

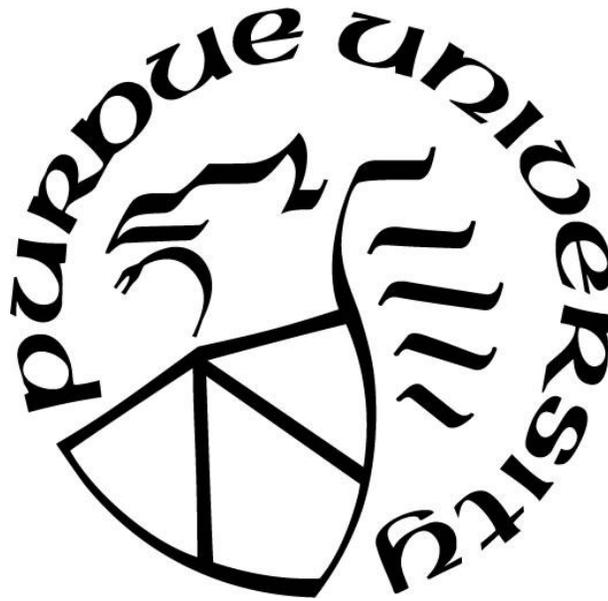
**THE ROLE OF THE MEDIAL PREFRONTAL CORTEX IN DELAY  
DISCOUNTING**

by  
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**A Dissertation**

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*Dedication:*

*To my family, whom are responsible for the person I am and all that I have done and will  
continue to do*

## ACKNOWLEDGMENTS

“No man is an island” – John Donne, Mediation XVII

I first read John Donne’s Mediation XVII at the start of Ernest Hemingway’s *For whom the Bell Tolls* when I was in high school (a library book I’m afraid to admit I still have yet to return). Whatever John Donne’s original meaning was, this poem has taken on a particular meaning for me. I am interconnected with everyone else, and owe a debt of gratitude to those around me for every way in which I am blessed. I want to express my thanks and deepest gratitude to all of the following individuals.

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## LIST OF ABBREVIATIONS

AA	Adjusting amount
ACC	Anterior cingulate cortex
AD	Adjusting delay
aPFC	Anterior PFC
BLA	Basolateral amygdala
BOLD	Blood oxygen level dependent signal
CDP	Cued delay period
Cg	Cingulate
CNDS	Competing neurobehavioral decisions systems theory
COMT	Catechol-O-methyltransferase
DA	Dopamine
DCS	Transcranial direct current stimulation
DCT	Discrete choice task
DD	Delay discounting
dHPC	Dorsal hippocampus
dIPFC	Dorsolateral prefrontal Cortex
DREADD	Designer receptor exclusively activated by designer drugs
DRT	Delayed reward task
DS	Discriminative stimuli
DTI	Diffusion tensor imaging
fMRI	Functional Magnetic resonance imaging
HPC	Hippocampus
IFG	Inferior frontal gyrus
IL	Infralimbic
IPL	Inferior parietal lobe
IOFC	Lateral orbitofrontal cortex
mOFC	Medial orbitofrontal cortex
mPFC	Medial Prefrontal Cortex

NAcc	Nucleus accumbens
OFC	Orbitofrontal cortex
PCC	Posterior cingulate cortex
PL	Prelimbic
PPC	Posterior parietal cortex
QA	Quantitative Analysis
rsFCI	Resting state functional connection intensity
SFG	Superior frontal gyrus
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
TMS	Transcranial magnetic stimulation
TTX	Tetrodotoxin
vHPC	Ventral hippocampus
vIPFC	Ventrolateral prefrontal cortex
vmPFC	Ventromedial prefrontal cortex
vOFC	Ventral orbitofrontal cortex
VP	Ventral pallidum
VS	Ventral striatum
VTA	Ventral Tegmental Area
WMI	White matter integrity
wOFC	Whole orbitofrontal cortex

## ABSTRACT

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Increased delay discounting (DD) has been associated with and is theorized to contribute to alcoholism and substance abuse. It is also been associated with numerous other mental disorders and is believed to be a trans-disease process (i.e., a process that occurs in and contributes to multiple different pathologies). Consequently insights gained from studying DD are likely to apply to many different diseases. Studies on the neurobiological underpinnings of DD have two main interpretations. The first interpretation is that two different neurobehavioral systems exist, one favoring delayed rewards (executive system) and one favoring immediate rewards (impulsive system), and the system with the greater relative activation determines choice made by an individual. Alternatively, a single valuation system may exist. This system integrates different information about outcomes and generates a value signal that then guides decision making. Preclinical investigations have steered clear of these two different interpretations and rather focused on the role of individual structures in DD. One such structure, the rat mPFC, may generate an outcome representation of delayed rewards that is critically involved in attributing value to delayed rewards. Moreover, there is evidence indicating the rat mPFC may correspond to the primate dlPFC, an executive system structure.

