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Zhijia Liang 04/16/2013

7,8-Dihydroxyflavone Facilitates the Consolidation of Reward-Related Learning

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Abstract

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Extensive research has been conducted to elucidate the role of Brain-Derived Neurotrophic Factor (BDNF) secretion and TrkB activation on a myriad of behavioral functions such as fear acquisition and consolidation, as well as morphological changes involved in neuroplasticity of key regions such as the hippocampus, cerebral cortex, and basal forebrain. However, most prior research focused on the effects of BDNF action in an aversive setting, and few studies have examined BDNF action in an appetitive context. As such, we utilized the BDNF analogue 7,8-dihydroxyflavone (7,8-DHF) and systemic administration of the agent to explore the effects of TrkB activation on appetitive conditioning. After acquisition of an instrumental response, mice undergoing reversal training showed evidence of strengthening of consolidation of response-outcome learning after 7,8-DHF treatment, as indicated by increased preference for the previously reinforced aperture. This effect was selective to reinforced responding since 7,8-DHF had no effects on response extinction. We also found that dendritic spine density in the orbito-prefrontal cortex (oPFC), a key area involved in response-outcome conditioning, was increased after 7,8-DHF treatment. Tracing studies also identified the existence of cortico-striatal projections in mice. Together, the data suggest distributed TrkB activation enhances consolidation of response-outcome contingency conditioning, and that the oPFC may be a critical neural substrate. Systemic 7,8-DHF administration may thus serve as a novel treatment option for disorders associated with deficiencies in reward-related learning.

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Introduction

Recent neurobiological research discovered the existence of 7,8-DHF, a small-molecule compound that acts as an agonist at the TrkB receptor, the high-affinity receptor for BDNF (Jang et al. 2010). Such a discovery is groundbreaking, as researchers can now mimic BDNF expression and ascertain its action in certain brain regions without the need of generating expensive genetic knockin mice. Importantly, 7,8-DHF has the ability to cross the blood-brain barrier, and can thus be administered systemically, minimizing technical skill and risks of intracranial delivery methods.

BDNF is a growth factor belonging to the neurotrophin family. Other neurotrophins include nerve growth factor, as well as neurotrophin-3 and neurotrophin-4. BDNF is expressed in the cortex, hippocampus, and basal forebrain, among other structures, where it has been implicated in neuronal growth, differentiation, dendritic arborization, and synapse formation as well as neurogenesis (Park and Poo 2013). BDNF is a high affinity agonist at the TrkB tyrosine kinase receptor, but also binds to the low-affinity nerve growth factor receptor (LNGFR) p75. It is its action at the high affinity TrkB receptor that has received the most research and attention, with studies elucidating the mechanism of action. The effects of BDNF have been thoroughly studied in the aversive context, and include regulating fear memory and extinction. For example, Andero et al. used standard fear conditioning and extinction protocols to test the effects of systemic 7,8-DHF injections in mice (2011). Compared to vehicle-injected mice, learning of cue-dependent fear conditioning was enhanced with systemic 7,8-DHF injections. Extinction was similarly enhanced. These results suggest the important role BDNF plays in fear learning and extinction, all forms of behavior conditioning, as well as the ability of 7,8-DHF to mimic BDNF expression.

7,8-DHF is a small molecule high affinity agonist active at the TrkB receptor. Due to its small size, it easily crosses the blood-brain barrier, and this has revolutionary implications for behavioral analysis of TrkB action. Now instead of generating knockin mice or conducting high-risk surgeries to manipulate BDNF expression in the brain, a systemic injection is sufficient to activate TrkB signaling pathways. For example, 7,8-DHF has been shown to activate amygdala TrkB receptors as visualized with standard immunostaining techniques; the drug also enhances fear acquisition and extinction. In addition, mice subject to immobilization stress, a common method to induce stress related behavioral rigidity, saw rescued extinction response as determined using cued fear conditioning tests (Andero et al. 2011). Despite extensive work on the effects of 7,8-DHF on fear and anxiety, few research efforts have shifted focus on the drug to appetitive settings. This is important because BDNF is a known regulator of emotional learning in both appetitive and aversive settings; for example, BDNF knockdown in the oPFC resulted in insensitivity to response-outcome contingency learning, in which mice learn to make an operant response to obtain a food reward (Gourley et al., in press). These and other results suggest that BDNF plays an active role in the regulation of reward-related learning.

In applying the BDNF agonistic activities of 7,8-DHF to appetitive settings, we hope to gain a better understanding of the neurobiological bases of behavioral inflexibility in psychopathological instances such as depression. Given its debilitating effects on emotional health and social function, depression is attributed as the number one cause of unemployment in the United States (World Health Organization). Sufferers fall into cyclic thought patterns and find themselves subject to the same daily routines. They no longer find previously rewarding activities enjoyable, and also cannot develop positive emotions associated with new experiences. Such anhedonia and ruminant thoughts are ways behavioral inflexibility can manifest. Our ultimate goal is to identify possible therapeutic effects of the drug for patients suffering from such disorders associated with behavioral inflexibility. Although one approach to

ascertaining the effects of 7,8-DHF in appetitive settings would be to deliver it to regions of interest such as the oPFC, we will mimic treatment in humans and administer it systemically. We hypothesize that mice treated with systemic 7,8-DHF injections will show enhanced appetitive conditioning, as measured by testing the effects of the compound on the consolidation of response-outcome contingency learning, wherein the dependent variable is response rate on an operandum that is reinforced with food.

In order to isolate the effects of 7,8-DHF as they relate to improvement of learning and memory processes, mice will be trained to perform a task in which they respond for food reinforcement in a standard operant conditioning chamber with 3 nose poke apertures (left (L), center (C), and right (R)). During initial response training, standard aperture reinforcer delivery settings will be in place such that both (L) and (R) apertures will deliver food reinforcers, while the (C) aperture will deliver no reinforcement. Sensitivity to the response-outcome contingency is then tested by uncoupling food delivery associated with one of the two active nose poke apertures. The drug will be delivered immediately after this "degradation" training has occurred, during the presumptive consolidation period. A probe test will be conducted to confirm no *adverse* effects on response-outcome learning have occurred. Next, a reversal learning task will test whether the drug had any *facilitative* effects on response-outcome learning, as determined by the animals' willingness to sample their surroundings. In this test, a preference for the previously reinforced aperture is interpreted as a strengthening of the consolidation of response-outcome conditioning.

For our reversal learning experiments, the only reinforced aperture will shift to (C), with the (L) and (R) apertures no longer reinforced. If 7,8-DHF enhances consolidation of appetitive conditioning, this task allows us the resolution to detect an enhancement since "normal" mice will sample all nose pokes (Gourley et al. 2010), while enhanced consolidation will be reflected by preferential responding on the previously reinforced apertures. These tasks are known to

depend on the prefrontal cortical regions such as the oPFC where BDNF is thought to act by facilitating activity-dependent neuroplasticity (Gourley et al. 2010; in press).

