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INFLUENCE OF DOPAMINE D2-TYPE RECEPTORS ON MOTOR BEHAVIORS AND CONDITIONING IN THE GREEN TREE FROG, *HYLA CINEREA*

by

ANNA E. CREIGHTON

Under the Direction of Walter Wilczynski

Dopamine modulates a range of behaviors that include motor processes, learning, and incentive motivation. Neuroanatomical research supports conservation of dopaminergic populations in the midbrain across vertebrate species, however, less evidence is available for dopamine receptor distributions and function. In order to test the behavioral role of dopamine in a conserved dopaminergic system, the effects of D2-type receptor manipulation on motor behaviors were examined in the anuran amphibian green tree frog, *Hyla cinerea*. In two different experiments, frogs were treated with a D2 receptor-specific drug, quinpirole or haloperidol, and exposed to a testing session to measure changes in motor behaviors. Quinpirole generally inhibited some motor behaviors, while haloperidol generally stimulated some motor behaviors, as predicted based on receptor mechanisms. A pattern of performance improvement also appeared in frogs in each experiment. Overall, the results support general conservation of DA in motor processes in vertebrate species.

INDEX WORDS: Dopamine, Anuran amphibian, Quinpirole, Haloperidol, D2 receptors, Motor behavior, Basal ganglia

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ANNA E. CREIGHTON

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2013

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Anna Elizabeth Creighton
2013

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LIST OF ABBREVIATIONS

-ir	Immunoreactive
AC	Adenylate cyclase
ARS	Amphibian Ringer's solution
DA	Dopamine
HAL	Haloperidol
IP	Intraperitoneal
LA	Lactic acid
NAc	Nucleus accumbens
QUIN	Quinpirole
SN	Substantia nigra
VTA	Ventral tegmental area
TH	Tyrosine hydroxylase

1 INTRODUCTION

Dopamine (DA) is a catecholaminergic neurotransmitter important for the modulation of processes in peripheral and central nervous systems of both vertebrate and invertebrate organisms (Yamamoto and Vernier, 2011). While a large amount of evidence points toward conservation of the DA neuroanatomical systems in vertebrates, notably in the basal ganglia nuclei (Smeets, 1995) (Reiner et al., 1998) (Maier et al., 2010) (Yamamoto and Vernier, 2011) (Callier et al., 2003), evidence also supports conservation for the influence of these systems in behavior. One challenge of interpreting the behavioral role of DA comes from the variation in behaviors that are exhibited by different organisms and modulated by the DA systems. However, it is possible to generalize behaviors across species into categories that include 1.) motor processes, 2.) social behaviors, and 3.) goal-directed behaviors. For example, singing behavior expressed by male songbirds is modulated by dopaminergic activity in the song and motor nuclei of the avian brain (Hara et al., 2007), and sexual behaviors in rats can be modified depending on the type of manipulation to the DA systems (reviewed by Hull et al., 2004). Because a vast amount of mammalian and avian data support a significant role for DA in motor processes, as well as goal-directed and social behaviors, a question arises that asks- How do DA processes in behavior compare across an evolutionary scale to simpler DA systems, such as those found in the anuran amphibian green tree frog, *Hyla cinerea*? In an attempt to answer this question, we first need to identify the evolutionary characteristics of structure and function across vertebrate DA systems, and determine how these comparisons relate to species-specific, as well as general, behaviors. To do so, we will review the conserved mechanisms of catecholamine synthesis and receptor processes, as well as receptor localization and functional networks. Finally, we will

consider how all these factors result in behavioral output through a motor test focused on DA receptor-specific manipulation on *H. cinerea*.

1.1 Tyrosine hydroxylase distribution

In support of physiological conservation of DA systems in vertebrates, catecholaminergic cell populations and projections to the basal ganglia have been identified by tyrosine hydroxylase (TH) immunohistochemical labeling in anuran neural tissue. TH is the rate-limiting enzyme for catecholamine synthesis, and labeling cells that use it is a common way to identify populations that produce dopamine, noradrenaline or adrenaline, based the presence or absence of other specific enzymes (ex. dopamine-β hydroxylase, phenylethanolamine *N*-methyltransferase) or neurotransmitters (DA and noradrenaline) (Gonzalez, 1994). Notably, the anuran TH-ir populations are comparable to some extent to avian and mammalian systems (Marin et al., 1997a). As in amniotes (reptiles, birds, and mammals), midbrain populations in anurans serve as the source of DA to the striatal regions, and there is a general pattern in TH-ir distribution across anuran species (Creighton, 2009) (Gonzalez and Smeets, 1991) (Gonzalez et al., 1993). In the amniote midbrain, TH-ir neurons are generally located in the substantia nigra (A9; SN) and ventral tegmental area (A10; VTA); SN neurons project to the dorsal striatum to form the nigrastriatal pathway, and VTA neurons project to the nucleus accumbens (NAc) to form the mesolimbic pathway (Yamamoto and Vernier, 2011). In the anuran midbrain, TH-ir neurons are distributed on a rostral-caudal plane through the posterior tuberculum and midbrain tegmentum (Marin et al., 1997a). While the anuran TH-ir neurons project to the striatum, there is less specificity in the terminal destination of the projections in the basal ganglia (Smeets, 1995)

(Reiner et al., 1998) (Marin et al., 1997a). However, the anuran midbrain exhibits stronger projections to the NAc than the striatum (Gonzalez, 1994) (Marin et al., 1998b).

1.2 Characteristics of DA receptors

Neurons are dependent on neurotransmitter receptors to initiate cell response to incoming signals by neurotransmitter binding. At least seven different DA receptor subtypes have been identified in vertebrate neural tissue (Yamamoto and Vernier, 2011). The different DA receptor subtypes are highly conserved in structure and function across vertebrates and invertebrates (Callier et al., 2003) (Yamamoto and Vernier, 2011) (Mustard et al., 2005). In general, all DA receptors are metabotropic, and can be categorized into two groups based on activation of adenylate cyclase (AC) upon binding (Beaulieu and Gainetdinov, 2011). D1-type receptors (referred to henceforth as D1 receptors; including subtypes $D_{1/1A}$, $D_{5/1B}$, D_{1C}, and D_{1D}) are coupled to G_s proteins, function post-synaptically, and activate AC to promote generation of EPSP. D2-type receptors (referred to henceforth as D2 receptors; including subtypes D_2, D_3, and D_4) are coupled to G_i proteins, function as autoreceptors and post-synaptically, inhibit AC, and initiate other processes that block either neurotransmitter release or the generation of EPSP, depending on location (Beaulieu and Gainetdinov, 2011) (Kebabian and Calne, 1979). Although mammals show five DA receptor subtypes, birds and anurans show six, but differ in one D1 receptor subtype (Yamamoto and Vernier, 2011). Functionally, D1 receptor activation increases cell excitability, while D2 receptor activation decreases cell excitability, in medium spiny neurons in both avian (Ding and Perkel, 2002) and mammalian (Nicola et al., 2000) basal ganglia tissue.

Although DA receptors have not yet been localized in anuran neural tissue, general similarities in receptor localization in other vertebrate neural tissue support conservation of receptor localization. Generally in the rat brain, DA receptors are located in the midbrain and at the terminal destinations of midbrain axons in the basal ganglia and forebrain regions. The dorsal striatum, nucleus accumbens, and olfactory tubercle show high levels of D1 receptors, while the hippocampus, cortex, thalamus, and hypothalamus express D1 receptors at lower levels (Palermo-Neto, 1997). D2 receptors are also found at high levels in the dorsal striatum, nucleus accumbens, olfactory tubercle, as well as the globus pallidus, periaqueductal gray, amygdala, cortex, substantia nigra, ventral tegmental area, and medulla (Palermo-Neto, 1997). Similar DA receptor localization patterns are found in birds (Kubikova et al., 2010).

While the same general neural regions express DA receptors across vertebrates, different classes show unique characteristics for receptor quantification. In particular, adult mammals have approximately twice as many D1 receptors compared to D2 receptors, while adult birds and turtles show the opposite with more D2 receptors in the basal ganglia (Richfield et al., 1987). This difference was supported more recently, when Kleitz et al. demonstrated a greater D2:D1 receptor ratio in quail neural tissue compared to rat neural tissue (Kleitz et al., 2009). However, autoradiographic labeling on anuran neural tissue tends to exhibit a ratio of DA receptors similar to mammals, i.e. more D1 receptors than D2 receptors, and similar, although not identical, receptor affinities to DA receptor-specific drugs between anuran and mammalian tissue samples (Chu et al., 2001). It is noted that all these comparisons are made on tissue samples collected from adult animals because receptor levels change through developmental stages in mammals,

making age a factor that can complicate comparisons (Rothmond et al., 2012) (Sobrian et al., 2003).

1.3 DA systems

While the functional processes for DA receptors are molecularly conserved across species, there are greater differences in the characteristics of neuronal populations that express and/or signal to DA receptors. Immunohistochemical labeling, chemical lesioning, and receptor-specific pharmacological testing have confirmed the presence of three general DA systems in vertebrates consisting of the mesolimbic system, the nigrastriatal system, and the tuberoinfundibular system (Nicholls, 2001). However, the tuberoinfundibular system will not be reviewed here, as it is primarily involved with modulating prolactin secretion from the pituitary gland (Lyons et al., 2012). As previously stated, DA signals originate in the midbrain, and communicate to populations in the basal ganglia and cortical regions. The VTA in the midbrain is the synthesis region for DA in the mesolimbic system. DA neurons project from VTA to forebrain regions and the NAc, i.e. ventral striatum, (Nicholls, 2001). The mesolimbic system is characterized by high levels of D1 receptors in the NAc, and is involved in processes for motivation, reward- and goal-oriented behaviors (Self, 2010). DA is also synthesized in the substantia nigra pars compacta in the midbrain, which projects to the dorsal striatum (caudate nucleus and putamen) to form the nigrostriatal system (Nicholls, 2001). The dorsal striatum expresses both types of DA receptors (Gurevich, 2010), and functions in motor behavior.

In amniotes, coordinated motor output is controlled through functional D1 and D2 receptor distributions on GABAergic neurons of the dorsal striatum to produce a disinhibitory effect on

the basal ganglia output pathways to the thalamus and brainstem (Purves, 2001). In particular, two output pathways originate in the dorsal striatum, and are dependent on modulation by DA signaling to function properly for motor production. The striatonigral pathway, also known as the direct pathway, is characterized by D1 receptors and substance P, and projects to the substantia nigra pars reticula and external globus pallidus, while the striatopallidal pathway, also known as the indirect pathway, is characterized by D2 receptors, A_{2A} receptors, and enkephalin, and projects to the internal globus pallidus. The internal globus pallidus also projects to the substantia nigra pars reticula and external globus pallidus, as well as the subthalamic nucleus (Deng et al., 2006) (Durieux et al., 2012) (Groenewegen, 2003). A balance between D1 receptor activation and D2 receptor inhibition on the GABAergic neurons in the basal ganglia is necessary for successful motor production. Interneurons also comprise three other neuronal populations in the striatum of amniotes, and function to modulate signaling in the basal ganglia with GABA or acetylcholine (Reiner et al., 1998).