The current body of work set about testing the hypotheses that the mPFC is necessary for attributing value to delayed rewards and that decreasing the activity in an executive system area, and thus the executive system, shifts inter-temporal preference towards immediate rewards. To this end the rat mPFC was inactivated using an hM4Di inhibitory designer receptor exclusively activated by designer drugs (DREADD; experiment 1) or microinjections of tetrodotoxin (TTX; experiment 2) while animals completed an adjusting amount DD task. Activation of the hM4Di inhibitory DREADD receptor caused a decrease in DD, opposite of what was predicted. Electrophysiological

recordings revealed a subpopulation of neurons actually increased their firing in response to hM4Di receptor activation, potentially explaining the unpredicted results.

Microinjections of TTX to completely silence neural activity in the mPFC failed to produce a change in DD. Together both results indicate that mPFC activity is capable of manipulating but is not necessary for DD and the attribution of value to the delayed reward. Consequently, a secondary role for the rat mPFC in DD is proposed in line with single valuation system accounts of DD. Further investigations determining the primary structures responsible for sustaining delayed reward valuation and how manipulating the mPFC may be a means to decrease DD are warranted, and continued investigation that delineates the neurobiological processes of delayed reward valuation may provide valuable insight to both addiction and psychopathology.

## INTRODUCTION

### **Impulsivity, Delay Discounting, and Psychopathology**

Poorly controlled and/or conceived behavior is often described as being impulsive. However the construct of “Impulsivity” does not appear to exist as a unitary entity. Rather the term describes a constellation of related but distinct behaviors all characterized as a lack of optimal behavioral control. Different operational definitions of impulsivity, both self-report and lab task, do not inter-correlate strongly (Cyders & Coskunpinar, 2011). Furthermore in a seminal review by Evenden (1999), it was demonstrated that different behavioral measures of impulsivity are pharmacologically dissociable.

One operational definition of impulsivity is high levels of delay discounting (DD). DD refers to the rate at which individuals devalue or discredit delayed outcomes. While attributing less value to delayed rewards is normally an adaptive process, it can become maladaptive. Exhibiting a strong preference for immediate gratification over later gains and rewards, even though the immediate rewards are considerably smaller or less desirable, constitutes acting without regard for future consequences and is often detrimental in the long term. As such, increased levels of DD are considered to be “Impulsive” (Ainslie, 1975).

As evidence of increased DD being maladaptive, it has been associated with a variety of psychopathologies. Indeed Bickel et al. (2012) have described high levels of DD as “trans-disease process.” Greater discounting of delayed rewards and reinforcers is seen in case-control comparison studies for disorders such as depression (Puluc et al., 2014), schizophrenia (Heerey et al., 2007), and attention deficit hyperactivity disorder (Demurie et al., 2012; Wilson et al., 2011). It has also been linked to greater levels of social anxiety (Rounds et al., 2007). Interestingly too little DD may also be problematic. Steinglass et al. (2012) recently found that the restricting subtype of anorexia nervosa is associated with decreased DD.

Increased DD has also been repeatedly associated with addiction and is theorized to play a causal role in substance abuse pathology (Perry & Carroll, 2008). From a

conceptual perspective, individuals are choosing immediate rewards and reinforcement (e.g., rush, euphoria, escape) in lieu of greater but delayed ones (e.g. better health, financial stability, improved interpersonal relationships, etc.). Case-control comparison studies have consistently shown that substance abusing individuals exhibit elevated levels of DD. This pattern has been demonstrated across multiple classes of substances from alcohol (Mitchell et al., 2005; Petry et al., 2001; Vuchinich & Simpson, 1998) and cocaine (Washio et al., 2011) to nicotine (Bickel et al., 1999; Mitchell & Wilson 2012) and heroin (Kirby & Petry, 2004). Across studies and classes of substances, the difference in DD from cases to controls has a moderate effect size, and inclusion of nonclinical samples (e.g. heavy versus light social drinkers, recreational illicit drug users versus abstainers, etc.) does not abolish this effect (MacKillop et al., 2011). Moreover, levels of DD are positively associated with other alcohol use and risky drinking variables that are continuous in nature (Claus et al., 2011; Kollins, 2003). Longitudinal studies have shown increased DD predicts later levels of smoking (Drain-McGovern et al., 2009), and mediates the protective effects of both working memory and religiousness on later alcohol and substance use respectively (Khurana et al., 2012; Kim-Spoon et al., 2015). In sum, DD has been shown time and time again to be related to substance abuse in correlational studies, and longitudinal evidence suggests increased DD occurs before substance use.