Finally, once the mice have fully acquired the new response (regardless of any effect of 7,8-DHF), they will be tested for extinction learning. In this case, all reinforcer delivery will be halted, and the time it takes for mice to inhibit responding on the previously active (C) aperture will be measured between groups.

After behavioral studies are conducted, post-mortem studies will be carried out focusing on the oPFC. The oPFC is a part of the prefrontal cortex with activity implicated in reward valuation, goal-directed action, and higher decision-making. For example, the oPFC mediates goal-directed decision making by updating the value of expected outcomes (Rushworth et al. 2007, Schoenbaum et al. 2009), and lesions of this region inhibit action-outcome (Gourley et al. 2010) and stimulus-outcome (Ostlund and Balleine 2007) learning.

Studies on the oPFC projections to the lateral striatum have revealed its importance in outcome-predictive relationships; additionally, *Bdnf* knockdown in the oPFC resulted in similar action-reinforcement insensitivity, while it simultaneously reduces striatal BDNF expression (Gourley et al., in press). The projections of the oPFC to the dorsal striatum (DS) have been implicated in reward valuation and the regulation of instrumental behavior by allowing an organism to learn and predict stimulus-outcome relationships (Ostlund and Balleine 2007). Thus, this connection is significant to the mechanisms involved in learning and behavioral flexibility. We used anterograde tracing techniques to identify the neural projections between the oPFC to the DS. While analogous projections have been identified in humans, non-human primates, and rats, it is still essential to identify and characterize such projections in mice, as these projections have not been explicitly outlined in peer-reviewed research. This is despite the widespread use of mice in biomedical research.

Finally, we also enumerated dendritic spines in the oPFC to assess morphological changes due to 7,8-DHF administration that may underlie behavioral findings. Given the correlation between spine density and neuronal activity, we hypothesized the oPFC will exhibit increased density measures compared to controls. Our results will add additional weight to the validity of the behavioral experimental findings.

Given the results of these experiments, future research could even more precisely probe the mechanism with which systemic 7,8-DHF leads to behavioral plasticity. The drug may also be injected into specific brain regions to mimic increased BDNF expression at key areas such as the oPFC, striatum, and amygdala. Tracing techniques may also be conducted on mice injected with the drug to determine if there were any effects on the strength of connection between key cortical and striatal areas. Although the drug has only been discovered for a short time, continuing research suggests that the potential applications of 7,8-DHF is vast and pioneering.

Methods and Materials

Subjects:

Adult male mice (N=15) between 16-28g originating from a line of thy-1 GFP-expressing mice backcrossed to the C57BL/6 background strain (Feng et al. 2000) were used for behavioral and postmortem studies. Tracing experiments used wild type C57BL/6 mice. Dendritic spine enumeration experiments utilized an additional control group of corticosterone-treated subjects as a negative control; these mice are discussed in more detail in the Results section and are described in Gourley et al. 2013. Animals housed in groups of 2 to 5 per cage were food restricted during the behavioral testing phase to motivate response for food reinforcement. All experiments were approved by Emory University Institutional Animal Care and Use Committee (IACUC) standards and Yerkes National Primate Research Center regulations.

Behavioral Studies

Drugs and Cohorts

The TrkB receptor agonist 7,8-DHF was administered using systemic intraperitoneal injections, at 5mg/kg. The drug is suspended in a vehicle solution of 17% DMSO and 1xPBS. Mice were separated into drug and vehicle groups. Agent administration always occurred immediately after a training session. Experiments were conducted at the same time of day.

Response Acquisition

Mice were first trained to acquire an instrumental task in a Med-Associates operant conditioning chamber using Med-Associates software. The testing chamber consisted of three

nose-poke apertures as well as a reinforcement (food pellet) delivery magazine. During acquisition, no agents were administered, and the mice were trained to acquire the response in which only the left (L) and right (R) apertures were active. Active apertures were defined by a single nose-poke response yielding one reinforcement delivery, a schedule of reinforcement termed fixed ratio 1 (FR1), or 'continuous reinforcement. Each active aperture resulted in 30 reinforcements maximum, and there was a time limit of 45 minutes for mice that fail to obtain maximum reinforcement from both apertures. Mice were trained daily until their responding became stable.

Response-Outcome Contingency Degradation

Response-outcome contingency degradation control consisted of three days of testing. On the first day, the response-outcome contingency was reinforced. Access to one of the previously reinforced apertures was physically blocked (*e.g.* L), while the other aperture remained unaltered (*e.g.* R). On the second day, response-outcome contingency degradation was established. The blocked aperture from day one became accessible (L), while the open aperture from day one was now physically blocked (R). The newly open aperture (L) underwent degradation in response-outcome relationship. When mice nose poked in this aperture, there was no longer fixed ratio reinforcement delivery. Rather, food was delivered on a random interval, independent of the nose poke response, that is yoked to the magnitude of total reinforcement received the previous day during contingency training (Gourley et al. 2012). Training during these first two sessions lasted 25 minutes each. Immediately after degradation training, 7,8-DHF was injected intraperitoneally in the drug group, while PBS + 17% DMSO was used as a control for the vehicle control group.

The following session, a probe test was used to determine aperture preference. Here, all apertures became exposed and available, and response rate on each aperture was measured for 15 minutes total.

Reversal and Extinction Learning

Reversal learning followed in which the previously active apertures L and R were inactivated, and the center (C) aperture was activated on FR1 reinforcement delivery schedule. Mice were subject to 25 min sessions, and response rates on all apertures were measured. Only the first session is shown here, however mice ultimately acquired the new response in four sessions, and extinction conditioning was tested. In this case, all apertures were inactivated. 7,8-DHF or vehicle were administered daily immediately after each 15-minute extinction training session.

Dendritic Spine Capture and Enumeration

As described (Gourley et al. 2012), fresh GFP-expressing brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 55 µm-thick sections on a microtome held at -15°C. Unobstructed dendritic segments running parallel to the surface of the section were imaged on a spinning disk confocal (VisiTech International, Sunderland, UK) on a Leica microscope. Z-stacks were taken with a 100x 1.4NA objective using a 0.1 µm step size, sampling above and below the dendrite. After imaging, we confirmed at 10X that the image was collected from the intended subregions.

Collapsed z-stacks were analyzed using ImageJ: Each protrusion <4 µm was considered a spine (Peters and Kaiserman-Abramof 1970). Individual planes were evaluated to detect protrusions perpendicular to the z-stack. Bifurcated spines were considered singular units. To

generate density values, spine number for each segment was normalized to the length of the segment.

From cortical neurons, 5-6 independent segments from secondary and tertiary dendritic branches within 50-150 µm of the soma were collected; the dendrites themselves ranged between 10-25 µm. Each group contained 4-6 mice, with each animal contributing a single density value to statistical analyses. Due to the stellate-like appearance of oPFC excitatory pyramidal neurons (Liston et al. 2006, Kolb et al. 2008), apical vs. basal branches were not distinguished.