Evidence supporting neuronal population specificity comes from experiments focused on DA receptors in different basal ganglia nuclei. In a classic study using in situ hybridization, Gerfen et al. showed that striatonigral neurons express D1 receptors, dynorphin, and substance P, while the striatopallidal neurons express D2 receptors and enkephalin (Gerfen et al., 1990). Robertson et al. demonstrated treatment with the D2 receptor antagonist, haloperidol, increased Fos-ir in striatopallidal and enkephalin-ir neurons, although the D1 receptor partial agonist, SKF-38393, did not affect Fos-ir in either striatopallidal or striatonigral neurons (Robertson et al., 1992). Finally, Matamales et al., using nuclear staining in bacterial artificial chromosome (BAC) transgenic mice, showed that all striatal medium-sized spiny neurons expressed either D1

receptors, D2 receptors, or both, and that striatonigral neurons are specific for expressing D1 receptors (Matamales et al., 2009). Together these results suggest functional specificity for the type of DA receptor that is expressed by neurons in the basal ganglia.

Identification of the DA systems is possible in non-mammalian species, and has been done to some extent. In general, the DA systems are conserved across vertebrates, although immuno-histochemical staining shows organizational differences and an increase in circuit complexity when progressing from invertebrates to amniotes (Marin et al., 1998a) (Reiner et al., 1998) (Smeets, 1995). Efferent neurons and axonal fibers from the striatum that correspond to the mammalian basal ganglia output pathways can be distinguished by protein expression patterns in anuran tissue (Endepols et al., 2004b) (Marin et al., 1997b). For example, retrograde labeling in the NAc reveals a mesolimbic-like pathway from the midbrain tegmentum in Perez's frog (*Rana perezi*) (Marin et al., 1995). Substance P-ir fibers are located in the NAc and ventral pallidum, while enkephalin-ir fibers are located in the NAc and dorsal pallidum in anurans (Marin et al., 1998b). Also immunohistochemical labeling for proteins associated with the striatal pathways in anuran tissue supports the presence of both direct and indirect pathways projecting from the dorsal striatal region (Endepols et al., 2004b). However, acetylcholinergic interneurons are much more sparse in anuran neural tissue (Marin et al., 1997c), as is the labeling for neuropeptides characteristic to the other interneuron populations, such as somatostatin, neuropeptide Y, and GABA (Endepols et al., 2007) (Reiner et al., 1998). Overall, data support the presence of projection pathways to and from the striatum in anuran neural tissue similar to those in other vertebrates, however the cell density of specific neuronal populations in the basal ganglia is much lower in anurans compared to the patterns seen in other amniotes.

1.4 Roles of DA in behavior

The modulatory role of DA on neuronal signaling can be seen directly in behavior and motor output, however it also acts in cognitive processes (Mink, 1996) (Gerfen, 1996). In mammalian research, a large amount of attention is directed to DA processes in the telencephalon and its influence on cognitive processes for attention, memory, and emotion, as well as sensory-motor integration. However, the fact that DA receptor-specific treatments result in similar behavioral effects across different species that lack cortical signaling supports the conservation of DA in behavioral processes at neural levels less complex than the cortex. Human diseases, such as schizophrenia and Parkinson's disease, have distinct behavioral symptoms that are due to different impairments in the central DA systems (Mink, 1996) (Groenewegen, 2003) (Muller et al., 2003). Animal models representing these neurobiological impairments and comparable behavioral effects demonstrate how well the DA systems are conserved in motor processing and initiation of action signaling. By testing the effects of manipulation of the DA system on motor behaviors in anurans, we can bypass the influence of cortical input on basal ganglia processes for motor behavior, and focus on receptor signaling in the thalamus and brainstem for behavior.

As previously stated, in mammalian basal ganglia, DA stimulates motor signals through D1 receptors in the direct striatonigral pathway, and inhibits motor signals through D2 receptors in the indirect striatopallidal pathway (Gerfen, 1996) (Morelli et al., 2007). Examples that support DA signaling in motor behaviors are present in the DA receptor-specific data from studies on locomotor behaviors in mammals. To start, ablation of D2 receptors in dorsal striatal tissue results in hyperlocomotion, while ablation of D1 receptors results in reduced locomotion (Durieux et al., 2012). The opposite effects occur when specific receptors are stimulated in the

dorsal striatum. In particular, D1 receptor-specific optogenetic stimulation increases locomotion, while D2 receptor-specific stimulation decreases locomotion (Kravitz et al., 2010). QUIN, the D2 receptor-specific agonist, reduces motor behaviors when administered at low doses by resulting in a decrease in DA release (Starke et al., 1989), although this effect of QUIN on motor behavior is not seen in D2 receptor total genetic knock-out mice (Usiello et al., 2000).

The behavioral effects of DA manipulation in anurans are consistent with the patterns found in mammal data. Lesions to DA-specific neurons with 6-OHDA in gray tree frogs (*Hyla versicolor*) result in severe motor impairments that correlate with the extent of damage (Endepols et al., 2004a). Also Ewert et al. have shown DA involvement in prey-catching behaviors in the common toad (*Bufo bufo*). In particular, treatment with the non-specific DA agonist, apomorphine (APO), alters a toad's "hunting strategy" to a "waiting strategy" by facilitating snapping behavior toward a target when within reach, although not tracking a target when moving out of the reaching field (reviewed in Ewert et al., 2001). APO also increases the time spent climbing in a motor task on northern leopard frogs (*Lithobates pipiens*, formerly known as *Rana pipiens*) (Chu and Wilczynski, 2007). Immediate-early gene activation in the midbrain tegmentum is more strongly correlated with locomotor behavior than with a social signal stimulus in female túngara frogs (*Engystomops pustulosus*, formerly known as *Physalaemus pustulosus*) when exhibiting a phonotaxic response to species-specific auditory stimuli (Hoke et al., 2007). Recent field data on *H. cinerea* showed IP treatment with QUIN inhibited calling behavior, while treatment with either a D1 receptor partial agonist, SKF-38393, or APO did not affect call rates (Creighton et al., 2013). However, no motor tests were conducted in the field,

and it is possible the inhibition of calling behavior could be due to a motor impairment following QUIN treatment.

1.5 Experimental focus

The goal of the following experiments is to contribute to the answer for the general question- Are the behavioral processes of the DA systems conserved across vertebrates? These experiments focus on manipulation of D2 receptors in *H. cinerea* for motor processes involved in a task that requires swimming, climbing, and crawling actions (Chu and Wilczynski, 2007) (Chu, 2008), with the hypothesis that DA modulates motor behaviors in *H. cinerea* as in mammals, and the D2 receptor has an inhibitory role in motor output. Because DA receptor distribution in the neural pathway for call output is unknown in anurans, we cannot determine the extent of the effect of QUIN treatment on call behavior that is due to motor inhibition based on D2 receptor signaling in a motor pathway, or due to an impaired ability to initiate calling based on D2 receptor signaling in the mesolimbic pathway. By examining the effects of D2 receptor-specific agonist QUIN, and antagonist, haloperidol (HAL), on motor behaviors, the following experiments aim to reveal the role of D2 receptors in different motor behaviors and incentive responses in *H. cinerea*. HAL is a typical neuroleptic prescribed to treat mental illness that acts mainly by blocking D2 receptors, among others. At a molecular level, HAL promotes c-Fos expression (Robertson et al., 1992) and phosphorylation of ERK pathways in striatopallidal neurons that express D2 receptors (Bertran-Gonzalez et al., 2008), while QUIN prevents phosphorylation of tyrosine hydroxylase in the DA synthesis pathway (Lindgren et al., 2001), and decreases DARPP-32 phosphorylation in dorsal striatal projection pathways (Lindskog et al., 1999). Based on the functional processes of the D2 receptor in mammalian basal ganglia and motor behaviors,

it is predicted that a D2 receptor agonist will decrease motor behavior (hypoactivity), while a D2 receptor antagonist will increase motor behavior (hyperactivity) in *H. cinerea* if the anuran basal ganglia relies on DA receptors for modulation of signal output, as in amniotes. Also evidence points to DA involvement in behavioral conditioning and learning in vertebrates (Smith et al., 2006) (Wise, 2004). Thus, if D2 receptors act similarly across vertebrates in the behavioral conditioning process, then behavior performance should improve following D2 agonist treatment in *H. cinerea*, however this may not be the case with D2 antagonist treatment. It is also possible that a balance between D1 and D2 receptor activation may be necessary for successful conditioning, as with motor output, and if this is the case, then either agonist or antagonist treatment will interfere with learning the motor procedure. Overall, these data will contribute to our understanding of how DA processes work to modulate behavior by demonstrating the behavioral effects of receptor-specific drugs in a more simplistic model of vertebrate DA systems in *H. cinerea*.

2 METHODS AND RESULTS

2.1 General procedures

2.1.1 Subjects

Adult green tree frogs, *H. cinerea*, were used in Experiments 1 and 2. Frogs were separated by sex, and housed in groups of 4-7 in glass aquaria. Each aquarium contained a shelter unit, water dish, and a small artificial plant for environmental enrichment. Water was provided *ad libitum*, and crickets (1cm) were provided twice per week as food. Feeding days were spaced so as to allow at least 1 day to pass after being fed before behavioral testing in order to avoid motor interference from any digestive processes.

2.1.2 Behavioral testing

All experiments implemented a motor test that requires swimming and climbing behaviors. The testing apparatus consisted of a swimming pool (122cm diameter) lined with red clay bricks (brick size: 45.6cmx9cmx5.5cm), and filled ~10-18cm deep with tap water (Figs 1a and 1b). Pool water was replaced every 3-4 days. A stationary camera was connected to a computer (Dell Opti-PLEX GX60), and directed to the pool. Digital recordings were made of each experimental trial for all subjects with Microsoft Windows Media Encoder 9 (version #9.00.00.2980).

