by inducing escape behaviors in response to noxious stimuli. The Vas heavily expresses DRD1 in both controls, but more so in amphetamine-treated fish, suggesting that these fish would likely exhibit more avoidance, stress, and vigilance behaviors, though these were not significantly increased in our fish.

Several other midbrain regions showed increased DRD1 labeling in amphetamine-treated fish versus controls. We recognize that DRD1 staining in controls and AMPH fish parallels TH staining in controls, however, not all areas that showed TH labeling were positive for DRD1 staining, suggesting that TH is not a reliable indicator of DA-specific signaling. Aside from the Vas, though, DRD1 punctae were evident in the periventricular nucleus of the posterior tubercle (TPp) a thalamic nucleus, along with the periventricular nucleus of the ventral tubercle (PVN), and the paraventricular organ (PVO) are thought to mediate DA synthesis for forward projecting connections to hypothalamic areas that govern reward-specific behaviors. DRD1 was also present in the rostral tegmental area (RT), a group of projections that mirrors the mammalian striatum for the nigrostriatal dopaminergic pathways controlling movement and motivation. Finally, DRD1 staining was especially strong in the dorsal hypothalamic nuclei (Hd), a group of cell bodies whose axons project anteriorly and serve to enable physiological drive, motivation, and satiety. Staining was sparse or absent in controls in these areas compared to amphetamine-treated fish.

Furthermore, for the locomotor behavioral study, we expected that treatment with a DRD1 agonist would increase activity and vigilance, for the same reasons we thought amphetamine-treated fish would show increases in activity and vigilance that would mirror its upregulation of DRD1. Triggering of DRD1 stimulates dopaminergic signaling, and though these DRD1 agonist-treated fish showed significant differences in vigilance, they did not have an upregulation of activity. These results contrast the increase in activity and absence of change in vigilance behavior in amphetamine fish for which we also see increased DRD1 signaling.
THC and CPP. Our initial THC behavioral studies were confounded by MS222, but we did condition several fish with THC using the cold water anesthetization protocol. THC is known to exert secondary effects on dopamine release to trigger reward, however, its initial effects on endocannabinoid receptors effectively slow down motor, perception, and cognitive responses through second messenger mediated neuronal membrane hyperpolarization\textsuperscript{82}. We expected reduced activity in the THC-treated fish, and these inhibited behaviors in fish were casually observed in reduced episodes of locomotion, absolute stillness during thigmotaxis, and the inability to respond to any sort of threatening stimuli, even handling. Aside from its initial inhibitory effects, THC did have a significant effect on CPP following treatment, suggesting that its stimulation of DAminergic pathways. THC is not only desired for its calming effects, but it also triggers the dopamine-driven reward system to give feelings of euphoria\textsuperscript{82}. Because of these initial inhibitory effects, we expected that THC-treated fish would show an increase in DRD2 signaling compared to DRD1, however, none of the brains we stained with DRD2 antibody showed any expression.

We can, however, draw parallels between DRD2 signaling and inhibitory behaviors. Though treatment with DRD2 agonist did not yield any locomotor behavior anomalies, DRD2-treated fish showed hypolocomotor behaviors, like tail dipping, and bouts of slowed or absent locomotion consistent with DRD2 stimulation. The reduction of dopaminergic neuronal activity by the inhibition of cAMP by DRD2, should result in reductions of motivators for movement and vigilance. Though these behaviors were not significantly decreased after DRD2 treatment, fish did show behaviors indicative of suppression of locomotion.

The significance of these combined behavioral and immunofluorescence data suggest that zebrafish are a viable model for studying both behavioral and neuroplastic changes brought about by drugs of abuse and other dopaminergic circuit-related neuropsychological disorders. These new findings, that DRD1 signaling is specific to the midbrain areas homologous to human dopaminergic
circuits and upregulated in amphetamine-treated individuals, invites many new questions regarding the differential effects of pharmaceuticals on dopamine signaling and behavior. By being able to recognize stereotypic responses to certain drug classes, and match them with specific DA receptor signaling in specific parts of the reward pathway, studies like this will help to inform on targeted treatments for addiction, anxiety, and compulsion disorders.

*Integrative Significance.* In recent years, the use of zebrafish to study the behavioral response to drugs of abuse has increased providing a rich platform with which to explore how these psychoactive drugs influence neurotransmitter systems. All addictive drugs are believed to increase dopamine transmission, beginning in the ventral tegmental area (VTA) of the midbrain and innervating the nucleus accumbens (NAc), amygdala, hippocampus and prefrontal cortex in humans. Although this mesolimbic dopamine system is not conserved among humans and zebrafish, the anatomical organization of the nervous system in similar among vertebrates, and the lateral and medial pallium in the zebrafish are analogous to the hippocampus and associated mesolimbic circuitry in humans. Dopaminergic receptors are expressed throughout these brain regions of vertebrates including zebrafish. Four classes of dopaminergic receptors, identified in zebrafish, correspond to the mammalian D1, D2, D3 and D4 orthologs. *Drd1* is the homolog of the mammalian D1-like receptor type, and *drd2a, drd2b, drd2c, drd3, drd4a, drd4b* and *drd4c* are homologous to the mammalian D2-like receptor type. From the literature we conclude that the interaction between dopaminergic signaling, specifically involving stimulation of *drd1*, is implicated in experiential learning and seeking behavior. Addictive behavior, motivated by amphetamines and other drugs of abuse, negatively influence the reward system by perpetuating reinforced learning towards drug related stimuli. Zebrafish display excellent cognitive abilities and complex decision-making, and, like rodents, show robust behavioral responses to stimuli, with high sensitivity to pharmacological manipulations [75]. Behavioral paradigms historically tested in rodents, such as conditioned place preference, have only recently been applied in zebrafish neurobehavioral
research but has already shown to be an excellent candidate for studying the rewarding effects and addictive potential of both well-classified and novel compounds. The zebrafish CPP model along with identifying differences in drd1 expression will likely increase our understanding of factors contributing to the pathogenesis of addiction and subsequently aid in the development of treatment strategies.

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