Tracing Studies

Surgery

Mice were anesthetized with intraperitoneal (i.p.) injections of Ketamine (75mg/kg)-Dormitor (1mg/kg), and mounted in a digital Just for Mice Dual stereotaxic apparatus (Kopf). Small holes were drilled above injection sites, and 50-100nl infusions of biotinylated dextran amine (BDA-10,000) were delivered at a rate of 50nl/min with a 32-gauge Hamilton microsyringe. Infusions were unilateral and delivered along mediolateral and rostrocaudal gradients. Coordinates from bregma, based on the mouse brain atlas of Paxinos and Franklin (2001), were as follows for the oPFC: AP=+2.22-2.8, ML=$\pm$1.2-1.75, DV=-2.8. The microsyringe remained in place for 10 minutes following infusions. Following the delivery of the tracer, the wound was sutured and mice were given injections of Antisedan (1mg/kg, i.p.). During surgery and at 24 and 48 hours following, mice were given subcutaneous injections of Meloxicam (1mg/kg) for pain management.

Histology

One week after surgery, mice were deeply anesthetized with an overdose of Ketamine-Dormitor and transcardially perfused with 4% paraformaldehyde. Brains were rapidly removed and stored in 4% paraformaldehyde at 4^{o}C for 48 hours before being transferred to 30% w/v sucrose. Following full immersion in sucrose (3-4 days), brains were mounted on a microtome and 55 µm coronal sections were collected. BDA signal was amplified with avidin-biotin complex and revealed by Ni-diaminobenzidine staining. Sections were then mounted on Superfrost Plus slides and lightly counterstained with Cresyl Violet before being coverslipped.

Qualitative Analysis

BDA signal was imaged by brightfield illumination using a Zeiss Axioscope. Maximum diffusion around the injection site was imaged, and the DS was examined for labeled axon terminals.

Results

For the majority of behavioral testing, response rate on an operandum was utilized as a measure for changes in behavior. The response rate was measured as responses per time spent in the testing chamber. This adjusted value ensures differences may be detected between an animal that obtained maximum reinforcement before the session timed-out and an animal that obtained maximum reinforcement at or close to the session time limit; in each case the animal obtained the same number of reinforcement, but the former has obviously a stronger acquisition of the measured response.

During the acquisition phase (Figure 1), mice were separated into “To Be Drug” and “To Be Vehicle” groups, given that no agents were administered post training sessions at this stage. Over the course of roughly two weeks, the response rates of both groups increased and stabilized. On day 12 of acquisition training, the drug group achieved a response rate (responses/min) of 1.55 (±0.11) and the vehicle group achieved a response rate of 1.55 (±0.18). The stabilization and convergence of response rate towards the end of this phase suggested the animals have successfully acquired this instrumental response. Two-factor ANOVA determined the main effect of day [$F(1,13)=10.4$, $p<0.001$], with no effect of group and no interactions ($p>0.05$).

After contingency degradation phase (Figure 2), the drug group achieved a response rate (responses/min) of 8.00 (±1.27) on the reinforced aperture and 3.35 (±0.82) on the degraded aperture; the vehicle group achieved a response rate of 7.54 (±0.60) on the reinforced aperture and 2.77 (±0.24) on the degraded aperture. As these data demonstrate, no difference was detected in responding on the non-degraded versus the degraded apertures. Both groups exhibited similar levels of responding in terms of absolute response rate measures, as well as comparable preference for the reinforced aperture. Two-factor ANOVA determined the main

effect of nose poke aperture [$F(1,13)=30$, $p<0.001$], with no effect of group and no interactions ($p>0.05$).

The reversal learning task followed (Figure 3), in which percent response on each aperture was used to determine response preferences. The drug group showed a significant preference for the previously reinforced aperture, as established during the contingency degradation phase; 57.7 (±3.69)% of responses were directed to the previously reinforced aperture, and 19.5 (±3.99)% for the newly reinforced aperture. There were no differences in the vehicle group; as expected, mice randomly sampled both apertures, and the percent of responses were 41.2 (±6.03) for the previously reinforced aperture, and 39.8 (±6.33) for the newly reinforced aperture. Two-factor ANOVA isolated an interaction effect between group and nose poke aperture [$F(1,13)=13.46$, $p=0.001$] with no main effects. Mice also responded on the degraded aperture, however unlike the previous measures, there was no statistical difference between groups.

Extinction conditioning (Figure 4) was initiated when acquisition of the reversal learning task was established in all mice (not shown). Over the course of one week, response rates of both groups decreased. The extinction curve for both groups overlapped on every day of training. Two-factor ANOVA analysis determined no significant effect of group, as expected, and no interaction between group and session ($Fs<1$).

Postmortem oPFC dendritic spine enumeration (Figure 5) was conducted as previously described (Gourley et al. 2012, 2013). In this experiment, we included a group of mice exposed to the stress hormone corticosterone and then euthanized immediately after the 3-week treatment; this group was included as a negative control since this treatment protocol is expected to have a modest or no effect on oPFC dendritic spines. Here, a main effect of group was identified [$F(2,12)=4$, $p=0.05$]. Post-hoc comparisons indicated that the 7,8-DHF group

exhibited significantly increased deep-layer excitatory neuron dendritic spine density, while the corticosterone group did not differ from control mice. Spine counts in the 7,8-DHF group were elevated relative to both control and corticosteroid-exposed mice ($p<0.05$).The 7,8-DHF group had a density value of 1.32 (±0.17) spines/μm; the corticosterone control and the vehicle control had a density value of 0.84 (±0.04) spines/μm and 0.91 (±0.04) spines/μm respectively.

All interactions of statistical analysis not specified for each of the above quantitative studies are insignificant ($p>0.05$).

Qualitative analysis of oPFC projections using anterograde tracing techniques revealed a marked prevalence of contrast in the DS (Figure 6), indicating presence of labeled axonal terminals. Projection patterns were organized topographically: for example, lateral infusions resulted in visualized terminals in the lateral striatum. BDA staining was prevalent in central regions of the caudate-putamen complex anteriorly and medially, as well as the lateral globus pallidus posteriorly.

Discussion

Clinically, depression diminishes motivation to perform even everyday tasks, attenuates reward sensitivity, and disrupts decision-making processes essential to accomplishing goals. A classic symptom of depression is behavioral rigidity, or the inability to alter previous behaviors, as well as difficulty acquiring new behaviors (Lewinsohn, 1974). Extensive research using animal models of depression has linked depression-associated behavioral inflexibility and amotivation to a number of neurophysiological factors, including BDNF expression, function of the hippocampal-pituitary-adrenal axis, and cortico-striatal as well as corito-amygdalo projection strength and plasticity (Pittenger and Duman 2008).