In general, 3-5 subjects were tested per day. Each frog underwent 14 experimental trials on 4 different days that consisted of an acclimation period and a test session. The acclimation period allowed the frog to adjust to the aquatic environment prior to treatment, and trial procedures were the same for the test session trials following treatment as trials in the acclimation period. Each trial began by releasing the frog closely above the water in the center of the pool, and allowing the frog to swim to and climb the bricks lining the inside of the pool. Trial timing started when the frog entered the water and continued to total 3 minutes. Following the fourth acclimation trial, the frog received an intraperitoneal (IP) injection of either a control or a test treatment, and was placed in a dark aquarium for 20 minutes (post-injection period). Control treatment was administered on the first and last day of testing, and each frog received a different test treatment on an intermediate day of test sessions. The order of treatment administration was balanced by dose and by sex. After the post-injection period, the test session was initiated, and the frog underwent 10 trials procedurally identical to the acclimation trials. After termination of the 10th trial in the test session, the frog was returned to its home tank. No test sessions extended

longer than 35 minutes, and at least 96 hours (4 days) passed before the next experimental trial for the same frog. Details for test sessions in Experiment 1 and Experiment 2 are presented below.

2.1.3 Statistical analysis

Time measures for behaviors were collected from 2 viewings of the digital recordings of the 10 post-injection test session trials using Media Player Classic (version #1.6.4.6052), and the time measures from each viewing were averaged for statistical analyses (inter-rater reliability Experiment 1 Pearson's r=0.68, Experiment 2 Pearson's r=0.99). The viewer was blind to treatment during the behavioral analysis. Table 1 lists the behaviors that were measured in Experiments 1 and 2. Measures are based on the average of all 3-minute trials per test session. *Trial time* is a measure of the time between entrance into the water of the testing arena and either when the frog exited the arena or when the trial ended at 3 minutes (180s), whichever came first. *Total time spent swimming* is a measure of the amount of time the frog exhibited swimming movements and motion in water. *Total time in water* is a measure of the amount of time the frog was in water in the arena. *Total time spent climbing* is a measure of the amount of time the frog was in a vertical position, attached to and/or moving vertically on either the bricks or the pool wall. *Vertical settle* refers to the total time the frog was immobile in a vertical position attached to the brick or pool wall. *Vertical settle in water* is a measure of the amount of time the frog was immobile in a vertical position attached to brick in water. *Total time spent in a horizontal position* is a measure of the amount of time the frog was immobile in a horizontal position on the bricks, or in Experiment 1, also on the top edge of the pool wall. *Total time in air* is a measure of the amount of time the frog was not in contact with a surface because of a jump or hop motion.

Due to small group sizes and balancing for sex and treatment order effects, the independent variable used in analysis for drug effect in either experiment was Treatment (k=3), with the average of the Pre-treatment and Post-treatment control measures as the control variable. Outliers (defined as measures at least 2 standard deviations from the mean) were removed before statistical calculations began. Treatment effects were analyzed with either a parametric one-way repeated measure (RM) ANOVA or a non-parametric Friedman RM ANOVA on ranks, depending on data distribution. In some cases of non-parametric distributions, data transformation by natural log normalized the distribution, and a parametric RM ANOVA was conducted. However if the distribution remained non-normalized after natural log transformation, the original measures were used in a non-parametric Friedman RM ANOVA on ranks. Post-hoc tests consisted of Tukey's HSD test (for parametric data) or Friedman post-hoc equation (for non-parametric data) (Experiment 1 Friedman critical difference CD_F=9.94; Experiment 2 Friedman critical difference CD_F=10.81) (Sheskin, 2007). Conditioning effects, defined as performance improvement based on trial repetition, were tested by a paired t-test or Wilcoxon signed rank test on measures from the control treatment days across all subjects in either experiment (Experiment 1 N=12; Experiment 2 N=25). Significance was confirmed at $P<0.05$. Statistics were calculated using SigmaStat 3.5, and data were plotted using SigmaPlot 10.0. Measures are reported as mean±standard deviation in the text.

Fig 1.1 Testing arena for Experiment 1

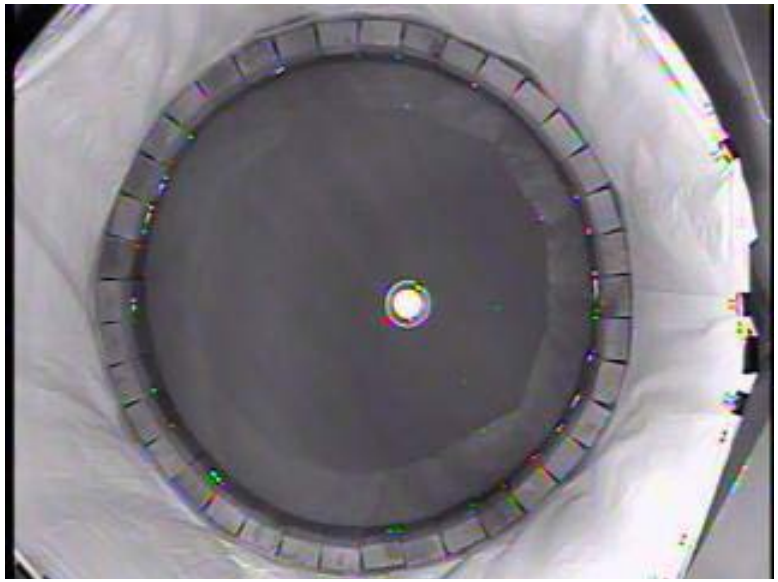

Fig 1.2 Testing arena for Experiment 2

Testing arenas were arranged differently for Experiments 1 and 2. In Experiment 1, a color-sensitive camera was assembled to record the pool at an angle, and bricks were arranged horizontally. In order to prevent subjects from traveling in poorly visible areas, bricks were not stacked to reach above water level on one third of the pool siding (Fig 1.1). In Experiment 2, bricks were arranged vertically to line the pool, thus limiting the amount of hiding space. A black-and-white camera was assembled over the pool, and a red-tinted light was attached near the camera to create enough light for clear recording (Fig 1.2). The brick and camera arrangement allowed for fewer hiding areas and better tracking of behaviors during the trials in Experiment 2, resulting in stronger inter-rater reliability.

Table 1. Treatment Tests and Effects Summary. Different behaviors were analyzed from the digital recordings of each subject following treatment, and the statistical results are summarized below. For parametric distributions, drug effects were tested by a one-way ANOVA with a post-hoc Tukey's HSD test, if significant, and conditioning effects were tested by a paired t-test. For non-parametric distributions, drug effects were tested by a Friedman's one-way ANOVA with a Friedman post-hoc test (QUIN CD_F=9.94; HAL CD_F=10.81), if significant, and conditioning effects were tested by a Wilcoxon signed rank on measures test. QUIN abbreviations: C, control, L, low dose 0.1mg/kg_{bw}, H, high dose, 1.0mg/kg_{bw}; HAL abbreviations: C, control, L, low dose 0.12mg/kg_{bw}, H, high dose, 1.2mg/kg_{bw}

Treatment tests and effects summary						
	QUIN			HAL		
Dependent variable (sec)	Drug effect N=12	Post Hoc	Conditioning effect N=12	Drug effect N=14	Post Hoc	Conditioning effect N=25
Trial time	No: F(2, 11)=1.11, P=0.35	-	Yes: t(10)=3.54, P<0.05	No: F(2, 13)=0.79, P=0.47	-	Yes: t(22)=4.42, P<0.05
Total time spent swimming	No: χ^2=4.22, P=0.15	-	No: t(10)=1.72, P=0.12	No: χ^2=1.08, P=0.58	-	No: z=-0.77, P=0.59
Total time in water	Yes: F(2, 11)=5.36, P<0.05	Tukey L vs H: q=4.31, P=0.02	Yes: z=-2.55, P<0.05	Yes: χ^2=12.67, P<0.05	$CD_F<CD_{\|C-L\|}$ $CD_F<CD_{\|C-H\|}$	Yes: t(20)=2.06, P=0.05
Total time spent climbing	No: F(2, 11)=3.42, P=0.053	-	Yes: t(10)=3.68, P<0.05	No: F(2, 13)=0.43, P=0.66	-	No: t(23)=1.76, P=0.09
Time spent in vertical settle position in water	-	-	-	Yes: χ^2=10.80, P<0.05	$CD_F<CD_{\|C-L\|}$ $CD_F<CD_{\|C-H\|}$	Yes: z=-1.99, P<0.05
Total time spent in vertical settle position	Yes: F(2, 11)=4.92, P<0.05	Tukey L vs H: q=4.30, P=0.02	Yes: t(9)=3.67, P<0.05	No: χ^2=1.86, P=0.40	-	No: t(23)=0.49, P=0.63
Total time spent in horizontal position on brick	No: F(2, 11)=0.32, P=0.73	-	Yes: t(10)=2.45, P<0.05	No: χ^2=3.00, P=0.22	-	No: t(22)=1.87, P=0.08
Total time in air	Yes: F(2, 11)=11.80, P<0.05	Tukey H vs L: q=5.92, P=0.001 Tukey H vs C: q=5.71, P=0.002	No: t(10)=-1.24, P=0.24	Yes: F(2, 13)=3.87, P<0.05	Tukey C vs L: q=3.75, P=0.04	Yes: t(23)=-2.26, P<0.05

2.2 Experiment 1 Details

2.2.1 Subjects

H. cinerea were collected (with permit) from the Charlie Elliott Wildlife Reservation (Mansfield, GA), and housed on Agnes Scott College campus (Decatur, GA) during the summer of 2010 in a greenhouse receiving natural light and temperature. Housing and testing procedures were reviewed and approved by the Agnes Scott College IACUC before initiation of the Experiment 1. A total of 12 frogs (5 females: 7 males) were used in Experiment 1. The average body weight of frogs taken prior to the first day of testing was 7.5±1.9g.

2.2.2 Treatments

Quinpirole hydrochloride (QUIN) was purchased in powder form from Sigma-Aldrich (product #Q102), and dissolved in amphibian Ringer's solution. It was administered in 140µl as a low dose (0.75µg, i.e. 0.1mg/kg_{bw}) or a high dose (7.5µg, i.e. 1.0mg/kg_{bw}) based on previous field data testing the effects of QUIN on calling behavior (Creighton et al., 2013). Control treatment consisted of 140µl of amphibian Ringer's solution. Treatments were delivered by IP injection following the acclimation period in behavioral testing. All frogs received either the high or low QUIN treatment on different days (see Table 2).

2.2.3 Details for Experiment 1 testing procedure

Testing started at 1900 each evening, and lasted until 0200. Experiment 1 utilized a color stationary color camera (Panasonic CCD model #KP-C550U-S2) for visual recording. Data from test days are referred to as control, low QUIN, and high QUIN, based on treatment.

Table 2. Experiment 1. QUIN Swim-Climb Test Subjects. Twelve frogs were used in Experiment 1, and received a low dose (0.1mg/kg_{bw}) or high dose (1.0mg/kg_{bw}) of QUIN, a D2 receptor agonist, on different intermediate test days. QUIN treatments were balanced by order and sex, while control treatment was administered on the first and last days of testing. At least 4 days passed between each behavioral test day for a subject.