Preclinical studies have also demonstrated that these elevated levels of DD are present in drug naïve animals before exposure and predict greater abuse liability. Higher levels of prior DD predict greater operant self-administration of cocaine and nicotine (Anker et al., 2009; Broos et al., 2012; Diergaarde et al., 2008; Koffarnus & Woods., 2011). In addition, rodent lines selectively bred for high intake and preference of alcohol show increased levels of DD (Linsenhardt et al., 2017; Oberlin & Grahame, 2009; Perkel et al., 2015; Wilhelm & Mitchell, 2008). Though discordant results for the selected lines exist (Wilhelm & Mitchell, 2012; Wilhelm et al., 2007), this disagreement is likely due to high levels of home cage intake and preference of alcohol being a pleiotropic phenotype. All of the possible gene networks underlying intake and preference may or may not be recruited in a given selection, and DD may only be influenced by a subset of those gene networks (Mitchell, 2011). The gene network underlying increased appetitive drive, is

one such candidate network. Indeed increased DD in alcohol naïve animals tracks with later seeking of alcohol, but not its consumption (Beckwith & Czackowski, 2014; Stein et al., 2015). Hence the preclinical literature builds on human correlational and longitudinal studies by demonstrating increased DD is indeed present before increased self-administration and is likely to be a genetically correlated trait with high levels of alcohol intake and preference.

There is also evidence to suggest exposure to drugs of abuse increases DD as well, but the evidence is not as congruent across different substances. In preclinical models, cocaine exposure increases DD (Dandy & Gatch, 2009; Mitchell et al., 2014; Paine et al., 2003; Roesch et al., 2007; Simon et al., 2007). Heroin and nicotine both have (Dallery & Loecy, 2005; Schippers et al., 2012) and have not (Anderson & Diller 2010; Counotte et al., 2009; Hart et al., 2011) been sufficient to increase DD with possible differences in exposure paradigms (contingent vs. non-contingent) and rate-dependent effects being possible moderators. Ethanol's effects are similarly mixed (Eviden & Ryan, 1999; Mejia-Tober et al., 2014; Olmstead et al., 2006; Pouls et al., 1998; Richards et al., 1999). Regardless, substance use causing an increase in DD is not mutually exclusive with DD causing increased abuse liability. Indeed if DD is both a risk factor and a consequence of substance abuse, then a potential positive feedback loop may be set-up. It is possible that DD confers risk for substance abuse, is increased by usage, and then confers additional risk for increased use.

There are undoubtedly more processes involved in psychopathology and addictive disorders than DD. Just to name several factors, religious affiliation, peers, and family relations are all thought to be involved in the development of substance abuse (Stone et al., 2012). That being said, it is clear DD is an important piece to the puzzle based on the reviewed correlational, longitudinal, and preclinical research. Given a disease such as alcoholism's large economic (\$223.5 billion in 2006; Bouchery et al., 2011) and public health impact (98,334 alcohol-attributable deaths from 2001-2005; CDC, 2011) multiple different approaches are warranted. Hence, developing interventions targeting DD is important, even though DD is not the end-all and be-all of addiction and psychopathology. In order to foster the development of these interventions, an understanding of the neurobiology of DD is needed. This knowledge is especially

important given the advent of new treatment strategies such as deep brain stimulation and site specific viral gene therapy. For example, LeWitt et al. (2011) used an adeno-associated virus (AAV) to overexpress glutamic acid decarboxylase (GAD) in the subthalamic nucleus of Parkinson's patients and saw improvement at 3 and 6 months without any major complications.

### **Delay Discounting as Decision Making & Mathematical Models**

While DD is formally defined as a discounting of outcome value as a function of the delay to its occurrence, it is often conceptualized and considered a form of decision making. Human and animal tasks assessing DD are based on a choice between two different events that differ in the time at which they occur. Indeed descriptions such as preferring a sooner-smaller reward over a larger-later one imply a choice. The discounted value of a delayed reward is typically expressed in units of the same commodity provided immediately. Value itself is an abstract construct often used to compare two different goods/outcomes in a common currency. A related term that is frequently used synonymously to DD is inter-temporal choice. Inter-temporal choice specifically refers to how time influences choices between outcomes which differ in time. As conceptualization and measurement of DD is so intimately intertwined to decision making, conceptualizations of decision making become pertinent.