While identifying therapeutic agents for depression is our ultimate motivation, our current behavioral analysis does not include animal models of depression, but rather focuses on behavioral rigidity present in a multitude of psychiatric disorders, including depression. Though previous studies have identified the role of 7,8-DHF in mediating aversive learning, ours is one of the first to examine the drug in an appetitive context. We begin by establishing that 7,8-DHF does not have an *adverse* effect on consolidating response-outcome contingencies. Contingency degradation tests (Figure 2) demonstrate similar response preferences between groups despite drug administration, suggesting 7,8-DHF does not undermine the fundamental mechanism of learning an action-outcome association. Mice were able to similarly develop response preferentially on the reinforced aperture.

Next, mice were tested in an instrumental reversal learning task (Gourley et al., 2010). We argue that the reversal learning task – as applied here – can possess a high degree of resolution in detecting whether the drug has facilitative or deleterious effects on acquiring a response-outcome contingency when it follows contingency degradation. Figure 3 demonstrates the significant preference of the drug group to maintain responding on the previously reinforced

aperture. This suggests that 7,8-DHF altered some underlying neuroplasticity to *enhance* response-outcome contingency learning and thus *increase* preference for a previous-reinforced aperture. This occurred, however, at the *expense* of sampling the newly reinforced aperture. This may seem counterintuitive to the behavioral plasticity previously associated with BDNF action, particularly given the complex relationship between BDNF expression in other brain regions and habit formation (Gourley et al. 2012). There are multiple interpretations as to why 7,8-DHF enhanced the consolidation of response-outcome contingency, but also decreased sampling and thereby hindered the development of a new behavioral strategy when the learned response failed to yield reinforcement. For example, 7,8-DHF is not selective for any single cell population, hence, a subset of neurons that may be activated by 7,8-DHF treatment is cortical interneurons that may impede or even silence baseline transmission in the oPFC-DS circuit in an exaggerated maintenance of native local homeostasis (Rutherford and Nelson 1998, Turrigiano and Nelson 2004). In all, the results presented here may be interpreted as a double-edged sword, in which 7,8-DHF enhances the strength of response-outcome learning, however at the expense of the speed of such learning.

Notably, extinction is unaffected by 7,8-DHF (Figure 4). While results from the reversal task may lead to a prediction of similarly enhanced responding on the previously reinforced aperture, it is important to keep in mind that extinction conditioning and reversal learning may not utilize the same neural pathways. For example, reversal learning in our paradigm involves the presence of a food reinforcer, only the location is altered; extinction conditioning on the other hand results in no food reinforcer for the animal. This seemingly small difference has profound impacts: Gourley et al. showed that an enhanced extinction in a genetically-modified mouse model can be nullified when alternative response options for food reinforcement were introduced (2009). We argue that the extinction results also lend validity to reversal learning results, as the increased preference for a previously degraded aperture does not stem from a

failure to extinguish response after contingency testing, and must then stem from a strengthened response-outcome relationship.

Depression

Depression is itself a difficult to define concept, encompassing etiological contributions from a number of disciplines including psychology, biology, and sociology. Currently, the most formal manifestation of the concept identifies major depressive disorder (MDD) as a form of mental disorder involving depressed mood and anhedonia; a more common use of the term refers simply to the symptom of depressed mood as characterized in MDD. Diagnostic requirements for MDD use a list of criteria outlined in the DSM-IV by the United States and ICD-10 by the World Health Organization; the criteria are identified by professional observation and self-report, and range from physical conditions such as fatigue and insomnia to psychological conditions such as loss of concentration and thoughts of death or suicide. Both depression as an underlying condition (MDD) and depression as a symptom (depressed mood) are also highly associated with a number of other mental disorders, such as anxiety, addiction, ADHD, schizophrenia, and bipolarism (Kessler et al. 1996, Hallowell and Ratey 2005) Due to the expansive degree of comorbidity, patients suffering from depression must often contend with multiple psychiatric disorders, further worsening adverse impacts on social function (Keller and Schatzberg 2007).

Exposure to stress and chemical agents that induce similar biological features in the body's response to chronic stress both result in behavioral rigidity and maladaptive decision-making (Schwabe and Wolf 2009, Gourley et al. 2012). Chronic stress exposure leads to depressed mood and lack of motivation, hallmark symptoms of clinical depression; and even acute stress exposure can disrupt reward-predictive decision-making. Evidence also exists

supporting the idea that action-outcome decision making is impaired due to cortico-striatal reorganization of neuronal circuitry and morphology in response to stressor exposure (Balleine and O'Doherty 2010, Dias-Ferreira et al. 2009, Gourley et al. in press). However, the mechanistic connection between goal-directed decision-making and behavioral inflexibility and amotivation characteristic of depression has not yet been firmly established.

BDNF

As a neurotrophin, BDNF exhibits a great variety and depth of actions associated with neuroplasticity, such as reorganizing cortical dendritic proliferation and spine density, as well as hippocampal size and function in the hippocampal-adrenal-pituitary (HPA) axis (Xu et al. 2006). Hippocampal volume has been shown to be significantly reduced, up to 10%, in patients suffering from unipolar depression (Videbech and Ravnikilde 2004). As such, given the depression-inducing and stress-resilience measures associated with hippocampal size, the hippocampus neurogenesis effects associated with BDNF expression is of great implication. Additionally, BDNF knockdown in the medial prefrontal cortex (mPFC) accounts for stress-related amotivation and increases vulnerability to prolonged stressor-related desensitization of the HPA axis, as modeled by glucocorticoid receptor (GR) blockade; furthermore, treatment with riluzole, a sodium-channel inhibitor, appears to reverse motivational deficits and restore BDNF expression in the GR desensitization stress model (Gourley et al. 2012). These results strongly promote the positive relationship between BDNF expression and behavioral flexibility.

Brunoni et al. conducted a systematic review of the literature relating to BDNF expression and MDD in 2008; the results were highly robust and suggested significant correlation between diminished BDNF expression and more severe MDD symptoms. Although mostly studied in the brain, BDNF is also present in the serum and serum concentrations have

thus been used by researchers as an indirect measure of neural BDNF expression; as such Karege et al. (2002) determined decreased BDNF in depressed patients vs healthy controls, and Aydemir et al. (2005) subsequently found increased BDNF levels after patients were subject to antidepressant treatments. Anatomical deficits of depression-like behavior in rodents include hippocampal volume reduction and decreased limbic structure activity (Warner-Schmidt and Duman 2006). Decreased BDNF expression in heterozygous knockout mice exhibits similar hippocampal atrophy. BDNF has also been linked to a multitude of other behavioral pathologies, including addiction, schizophrenia, epilepsy, and additional mood disorders; these findings are not at all surprising given the extensive comorbidity of MDD with many of the same psychiatric disorders (Gratacos et al. 2007, Koyama and Ikegaya 2005, Ren and Dubner 2007).