Experiment 1. QUIN Swim-Climb Test Subjects						
	Subject	Sex	Day 1	Day 2	Day 3	Day 4
1	1A	M	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
2	2A	M	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control
3	2D	M	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
4	3A	F	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
5	4B	F	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
6	4E	F	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
7	4F	F	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control
8	4G	F	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control
9	5C	M	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control
10	5D	M	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control
11	5E	M	Pre-treatment Control	Low QUIN	High QUIN	Post-treatment Control
12	5G	M	Pre-treatment Control	High QUIN	Low QUIN	Post-treatment Control

2.3 Experiment 1 Results

2.3.1 Treatment effects

While no significant differences were present in the swimming measures across QUIN treatments (Friedman χ^2=4.22, P=0.15) (Fig 2a), there was a treatment effect on the total amount of time a frog spent in water per trial ($F_{(2, 11)}$=5.36, P<0.05, adjusted to natural log) (Fig 2b). Frogs spent significantly more time in water following high QUIN treatment (30.93s±26.10s) compared to low QUIN treatment (7.75±6.05s) (Tukey's q=4.31, P=0.02), and although similar to low QUIN results, there was no effect of control treatment (9.48±5.23s). Overall, frogs remained in the water for a longer amount of time following the high QUIN dose.

The total time spent climbing (i.e. in a vertical position) was greatest following high QUIN treatment, although not significant ($F_{(2,11)}$=3.42, P=0.053) (Fig 2c). In particular, time spent in a vertical settle position was significantly increased with high QUIN treatment (55.62±37.67s) compared to low QUIN treatment (19.55±20.44s) ($F_{(2, 11)}$=4.92, P<0.05; Tukey's q=4.30, P=0.02) (Fig 2d). However, it is noted there were no significant treatment effects on the time to reach the top of the bricks (Friedman χ^2=3.46, P=0.18) (data not shown), and there were no significant differences in the amount of time spent in a horizontal position (i.e. sitting) following any QUIN treatments ($F_{(2, 11)}$=0.79, P=0.47) (Fig 2e). These results suggest that while frogs showed no influence of QUIN on the ability to reach the bricks by swimming, the high QUIN treatment resulted in a preference to remain in a vertical position, even upon reaching the top of the bricks.

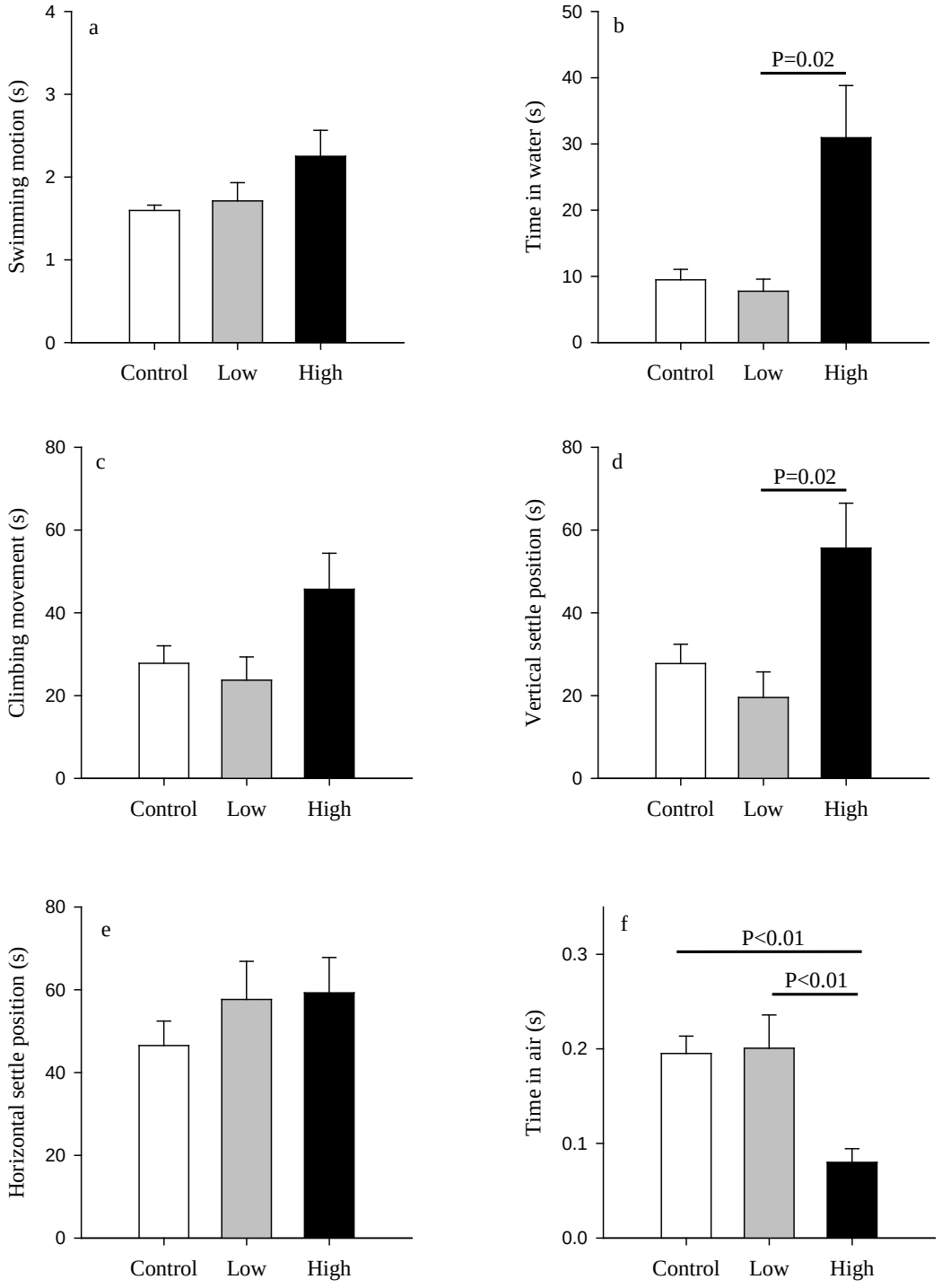

Fig 2. Behavioral changes following QUIN treatments. While QUIN treatment did not affect the amount of time frogs exhibited the swimming motions (a), the high QUIN dose (1.0mg/kgbw) significantly increased the total amount of time the frogs remained in the water per trial on average (b). A trend appeared for general climbing movements ($F_{(2, 11)}$=3.42, P=0.053) (c), and frogs spent significantly more time in the vertical settle position when treated with the high QUIN dose compared to the low QUIN dose (d). No significant effects on the amount of time spent in a horizontal settle position were

found (e), although the amount of time for air travel decreased significantly following high QUIN treatment compared to low QUIN and control treatments (f). Vertical bar values represent the mean±SD. P-values represent the post-hoc results.

A measure of travel used in this analysis was the size of a frog's jumps, determined by the amount of time the frog spent in the air (air travel). This was chosen based on the jumping movement of the frog being a readily identified action. QUIN treatment decreased the time in air ($F_{(2, 11)}$=11.80, P<0.05) (Fig 2f). In particular, high QUIN treatment (0.08±0.05s) resulted in significantly less time in air than low QUIN treatment (0.20±0.12s) (Tukey's q=5.92, P<0.01) and control treatment (0.19±0.06s) (Tukey's q=5.71, P<0.01). Overall, the amount of time the frogs were not in contact with a surface was similar between the low QUIN and control treatments, and both were greater than the high QUIN treatment, suggesting extended immobility on a solid surface after high QUIN treatment, since jumping and hopping are the typical forms of travel for these frogs.

2.3.2 Conditioning effects

Improvement in behavioral task performance is often seen after repeated trials (conditioning effect) in other organisms, including other anurans (Muzio et al., 1992), and the question was asked whether *H. cinerea* would show improvement even under drug manipulation on D2 receptors. Conditioning effects were measured in Experiment 1 as significant differences in behavioral measures from the days of control treatment, which were given on the first day (Pre-treatment control) and last day (Post-treatment control) of testing. Trial time measures decreased significantly from Pre-treatment control (158.62±16.71s) to Post-treatment control (136.37±15.49s) ($t_{(10)}$=3.54, P<0.05) (Fig 3a). This means frogs exited the arena faster on the last day of testing compared to the first day. The total amount of time in water decreased

significantly between Pre-treatment control (14.51±11.67s) and Post-treatment control (2.18±0.72s) (Wilcoxon signed rank, Z=-2.55, P<0.05) (Fig 3b). The total amount of time spent climbing decreased between Pre-treatment control (52.37±35.01s) and Post-treatment control (9.09±8.33s) ($t_{(10)}$=3.68, P<0.05) (Fig 3c), particularly time spent in the vertical settle position ($t_{(9)}$=3.67, P<0.05) (Fig 3d). Also the amount of time frogs spent immobile in the horizontal settle position decreased significantly between Pre-treatment control (57.13±23.53s) and Post-treatment control (46.34±24.46s) ($t_{(10)}$=2.45, P<0.05) (Fig 3e). Measures for time of air travel increased between Pre-treatment control and Post-treatment control, although not significantly ($t_{(10)}$=-1.24, P=0.24) (Fig 3f). Overall the conditioning effects point toward increased movement and improved behaviors generally directed toward exiting the arena, thus resulting in decreased trial times for all subjects on Post-treatment control day compared to Pre-treatment control day.

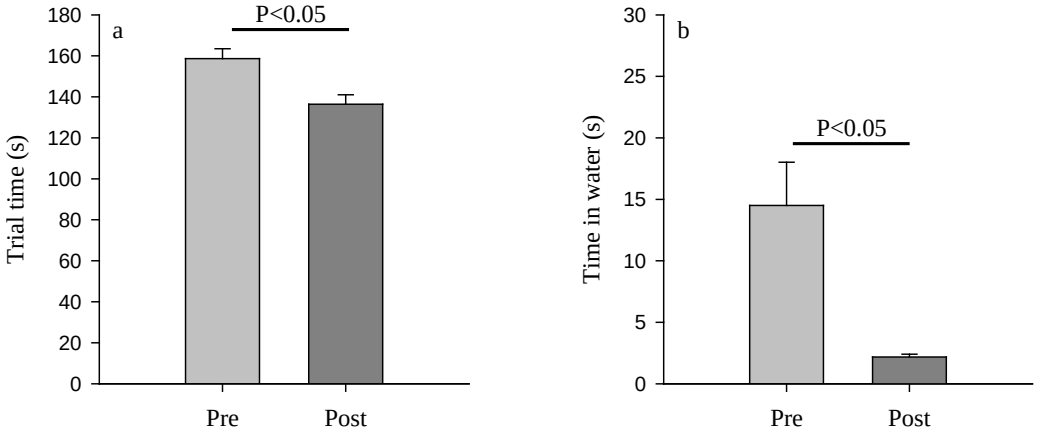

Fig 3.1 Conditioning effects of repeated trials with QUIN treatment. QUIN treatment resulted in improved performance in some tasks, measured as a decrease in time from Pre-treatment measures (Pre) and Post-treatment measures (Post). In general, frogs showed a decrease in the amount of time to exit the arena in the Post-treatment control measures compared to the Pre-treatment control measures (a). In line with decreased trial time are fewer displays of other behaviors while in the arena on the final day of testing, including time in water (b). Vertical bar values represent the mean±SD. P-values represent significant differences in the paired t-test or Wilcoxon signed rank test.