7,8-DHF

7,8-DHF is a small-molecule high-affinity TrkB agonist recently discovered by Jang et al. through assay of an extensive chemical library for compounds with high probability of inducing receptor dimerization and autophosphorylation. Due to its small size, 7,8-DHF can effectively reach neural tissues through systemic administration, with a peak of action roughly 2hrs after delivery. Given the poor pharmacokinetics of BDNF, such a discovery is groundbreaking in providing researchers novel and effective tools in assessing BDNF action. Not only does 7,8-DHF possess high affinity for TrkB receptor agonism, extensive evidence corroborate its action in mimicking BDNF expression. 7,8-DHF induces neuronal survival in wild type but not TrkB-deficient mice, inhibits kainic acid-induced toxicity, decreases infarct volume depending on levels of TrkB expression, and exhibits neuroprotective activity in an animal model of Parkinson's (Jiang 2010). The drug also rescues behavioral deficits of a fear conditioning mouse model in selective BDNF cortical knockouts (Choi et al. 2010). Finally, 7,8-DHF

enhances both fear acquisition and extinction, as well as rescues extinction deficits (Andero et al. 2011). Thus a great surge of evidence is present to confirm the analogous activity of 7,8-DHF to BDNF in activating TrkB signaling pathways, and emotional learning, especially in the aversive context, is similarly mediated by both molecules.

oPFC

The oPFC is a region of the frontal lobe implicated in learning, cognitive processing, reward valuation, emotion regulation, and higher-order decision-making (Murray and O'Doherty 2007). Reversal learning studies designed to assess behavioral adjustment to environmental perturbations reveal retardation of acquisition of new behaviors as appropriate in oPFC lesioned rodents, humans, and non-human primates (Hornak et al. 2004, Izquierdo et al. 2004, Bissonette et al. 2005). Reinforcer devaluation studies designed to assess action-outcome contingency learning, or the ability of an animal to associate a specific action with a specific outcome, reveal similar deficiency in inhibiting responses under devalued reinforcement conditions (Izquierdo et al. 2004). Lesions in the oPFC at any time after acquisition of stimulus-outcome associations, including post outcome devaluation similarly inhibit appropriate response inhibition (Pickens et al. 2003). Thus the oPFC is implicated in updating the expected value of rewards and integrating appropriate memory with current information, wherein lesions to the region induce a behavioral inflexibility akin to habit formation.

The oPFC is also highly associated with depression and mood disorders. Various cytoarchitectonically separate subregions of the oPFC have shown distinct effects on mood regulation: posterior lateral and medial oPFC activity is inversely correlated with depression severity, whereas anteromedial oPFC activity directly correlates with depression severity (Drevets 2007). Behavioral rigidity associated with oPFC malfunction is further corroborated by

functional and structural deficits of the region in addicted individuals (Fowler 2000). BDNF-associated neuroplasticity also mediates oPFC activity, as oPFC BDNF knockdown results in insensitivity to response-outcome contingency learning (Gourley et al., in press). Together a large amount of evidence suggests oPFC activity underlies certain fundamental mechanisms in updating and adjusting behavior as consistent with the environment.

Figure 5 demonstrates increased oPFC spine density for subjects treated with 7,8-DHF. In interpreting such findings it is important to keep in mind the oPFC deep-layer excitatory neurons imaged both project to and receive projections from target brain regions. The elevated spine density count could thus increase the sensitivity of these neurons to projections from various regions, such as the DS. This may serve to aid interpretation of behavioral findings. The oPFC is critical to adjusting and maintaining action-outcome relationships; it may be possible oversensitivity of the oPFC leads to abnormally high function in establishing response-outcome relationship, and thus strong preference for reinforced responses (Figure 3). BDNF activity has been associated with dendritic spine density: late-onset forebrain-specific BDNF knockout mice exhibited reduced spatial learning and increased behavioral markers of depression, and cortical spine density saw marked reduction up to 30% compared to wildtype mice; as such it can be concluded that sustained BDNF expression in adulthood is required for maintenance of normal dendritic spine profiles and behavior (Vigers et al. 2012). The addition of the corticosterone control offers a negative control not expected to have a proliferative effect on oPFC dendritic spines (Gourley et al. 2013), and given that the proliferative phenotype was unable to be recapitulated in this group, the significance in 7,8-DHF treatment is quite specific.

Cortico-Striatal Connections

Orbitofronal-striatal pathway abnormalities have been linked to psychiatric disorders such as OCD and addiction (Saxena and Rauch 2000, Fowler 2000). And given the importance of the oPFC and DS, cortico-striatal projections of the circuit become vital to characterize. While topographical projections from distinct subareas of the oPFC to those of the striatum have been identified, little has been revealed concerning rodent neuroanatomy. Schilman et al. conducted extensive anterograde tracing techniques from several cytoarchitectonically distinct oPFC subareas, including the medial orbito, ventral orbito, vetrolateral orbital, and lateral orbital areas to striatal areas and specifically, parts of the caudate-putamen complex (2008). Although considerable terminal field overlap exists, certain cortical areas also display preferential connections to target striatal subregions. Schilman et al. also discovered a key distinction between rats and nonhuman primate circuits in that the remaining areas of the rat accumbens contain no substantial projections (Schilman et al. 2008). This may underlie key neurobehavioral differences across species.

The connection between the oPFC and DS is similarly important in outcome-predictive relationships, and functional disconnection of the circuit inhibits behavioral adaptation to new response-outcome associative contingencies (Gourley et al., in press). The dorsomedial striatum (DMS) is implicated in establishing action-outcome, or response-outcome, contingencies; *e.g.* NMDA receptor blockade with APV inhibited outcome-predictive relationship devaluation (Yin and Knowlton 2005). The experiment was also able to localize effects to the DMS, as dorsolateral infusions did not prevent learning. Furthermore, excitotoxic lesions and muscimol-induced inactivation of posterior, but not anterior DMS yielded insensitivity to outcome devaluation and contingency degradation; however, outcome identification and instrumental action were not affected (Yin and Ostlund 2005). These findings demonstrate the importance of

areas of the DS in mediating behavioral flexibility as revealed through response-outcome predictive paradigms.

Qualitative analysis using BDA anterograde labeling discovered projections to the DS (Figure 6). The purpose of this procedure is simply to map cortico-striatal projections that have yet to be characterized in mice. Their formal identification strongly indicates that similar pathways implicated in outcome-predictive conditioning overlap between humans, nonhuman primates, rats, *and* mice.

Limitations and Future Direction

Our study succeeds as one of the first to utilize 7,8-DHF to induce TrkB agonism in an appetitive setting, as well as characterize projections from the oPFC to the striatum in the mouse. However, given the limited time-frame, many questions extending from our findings remain unanswered. Systemic 7,8-DHF administration is received by all parts of the brain, given the drug's ability to cross the blood-brain barrier; but rates of uptake and metabolism in distinct brain regions and distinct neuron subsets, as well as specific distributions of binding-densities, are unknown, and activation of additional systems that may augment or counteract the oPFC-striatum pathway is left ambiguous as well.

Systemic 7,8-DHF administration also precluded us from ascertaining which prefrontal cortical projections are being activated by the TrkB agonsim. While limiting, systemic 7,8-DHF delivery can also however be a great strength. Human treatment of neuropathologies is often best conducted through noninvasive procedures, and our findings thus provide basis for potential pharmacological applications of the drug in the human population.

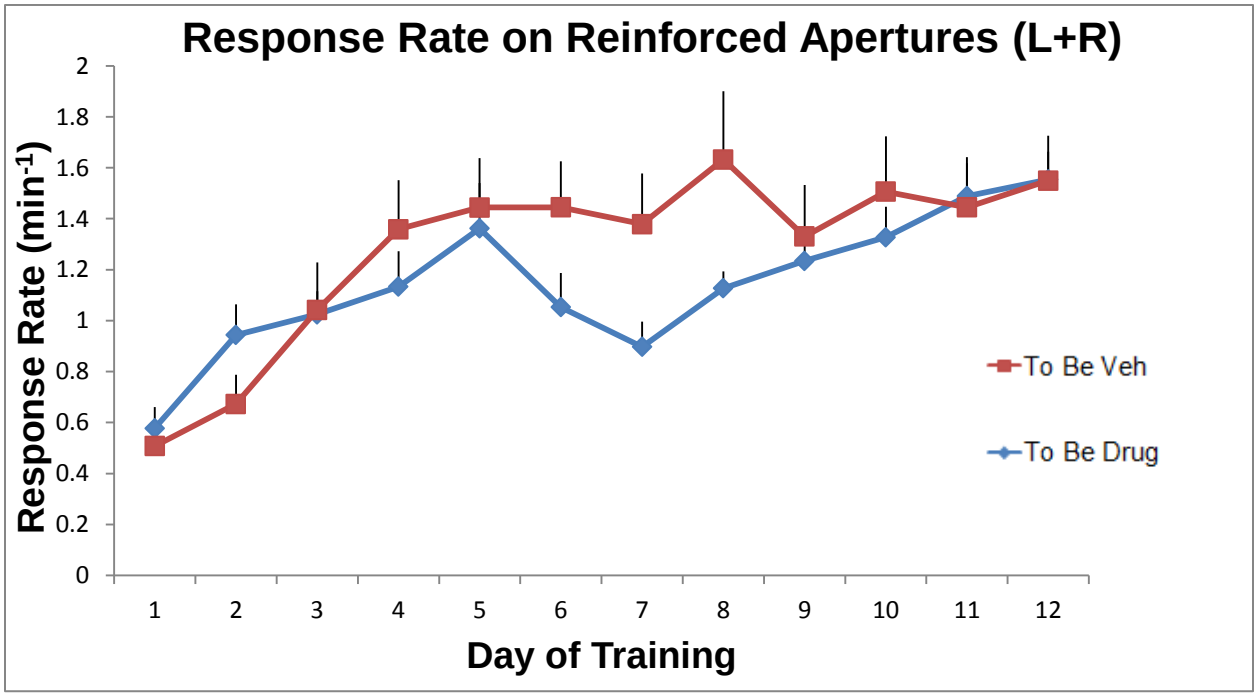
In all, our experiments pave the way for future research to better understand the pharmacokinetics of 7,8-DHF, including half-life as well as diffusion and metabolism rate. The drug may be administered to specific brain regions to determine whether it induces dendritic spine proliferation locally or downstream of the infusion region, in addition to assessing effects on distinct brain areas. Future directions may also utilize the drug in more incorporative paradigms such as animal models of depression in order to fully assess behavioral benefits.

Figures

Behavioral Testing

Figure 1: *Acquisition*

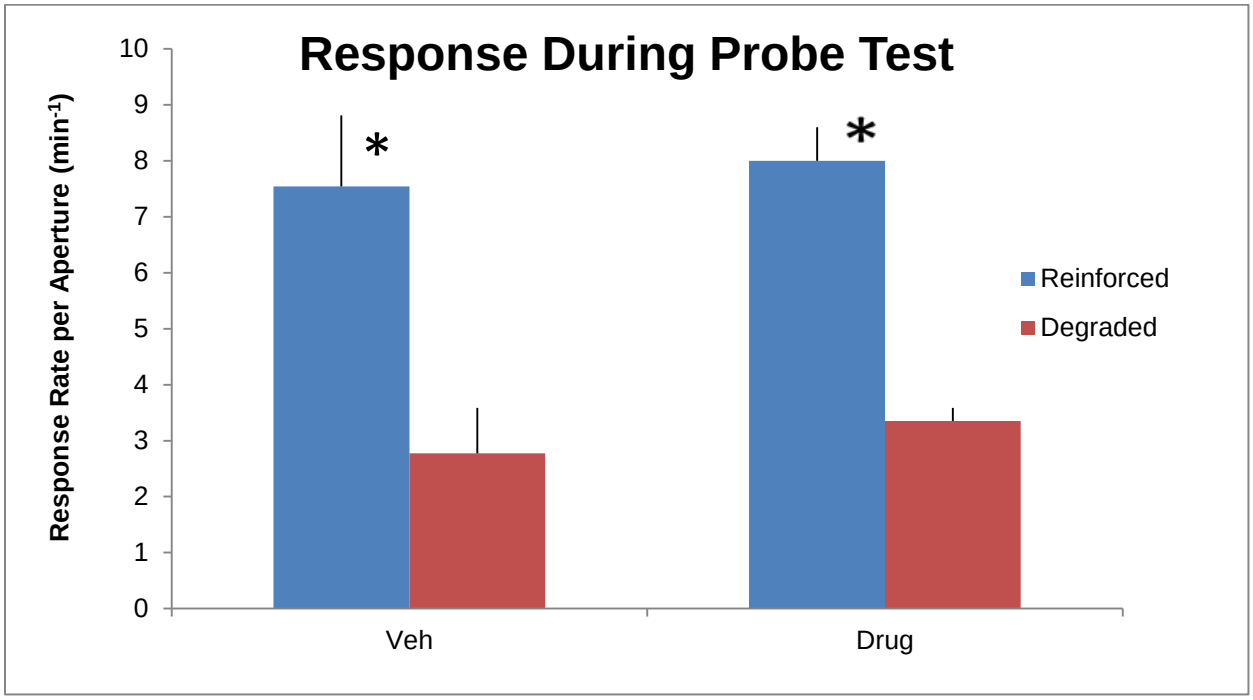
Initial instrumental response acquisition was analyzed by two-factor ANOVA; $F(1,13)=10.4$, $p<0.001$ (main effect of day).