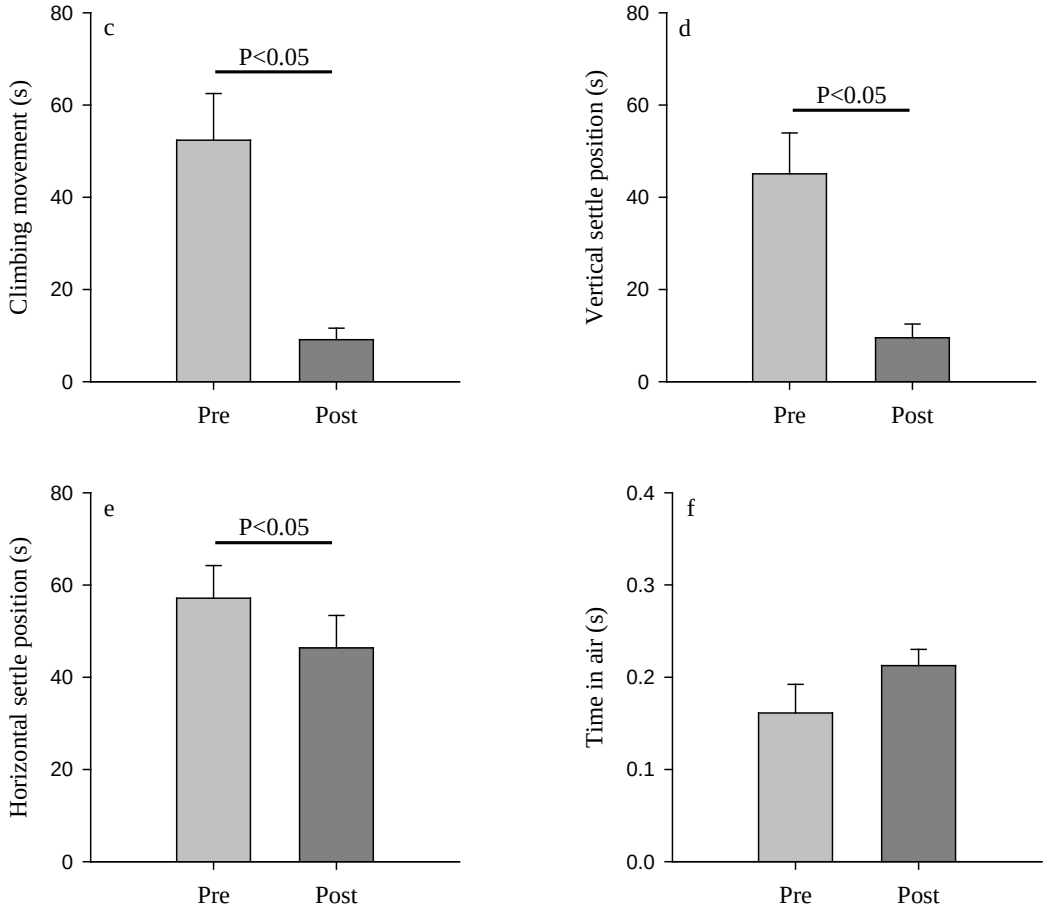

Fig 3.2 Conditioning effects of repeated trials with QUIN treatment. QUIN treatment resulted in improved performance in some tasks, measured as a decrease in time from Pre-treatment measures (Pre) and Post-treatment measures (Post). Other behaviors that decreased between Pre-treatment control measures and Post-treatment control measures include climbing movement (c), vertical settle position (d) and horizontal settle position (e). While air travel increased in Post-treatment measures (f), it was not significant. Vertical bar values represent the mean±SD. P-values represent significant differences in the paired t-test or Wilcoxon signed rank test.

2.4 Experiment 2 Details

2.4.1 Subjects

H. cinerea were purchased from Charles D. Sullivan Co. Inc., and housed in aquaria on a reverse long-day light cycle (14L:10D) in a room designated for amphibian housing at Georgia State University Neuroscience Institute in late summer of 2011. Temperature was maintained between 20-25°C. All housing and testing procedures were approved by Georgia State University IACUC

before testing was initiated for Experiment 2. A total of 25 frogs (15 females: 10 males) were used in Experiment 2. Feeding was decreased to once per week after an observation that the body weights of frogs were increasing considerably before testing began. The average body weight of frogs in Experiment 2 taken prior to the first day of testing was 8.4±1.4g.

2.4.2 Treatments

Haloperidol (HAL; 50mg) was purchased in stock solution with lactic acid from SAGENT Pharmaceuticals (Schaumburg, IL). Treatment doses (based on $8.4g_{bw}$) were prepared using a working stock solution in amphibian Ringer's solution. A low dose (1µg, i.e. $0.12mg/kg_{bw}$) or a high dose (10µg, i.e. $1.2mg/kg_{bw}$) of HAL was diluted from the working stock and administered in 100µl IP injections. A vehicle group (LA; n=5) received a 100µl IP injection of a 2% lactic acid solution on experimental treatment days. To serve as a conditioning control in Experiment 2, a group of frogs (ARS; n=6) received only control treatment of amphibian Ringer's solution on all behavioral test days (see Tables 2.1 and 2.2 for all groups). Doses were based on mammalian behavioral data (Huang et al., 2010) (Salamone et al., 1996).

2.4.3 Details for Testing apparatus and procedure for Experiment 2

The same testing apparatus as used in Experiment 1 was constructed in the housing room for Experiment 2, however there were some differences in the arrangement of bricks and lighting (Fig 1b). Bricks were arranged vertically to line the swimming pool siding. This allowed better visibility of the frog in the pool, and it resulted in less enclosed vertical surface area. White, plastic-covered cardboard surrounded the pool to prevent test subjects from escaping recapture in

Table 3.1 HAL Swim-Climb Test Subjects. Treatment Group 1. A total of 25 subjects were used in Experiment 2. Treatment group 1 consisted of 14 subjects that were administered either a low dose (0.12mg/kg_{bw}) or high dose (1.2mg/kg_{bw}) of HAL, a D2 receptor-specific antagonist. Treatment was balanced by order and sex on intermediate test days, while control treatment was administered on the first and last days of testing.

Experiment 2. HAL Swim-Climb Test Subjects						
Treatment Group 1						
	Subject	Sex	Day 1	Day 2	Day 3	Day 4
1	S23	F	Pre-treatment control	High HAL	Low HAL	Post-treatment Control
2	S24	F	Pre-treatment control	Low HAL	High HAL	Post-treatment control
3	T17	M	Pre-treatment control	High HAL	Low HAL	Post-treatment control
4	T18	M	Pre-treatment control	Low HAL	High HAL	Post-treatment control
5	U30	F	Pre-treatment control	High HAL	Low HAL	Post-treatment control
6	U31	F	Pre-treatment control	Low HAL	High HAL	Post-treatment control
7	W13	M	Pre-treatment control	High HAL	Low HAL	Post-treatment control
8	W16	M	Pre-treatment control	Low HAL	High HAL	Post-treatment control
9	X11	M	Pre-treatment control	High HAL	Low HAL	Post-treatment control
10	X12	M	Pre-treatment control	Low HAL	High HAL	Post-treatment Control
11	Y5	F	Pre-treatment control	Low HAL	High HAL	Post-treatment Control
12	Y6	F	Pre-treatment control	High HAL	Low HAL	Post-treatment Control
13	Z2	F	Pre-treatment control	High HAL	Low HAL	Post-treatment Control
14	Z4	F	Pre-treatment control	Low HAL	High HAL	Post-treatment Control

Table 3.2 HAL Swim-Climb Test Subjects. Treatment Groups 2 and 3. Treatment group 2 served as a vehicle control group, and subjects were treated with a 2% lactic acid solution (LA) on intermediate test days. Treatment group 3 served as a conditioning test group, and subjects received the control amphibian Ringer's solution (ARS) on all test days. All subjects went through a motor test measuring the treatment effects on swimming and climbing behaviors, and at least 4 days passed between each test day in Experiment 2.

Experiment 2. HAL Swim-Climb Test Subjects						
Treatment Group 2						
	Subject	Sex	Day 1	Day 2	Day 3	Day 4
1	S21	M	Pre-treatment control	LA	LA	Post-treatment control
2	U29	F	Pre-treatment control	LA	LA	Post-treatment control
3	V28	F	Pre-treatment control	LA	LA	Post-treatment control
4	X10	M	Pre-treatment control	LA	LA	Post-treatment control
5	Y8	F	Pre-treatment control	LA	LA	Post-treatment control
Treatment Group 3						
	Subject	Sex	Day 1	Day 2	Day 3	Day 4
1	S22	F	Pre-treatment control	ARS	ARS	Post-treatment control
2	T19	M	Pre-treatment control	ARS	ARS	Post-treatment control
3	V27	F	Pre-treatment control	ARS	ARS	Post-treatment control
4	X9	M	Pre-treatment control	ARS	ARS	Post-treatment control
5	Y7	F	Pre-treatment control	ARS	ARS	Post-treatment control
6	Z1	F	Pre-treatment control	ARS	ARS	Post-treatment control

the room after exiting the arena. A stationary black-and-white camera (Panasonic CCTV model WV-BP330) was connected to a computer (Dell Opti-PLEX GX60), assembled on a pole over the pool, and directed toward the water. Anuran eyes lack receptors to red light (Stebbins, 1997), thus in order to generate enough light to record the behavioral tests without disrupting the reverse light-dark cycle of the room, a red light was fixed near the camera for filming. Experiment sessions followed the same behavior protocol as Experiment 1. Lights went off at 0700; experiment sessions started between 0800-1000, and lasted until 1500-1800.

2.5 Experiment 2 Results

2.5.1 Treatment Effects

Analysis 2a

There were no significant changes in swimming behaviors following any HAL treatments (Fig 4a). However, the total amount of time spent in water decreased significantly with each HAL dose compared to control treatment (Friedman χ^2=12.67, P<0.05; $CD_{|C-L|}$=15, $CD_{|C-H|}$=18) (Fig 4b). This means that while the frogs did not differ in the amount of time to reach the brick siding, they exited the water faster and remained on a dry surface longer when treated with either HAL dose than when treated with control.