The response rate increased, indicating acquisition of the response, with no differences between groups ($p>0.05$).

Figure 2: *Probe Test*

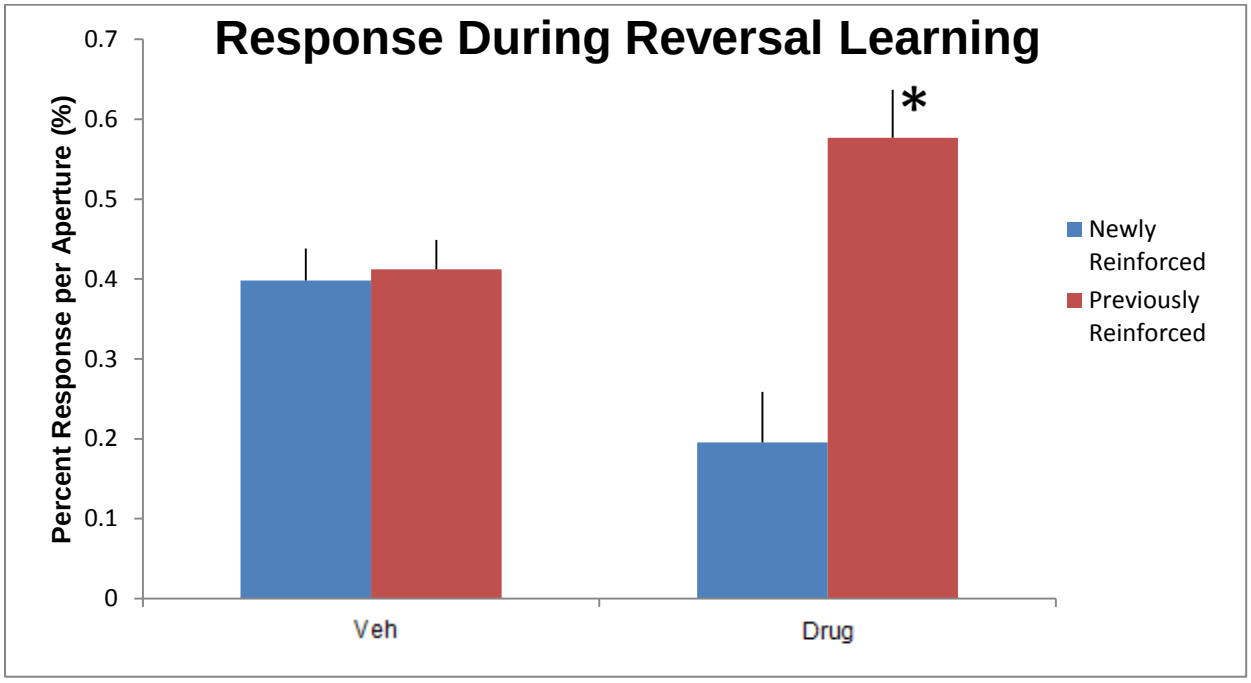
Responding after response-outcome contingency degradation was analyzed by two-factor ANOVA; $F(1,13)=30$, $p<0.001$(main effect of nose poke, no effect of group).



Both drug and vehicle group exhibited preference for the reinforced aperture.

Figure 3: *Reversal Learning*

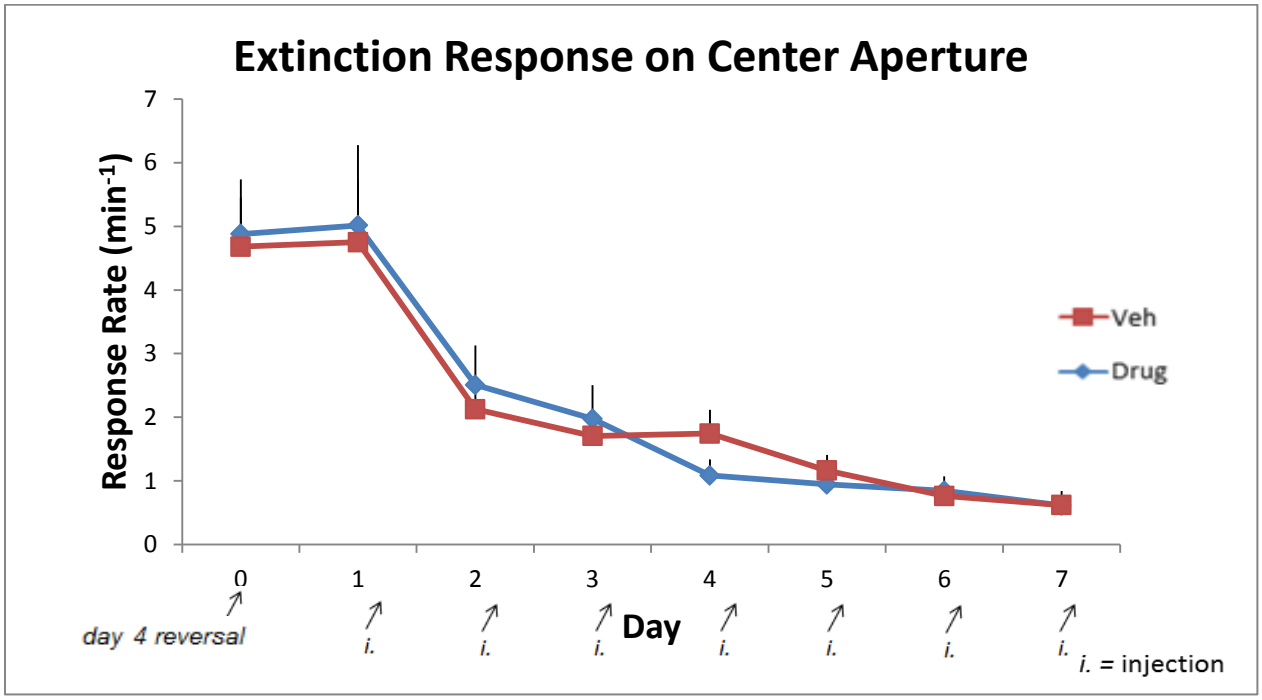
Percent response per aperture was analyzed with two-factor ANOVA; F(1,13)=13.46, p=0.001(interaction between group and nose poke).



The strong preference of the drug group for the previously reinforced aperture suggests strengthened consolidation of appetitive conditioning after response-outcome contingency degradation. (As a reminder, the newly reinforced aperture represents center aperture for all mice, while the previously reinforced aperture represents the non-degraded aperture during response-outcome contingency degradation).