Neither HAL treatment had any effects on the total amount of time spent climbing ($F_{(2, 13)}$=0.43, P>0.05) (Fig 4c). However, HAL treatments did result in changes to time spent in the vertical settle position in water (Friedman $\chi^{`}$=10.80, P<0.05) (Fig 4d). Post-hoc testing shows both HAL treatments (low HAL: 6.88±10.71s, $CD_{|C-L|}$=15.5; high HAL: 7.74±10.86s, $CD_{|C-H|}$=15.5) decreased the time spent in vertical settle position in water compared to control treatment

(13.37±13.38s), although there were no differences in the total amount of time spent in vertical settle position when including time on a dry surface (Friedman χ^2=1.86, P>0.05) (Fig 4e). This behavioral difference supports the general decrease in time spent in water following HAL treatment. Both HAL treatments showed similar measures for time in air (high HAL: 0.11±0.06s; low HAL: 0.13±0.07s) (Fig 4f), although only the low dose was significantly greater than the control treatment (0.08±0.05s) ($F_{(2, 13)}$=3.87, P<0.05; Tukey's q=3.75, P=0.04). However this effect is viewed with caution after vehicle control analysis (see Analysis 2b).

Analysis 2b

Because HAL must be dissolved in an acidic solution for administration, it is possible the acid may have a molecular effect that results in behavioral changes. For this reason, a second test group received a 2% lactic acid solution (LA) on the intermediate test days (Treatment group 2). The second analysis predicted if the HAL treatments were ineffective and behavioral changes were due to LA in the treatments, then the differences (measured by percent of change) between measures of the HAL-treated frogs from the LA-treated average should not be significant when tested in a one-way ANOVA by treatment. Analyzing the percent of change between the LA-treated vehicle group mean and HAL-treated subjects across the significant behavioral data reveals evidence for an effect of LA in certain behaviors. Specifically, the percent of change between the vehicle group mean and treatment groups in the total amount of time in air did not differ significantly across treatment days (one-way RM ANOVA, $F_{(3,13)}$=0.50, P=0.68). Overall, these data support the possibility that the changes seen in jumping behaviors following HAL treatment are no different than the changes due to LA treatment only. Other tests on the measures that had significant treatment effects (total time in water and vertical settle in water) showed

significant differences in percent of change from the vehicle group mean on treatment days, which supports the behavioral differences in the treatment group were due to HAL and not lactic acid in the treatment solutions.

2.5.2 Conditioning Effects

In order to determine whether the changes in behaviors between the Pre-treatment control and Post-treatment control tests were due to conditioning alone or included drug effects, a test group (n=6) was included in Experiment 2 that received only control treatment for all test sessions. Data from this group were analyzed in a one-way RM ANOVA by Day (k=4). No significant differences were present in the behaviors of interest, although trends in the data suggest this may be due to the small sample size and high variability among individuals in the group.

Still, significant differences were present between Pre-treatment control and Post-treatment control measures when analyses included all subjects (N=25). Trial time decreased between Pre-treatment control (156.94±33.83s) and Post-treatment control (118.57±38.32s) (paired t-test, $t_{(22)}=4.42$, $P<0.05$) (Fig 5a). The amount of time to first exit the water decreased significantly from Pre-treatment control (20.08±18.39s) to Post-treatment control (11.413±11.28s) ($t_{(22)}=2.52$, $P<0.05$) (Fig 5b). However, when including any re-entry into water throughout the 3-minute trial, the post-treatment measures rose and lost significance, although keeping a trend for total time in water ($t_{(20)}=2.06$, $P=0.053$) (Fig 5c). This means on the final day of testing, while taking less time to exit the water upon first entry, frogs were more likely to re-enter the water during a trial. Also while a trend was present for time spent climbing ($t_{(23)}=1.76$, $P=0.09$) (Fig 5d), further analysis shows frogs spent significantly less time in the vertical settle position in water in Post-

treatment control (10.15±11.16s) compared to Pre-treatment control (14.81±15.79s) ($t_{(20)}$=2.15, P<0.05) (Fig 5e). However analysis of the total time in vertical settle position did not reveal a significant effect ($t_{(23)}$=1.71, P=0.10) (Fig 5f). These data support improved behavior to exit water, but no change in preference for a vertical position across trials in general. Notably, the amount of time spent in air increased significantly between the Pre-treatment control (66.4±58.1ms) and Post-treatment control (98.6±52.2ms) ($t_{(23)}$=2.26, P<0.05). Similar to the conditioning effects seen in Experiment 1, these results suggest behavior improvement still occurred in movements directed toward exiting the arena even under D2 antagonist treatment.

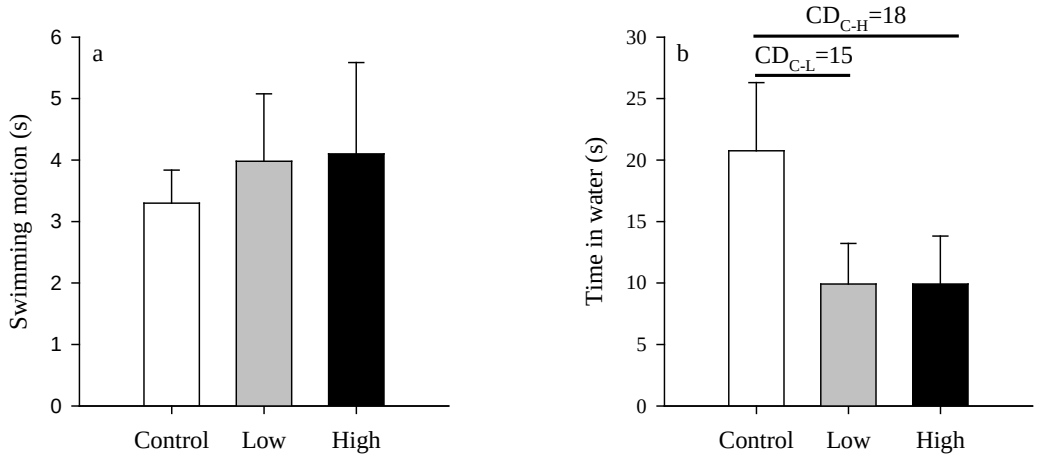

Fig 4.1 Behavioral changes following HAL treatment. HAL treatments tended to decrease measures for behaviors associated with being in water. While neither treatment has any effect on time spent swimming (a), both treatments significantly decreased the total amount of time in water per trial (b). Friedman's critical difference value (CD_F)=10.81. Vertical bar values represent the mean±SD. P-values represent the post-hoc results.

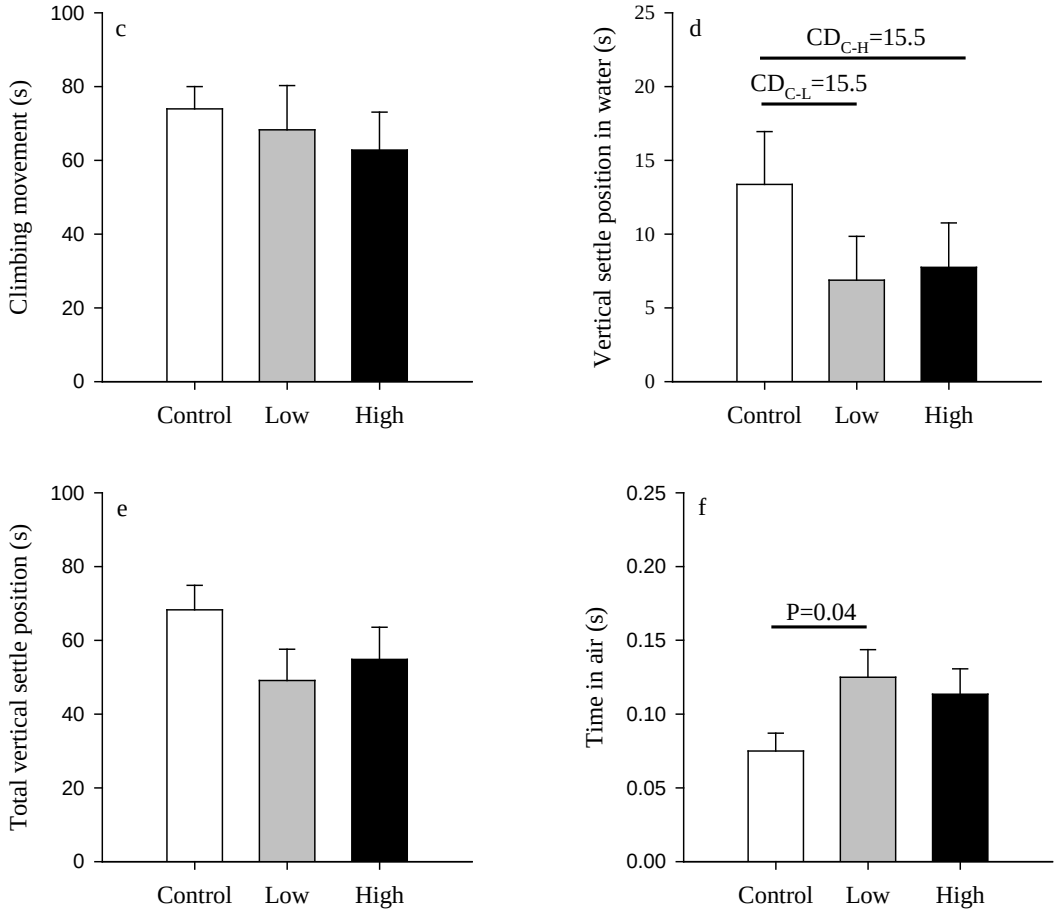

Fig 4.2 Behavioral changes following HAL treatment. HAL treatments tended to decrease measures for behaviors associated with being in water. Although there were no differences in general climbing movements (c), both HAL treatments decreased the time frogs spent in a vertical settle position in water (d). However no differences were present in the total time spent in vertical settle position (e). Low HAL treatment significantly increased air travel ($F_{(2,13)}$=3.87, P<0.05) (f), although a second analysis of the change between HAL and LA-vehicle group suggests this effect may be due to LA. Friedman's critical difference value (CD_F)=10.81. Vertical bar values represent the mean±SD. P-values represent the post-hoc results.

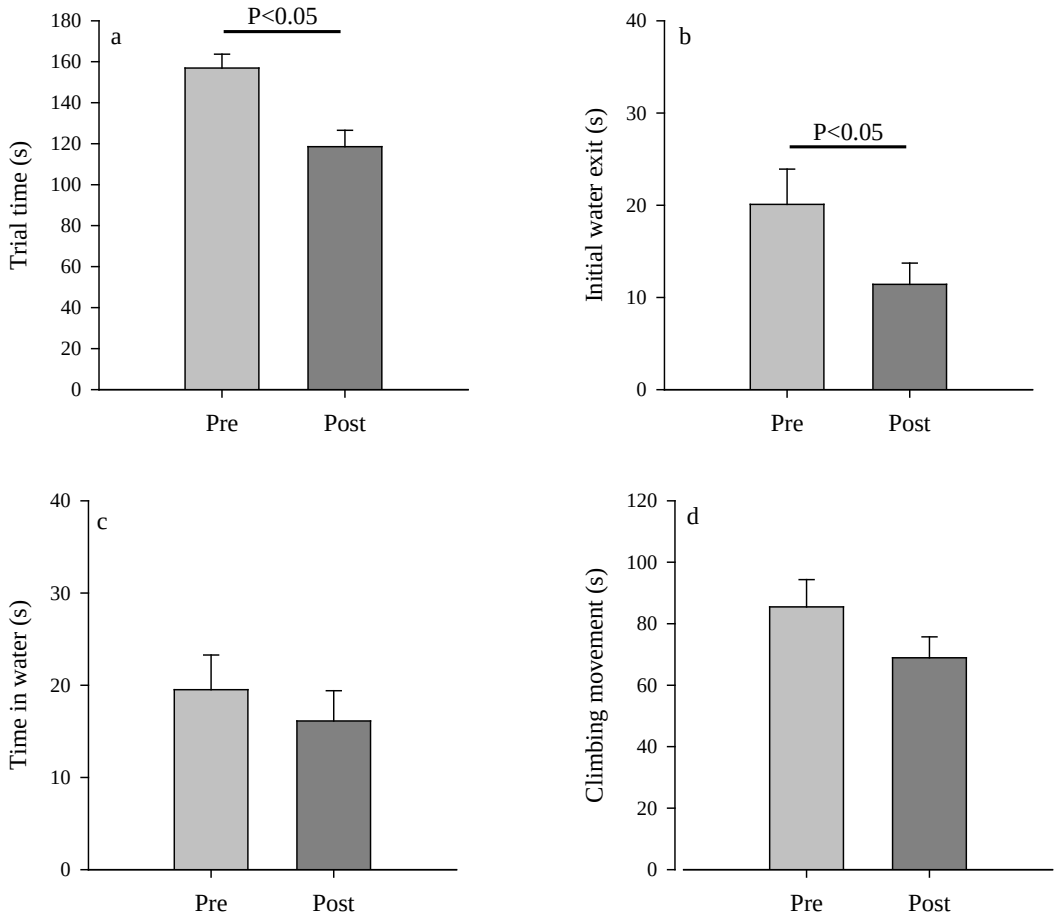

Fig 5.1 Conditioning effects of repeated trials across groups in Experiment 2 HAL treatment resulted in improved performance in some tasks measured by a decrease from Pre-treatment control measures (Pre) and Post-treatment control measures (Post). In general, frogs showed improvement in behaviors directed toward exiting the arena to result in decreased trial time (a) and in exiting water (b). However, neither total time in water (c) nor climbing movement (d) showed significant conditioning effects. Vertical bar values represent the mean±SD. P-values represent significant differences in the paired t-test.

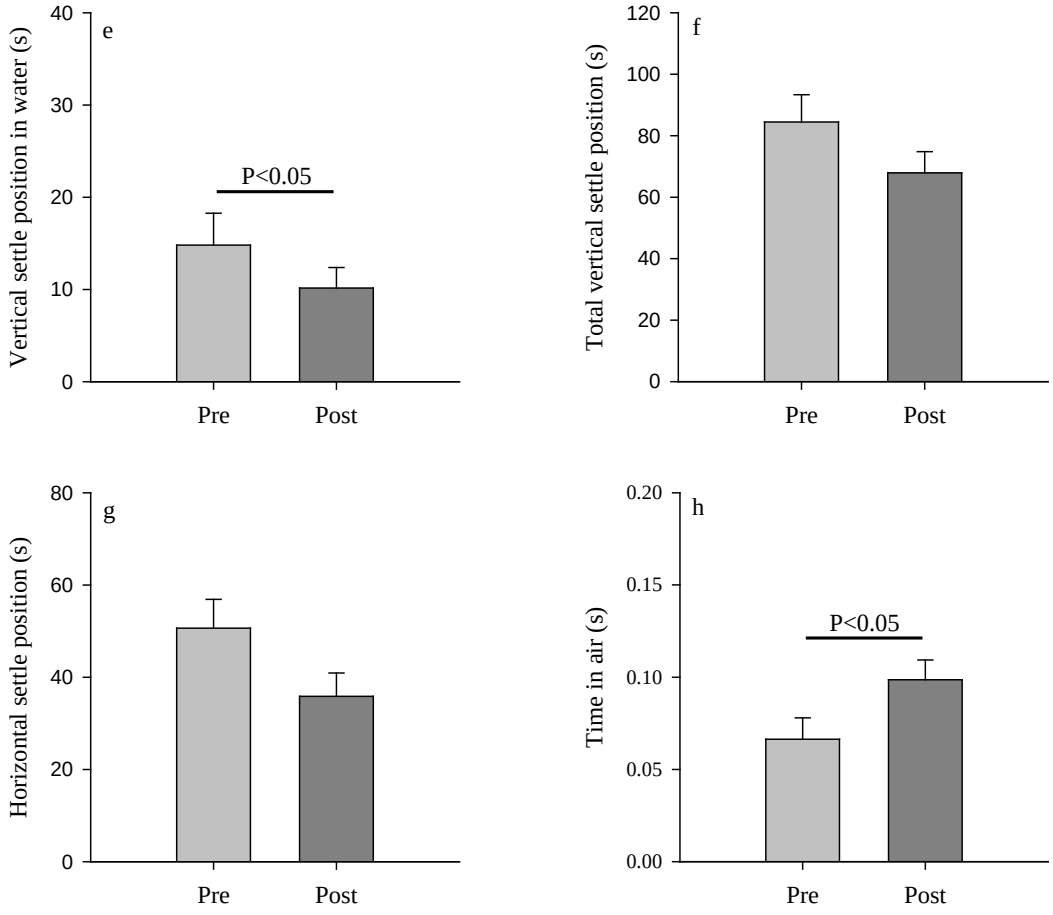

Fig 5.2 Conditioning effects of repeated trials across groups in Experiment 2 HAL treatment resulted in improved performance in some tasks measured by a decrease from Pre-treatment control measures (Pre) and Post-treatment control measures (Post). The time spent in a vertical settle position in water significantly decreased in the Post-treatment measures across all subjects (e). Although time in vertical settle position (f) and horizontal settle position (g) did not differ significantly, air travel increased significantly in the Post-treatment measures (h). Vertical bar values represent the mean±SD. P-values represent significant differences in the paired t-test.

3 DISCUSSION

The neuroanatomical conservation of DA systems across species supports the significance of DA in vertebrate behavior, however whether and how such behaviors are related at the functional level is largely undetermined, especially in non-mammalian systems. The aim of the current experiments was to examine the role of DA in a complex motor task by manipulating D2 receptor activity in the anuran amphibian green tree frog, *H. cinerea*. Based on DA receptor mechanisms, it was predicted the D2 receptor agonist, quinpirole (QUIN), would decrease motor behaviors, and the D2 receptor antagonist, haloperidol (HAL), would increase motor behaviors if anuran basal ganglia function similarly to amniote basal ganglia. Understanding the behavioral effects of DA receptor-specific drugs in anurans contributes to our knowledge of DA modulation in behaviors of different vertebrate species.

In general, the results meet the predicted outcome, in that QUIN tended to inhibit some behaviors, and HAL tended to facilitate some behaviors. For example, frogs that received the high QUIN dose spent more time in water compared to the low QUIN dose and control measures (Fig 2b), while frogs that received either HAL dose spent less time in water compared to the control (Fig 4b). Treatment with high QUIN dose increased time spent in a vertical settle position (Fig 2d), while treatment with HAL specifically decreased time to climb out of water (Fig 4d), and had no effect on the total amount of time in a vertical settle position (Fig 4e). The high QUIN treatment resulted in less air travel (hops and jumps) than the low QUIN or control treatments (Fig 2f). Although behavioral testing suggested HAL increased air travel, analysis of the vehicle-treated group suggested that this effect, in particular, may be due to lactic acid in the HAL solution. Overall, motor behaviors were not completely impaired following any treatments,

in that all frogs were able to swim to and climb to the top of the bricks following all drug or vehicle treatments. These experiments reveal treatment-specific behavioral effects that support D2 receptor signaling in motor processes of anurans, although other factors may also influence behavior expression. Notably the dose differences with QUIN treatment and similar effects with either HAL treatment support specificity of QUIN to the D2 receptor and a more variable effect of HAL on different neurotransmitter receptors.

3.1 Inhibitory effects of D2 receptor agonist

The prediction of opposite effects of the D2 receptor-specific treatments was based on previous behavioral and molecular data focused on DA receptors. In the field, QUIN treatment inhibited calling behavior of *H. cinerea* (Creighton et al., 2013). On similar lines, both IP and IV administration of QUIN inhibits sexual behaviors in quail (Kleitz-Nelson et al., 2010) (Balthazart et al., 1997) and mammals (reviewed in Hull, 2006), and aggressive behaviors for mate competition in zebra finches (Kabelik et al., 2010). It is noted that none of these experiments show impairment of movement per se, but rather a decrease in production of the behavior of focus. However, inconsistent effects of QUIN on locomotor behaviors in rodents confound the interpretation of what may be taking place. For example, QUIN increased general locomotor behaviors in rats (Stuchlik et al., 2007) and mice (Jung and Shim, 2011). However, Schindler et al. and Starke et al., using a lower dose, presented data for the opposite effect of QUIN treatment, in that it reduced locomotor behaviors (Schindler and Cannona, 2002) (Starke et al., 1989). In fact, a biphasic effect is often seen following QUIN treatments in mammals, with locomotor behaviors decreasing for the first 5-15 minutes and increasing after 35 minutes (Horvitz et al., 2001) (Eilam and Szechtman, 1989) (Jesus Luque-Rojas et al., 2013) (Van Hartesveldt et al., 1992). Overall the mammalian data suggest different processes that involve

DA receptors may be acting to produce locomotor and goal-directed behaviors in the mammalian nervous system than in either the avian or anuran systems, where QUIN generally inhibits behaviors. It is already accepted that the avian nervous system contains more D2 receptors than D1 receptors (Kubikova et al., 2010) (Richfield et al., 1987), which may produce more consistent behavioral results following receptor-specific treatments, while the mammalian nervous system may be more susceptible to individual variation in D2 expression. Future work should identify and localize DA receptors in anuran tissue before confirming qualitative differences in DA receptors that could explain variances in the behavioral effects of QUIN treatment when compared with mammals.