Figure 4: *Extinction Conditioning*

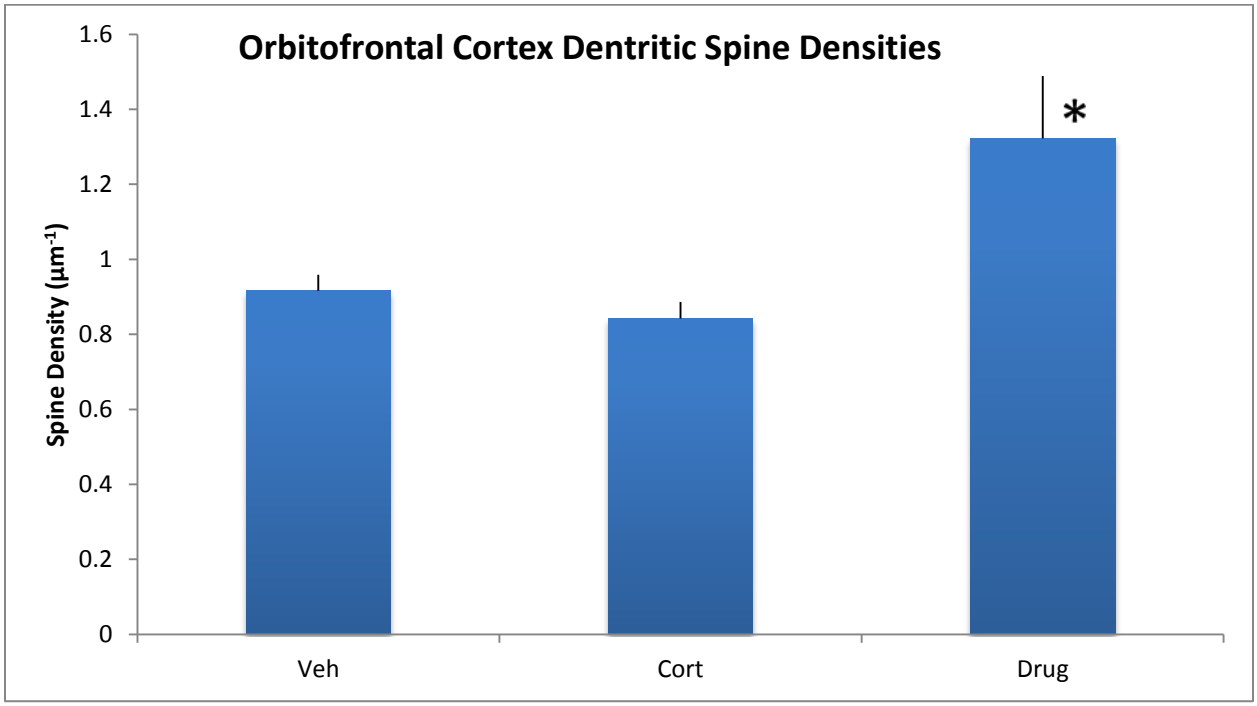
Total response during extinction training analyzed by two-factor ANOVA revealed no significant effect of group; $p>0.05$.



Extinction does not appear to be affected by drug injections. Though many interpretations are possible, this does lead to a stronger interpretation of the reversal learning data. In other words, the difference between drug and vehicle groups is likely due to stronger consolidation of a newly acquired behavior, and not due to deficiency in extinguishing a previously learned behavior. Day 0 denotes the last day of reversal learning (day 4 reversal); and response rate of both groups are comparable on this day ensuring all extinction learning originates from the same level of responding.

Figure 5: Dendritic Spine Enumeration

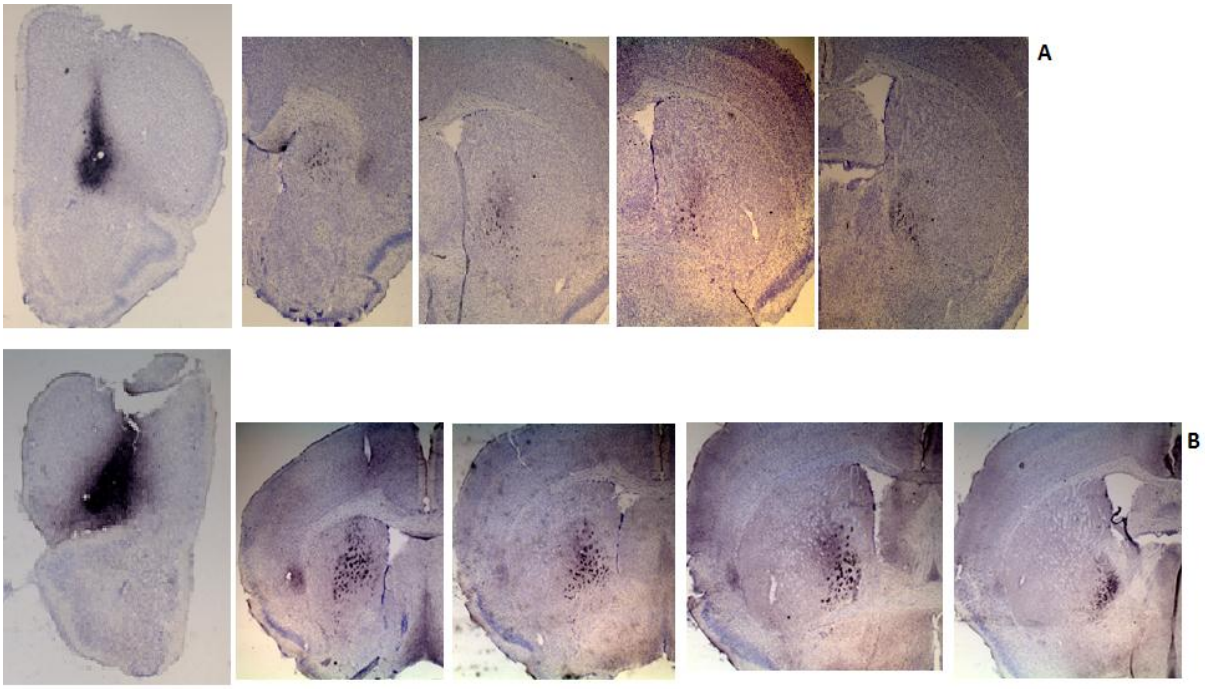
Dendritic spine densities in the oPFC were analyzed with two-factor ANOVA; $F(2,12)=4$, $p=0.05$ (main effect of group).



The 7,8-DHF (Drug) group showed significant increase in dendritic spine densities in deep-layer excitatory neurons of the oPFC. While the corticosterone (Cort) control group exhibits trending of lower density value compared to vehicle control (Veh), statistical significance was not established.

Figure 6: Tracing Studies

Staining for BDA in cortical injection sites and subsequent detection in downstream axonal terminals of the striatum.



Enlarged image at left represents BDA injection site in the oPFC. Four images on the right represent BDA labeling in downstream axonal terminals. Coronal sections are collected from most anterior to most posterior, and BDA transmission can be detected in areas of the lateral and medial striatum. Axons project ipsilaterally, where A and B represent anterograde staining in left and right hemispheres respectively.

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