Field data on *H. cinerea* suggest QUIN may inhibit motivated behaviors, i.e. behaviors that are expressed for an intended goal. Anuran males produce advertisement calls to attract females during the mating season. Based on a generally low success rate and an increased risk of predation, one may question what is behind the strong innate drive to exhibit a costly behavior, such as calling, when the likelihood of an immediate reward is low. We found that active male green tree frogs treated with 5µg or 50µg of QUIN cease to exhibit any calling behaviors (Creighton et al., 2013). The present behavioral data suggest this inhibition of calling is not due to a general inhibitory effect of the drug on the motor system because frogs showed no differences in performance of some motor behaviors on control days and QUIN treatment days (e.g. swimming). However, behaviors that were affected by QUIN suggest a decreased drive to escape unpleasant conditions, thus spending more time in water even though this species is land-oriented as adult. Together these behavioral data support that DA acts in the production of effortful behaviors, such as calling and climbing out of water, in *H. cinerea*.

3.2 Excitatory effects of D2 receptor antagonist not present

The prediction of hyperactivity in motor behaviors was not seen following D2 receptor antagonist treatment, although frogs exited water faster on HAL treatment days compared to control treatment days. In fact, HAL often causes catalepsy, or muscle rigidity and freezing, in mammals at doses (by bodyweight) only slightly higher than those used in this study (Boulay et al., 2000) (Huang et al., 2010) (Salamone et al., 1991). The current behavioral measurements do not suggest HAL induced a cataleptic-like response in frogs. However, body-freezing and tonic, rigid movements similar to catalepsy can be induced in frogs by a HAL metabolite, CPPO, although the behavioral effects are not reliably visible until at least 2 days after IP treatment (Ablordeppey et al., 1992). The differences in the occurrence of catalepsy following HAL treatment, along with the differences in the effects of QUIN on locomotor behaviors, between anurans and mammals suggest dissimilarities in D2 receptor sensitivity, or DA system in general, between these classes of vertebrates.

Retesting anurans with a greater range of HAL doses may produce results that clarify what is occurring at the receptor level to produce the behavioral differences compared to mammals. While the selected doses were presumed to be low on mammalian standards, if the anuran D2 receptor is overly sensitive to HAL, then even the low dose used here could have a saturation effect. Also if multiple types of anuran receptors are sensitive to HAL, then many systems could be affected by IP treatment, causing a non-specific, generalized effect. Considering there was no dose effect, a greater range of doses may reveal HAL-sensitive receptor properties either unique to anurans or masked in anurans by oversaturation in the synapse by the current doses. It is worth noting very few published experiments using HAL have examined the impact of the acidic disso-lvent in the HAL treatments compared to a saline-based control treatment. The finding that lactic

acid alone enhanced air travel behaviors questions the extent of HAL acting molecularly, rather than the dissolvent solution, to produce the behavioral effects in other experiments using this combination as treatment. Also what does it say about the anuran system to respond to LA with behavioral changes? One explanation may be based on the ability of anurans to adapt to different environments, and as a survival mechanism, their bodies are responsive to potentially dangerous substances found in the wild, such as LA. Thus, the increase in air time would be an indirect escape mechanism automatically triggered by the presence of LA in the body. Research needs to determine where LA is acting in anurans before its behavioral impact can be explained.

3.3 Conditioning effects and performance improvement followed both treatments

Behavioral conditioning is a term that refers to improvement of behavior performance, and the conditioning effect refers to the level of improvement that occurs with trial repetition. Both QUIN and HAL treatments resulted in a significant decrease in trial time for Post-treatment control compared to Pre-treatment control, suggesting the conditioning effect of repeated trials still took place even in the presence of drugs acting on D2 receptors in different ways. Other tests have shown anurans exhibit instrumental conditioning effects in a goal task without drug treatment, although conditioning effects are not quite the same as those of mammals and birds (Bilbo et al., 2000) (Muzio et al., 1992). However, it is striking that a conditioning effect was not present in the control group alone for Experiment 2, even though it is seen in the vehicle group that received LA on intermediate test days. More behavioral testing with a larger sample size focused on the number of trials necessary for performance improvement to appear may better explain what is taking place in the control group.

A second result from Experiment 2 that contradicts mammalian data is the presence of a conditioning effect following D2 antagonist treatment. Evidence from rats demonstrates D2 antagonists prevent the development of conditioned place preference (Tzschentke, 1998) (Wright et al., 2013) and instrumental incentive learning (Dickinson et al., 2000). One hypothesis for these differences in results between anurans and mammals is that the level of D2 receptors responsive to antagonist treatment is altered in anurans compared to mammals. Differences in the effects of D2 receptor inhibition could be due to a lower total quantity of receptors or a decreased efficacy of the drug in anuran tissue compared to mammalian tissue. Also mammals have more neural tissue with DA receptors in general, and particularly in regard to cortical tissue in the forebrain. The presence of DA receptors in cortical tissue allows for potential synaptic connections that could influence mammalian behavior, but such cortical connections do not exist in the anuran brain. However non-cortical areas that process DA are also involved in behavioral conditioning in mammals. DA receptor density and localization need to be determined in anuran neural tissue to confirm what is taking place at the functional level to result in task performance improvement. In any case, the conditioning process differs to some extent between mammals and anurans.

The basal ganglia direct and indirect pathways are significant in motor signaling and movement execution in mammals. Nishizawa et al. showed that elimination of the indirect striatopallidal pathway signaling specifically reduced motor accuracy with no effect on response time in an auditory conditioning task (Nishizawa et al., 2012), while Fukabori et al. revealed that direct striatonigral pathway lesioning impairs response time with no effects on motor accuracy (Fukabori et al., 2012). Finally Durieux et al. selectively damaged D2 receptor-expressing

medium spiny neurons, which resulted in overall diminished performance on a rotarod task (Durieux et al., 2009). However, in all cases, behavior performance of the test animals improved to control levels with repeated trials. The effect of performance improvement across trials when lacking at least one functional dorsal striatal pathway suggests the D2 receptor is not a determining factor for effective behavioral conditioning. Data from the current experiments support this suggestion by demonstrating the presence of conditioning effects from repeated trials while using treatments affecting the D2 receptors in opposing ways, i.e. both QUIN and HAL treatments resulted in behaviors that improved between the Pre-treatment control and Post-treatment control measures.

However, whether the behaviors that exhibited significant changes are considered "rewarding" from a frog perspective, and function through processes similar those in the mammalian mesolimbic reward pathway remains undetermined. Based on differences in neuroanatomical populations and striatal pathways in anuran neural tissue, it is possible the conditioning effects exhibited by frogs in these experiments may be through habit learning, which is not necessarily as dependent on DA as incentive-based learning (Berridge, 2007). Other behavioral evidence on water-deprived toads (*Chaunus arenarum*, formerly known as *Bufo arenarum*) supports the possibility of a different learning process based on habit formation, rather than behavior sensitive to incentive-based reward. Water-deprived toads conditioned in a runway task to obtain a water reward do not show similar conditioning effects as exhibited by mammals. In particular, toads show impairments in acquisition of a task, but not extinction, when receiving only partial reinforcement, while continuous reinforcement results in better acquisition of the task with reward and faster extinction when the training reward was large. However, rats show

impairments in extinction when conditioned on a partial-reward schedule or trained with a small reward (Muzio et al., 1992). Behavioral effects similar to anurans are also exhibited by goldfish (reviewed in Muzio et al., 1992). These results suggest anurans learn through habit formation and not by incentive value, which relies on DA in the mesolimbic system (Berridge and Robinson, 1998) (Muzio et al., 2011). For example in toads, adjustment to behaviors to obtain a reward, as well as reward intake, are based on previous reward size (Papini et al., 1995). This suggests habit formation because the behavior response in extinction is strongly related to the reward size during acquisition (Muzio et al., 2011). Together these differences in behavior support the possibility of another process that may be occurring to result in performance improvement in anurans that does not involve the DA systems.

It is noted that other work on anurans has shown conditioning effects in a swimming task in the northern leopard frog (*Lithobates pipiens*) following anticholinergic treatment (Bilbo et al., 2000). In particular, the anticholinergic treatment, atropine sulfate, impaired the ability of frogs to use a visual cue to locate the target stand in a dose-dependent pattern compared to control treatment. These results suggest acetylcholinergic systems are more strongly involved in the behavioral conditioning process of frogs than DA systems. The anuran acetylcholinergic system shows general similarities in cell populations in the basal forebrain when compared to amniotes, although notably different from other anamniote systems (Gonzalez et al., 2002). When considering the differences in neuroanatomy of the anuran basal ganglia system, it is possible that acetylcholine (ACh) has a greater role in behavior because anurans may not share the same functional disinhibitory basal ganglia system modulated by DA and ACh as accepted for amniotes. Although research has shown ChAT-ir neurons in the anuran forebrain (Marin et al.,

1997c), more testing needs to be conducted to determine if there is any interaction between ACh and DA, such as DA receptor colocalization with ACh neurons.

4 CONCLUSION

The amniote striatum receives DA signals from the substantia nigra pars compacta, and relies on a functional balance between D1 and D2 receptors for accurate motor production (Choi et al., 2009) (Robertson et al., 1992). There is also a large amount of glutamatergic input to the striatum from cortical areas involved in sensory and motor processing in the amniote brain (Yelnik, 2002) (Groenewegen, 2003). Evidence supports the presence of DA receptor-specific populations in the amphibian striatum (Marin et al., 1998b), and a dorsal striatopallidal pathway in anurans (Endepols et al., 2004b). However the anuran brain does not show the complexity of connections and feedback circuits with cortical tissue that produce behavioral signaling in mammals. How can receptor-specific drugs cause comparable behavioral effects in neural systems that differ in circuit complexity, such as in the mammalian and amphibian brains? To answer this question, we need to consider the significance of the conserved features in the vertebrate nervous system, and the processes that produce behaviors. While receptor localization is unknown in the anuran brain, data from Experiments 1 and 2 support functional conservation of DA systems and basal ganglia motor pathways in the production and improvement of behaviors without cortical input. Also the presence of conditioning effects following either D2 agonist or antagonist treatment suggests DA may not play as significant a role in learning in anurans as in mammals. In fact, research on toads supports anuran learning processes are limited to habit formation, and do not extend to the generation of incentive value of a reward, as expressed by mammals (Muzio et al., 2011). In conclusion, *H. cinerea* serves as a useful model

for investigating the role of DA in regulating motor behavior because anurans lack executive processing that can complicate interpretation of pharmacological manipulations. The results from the current experiments support conservation of DA processes in behavior across vertebrate species, although future research is needed to determine the distribution of DA receptors in anuran neural tissue.

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