In both psychology and economics as well as their intersection, behavioral economics, there is a rich history of studying decision making. Models of decision making generally fall into two broad categories, normative and descriptive. Normative, also known as proscriptive, models specify what an individual *should* choose (Anderson, 1990). In other words if a person is a rational agent, what choice should they make. By contrast descriptive models do not make assumptions about what factors make the best alternative. Rather as their name implies, they simply describe observed patterns of behavior (Anderson, 1990). Mathematical models of DD are no exception and can be categorized as normative or descriptive.

The earliest models of DD, or rather inter-temporal choice, were normative and derived from economics. These models are based upon a discounted utility model. The

utility<sup>1</sup> of an outcome declines at a constant rate as a function of time due to a constant hazard rate (Samuelson, 1937; Strotz, 1956; Hull, 1943). In other words, for each unit of time there is a constant probability that the delayed outcome will no longer occur. Consequently, the decline in subjective value, or utility as it is defined, decreases at an exponential rate as a function of time. The equation below is Hull's model where V is the value/utility, A is the amount, K is the discount rate, and D is the delay to the outcome.

$$V = Ae^{-KD}$$

However, simple exponential models are unable to account for "preference reversals" (Ainslie, 1975)<sup>2</sup>. When individuals are faced with a choice between two different delayed outcomes they typically will choose the larger delayed one. However, as the time to receipt comes closer and the sooner outcome essentially becomes immediate, they will change their choice to the sooner-smaller outcome. Put a different way, individuals have inconsistent preferences over time (Ainslie, 1974; Rachlin & Green 1972; Green et al., 1981). Quasi-hyperbolic models, have attempted to account for this dynamic inconsistency in choice. Laibson (1997) proposed a model where DD results from two competing systems. The first corresponds to an impulsive  $\beta$  system that discounts rewards faster than the actual decline in subjective value. This system attempts to capture the extra preference for immediate rewards via quasi-hyperbolic discounting. The second system, the  $\delta$  system, is the rational system that discounts at a slower rate than is actually observed. In its simplest form<sup>3</sup>, the model is below where u is the undiscounted value of the outcome and t is the delay (McClure et al., 2004).

$$V = \beta\delta^t u$$

One of the most prevalent models in psychology is the hyperbolic model proposed by James Mazur (1987). This is a purely descriptive model that was worked out in pigeons via testing predictions about the delays needed to equate the value of two different rewards. Mazur's hyperbolic model has since been seen to hold across species and explain more variance in DD than other equations (Ainslie, 1992; Green et al, 1994;

<sup>1</sup> The satisfaction acquired from a good or service.

<sup>2</sup> Myerson & Green (1995) make the argument that exponential models can actually account for preference reversals once one accounts for magnitude effects on the rate of discounting.

<sup>3</sup> Both  $\beta$  and  $\delta$  have been simplified.

Rachlin et al., 1991; but see Kable and Glimcher 2010; Myerson & Green 1995). Below  $V$  corresponds to the subjective value of the delayed reward,  $A$  the amount of the reward,  $k$  is the discount parameter, and  $D$  is the delay.

$$V = \frac{A}{1 + kD}$$

Ho et al. (1999) formally proposed another model that has been influential on neuroscience research, the multiplicative hyperbolic model. This model is often used in preclinical studies seeking to manipulate DD because in theory it allows one to disentangle differing effects of sensitivity to reward magnitude, probability discounting, and DD all of which may alter choice behavior. This model postulates that the value of a reward is a function of hyperbolic delay, magnitude, and probability discount functions that are multiplicatively combined. This is expressed mathematically below where  $q$  is the reward magnitude,  $Q$  is a discounting parameter for magnitude,  $d$  is the reward delay,  $K$  is the discount parameter for delay,  $\theta$  is the odds-against ratio ( $[1/p]-1$ ), and  $H$  is the discount parameter for the odds against ratio (Ho et al., 1999).

$$V = \frac{1}{1 + Q/q} \cdot \frac{1}{1 + Kd} \cdot \frac{1}{1 + H\theta}$$

Using a series of null equations based on when two rewards (A & B) have equal value, this model predicts a linear relationship between the delay to the sooner-smaller reward and the delay to the larger-later reward needed to equalize the value of the two rewards (da Costa Araujo et al., 2009; Ho et al., 1999; Kheramin et al., 2002). Below  $d_{B(50)}$  is the “indifference delay” when the larger-later reward (B) has an equal subjective value to the sooner-smaller reward (A) delivered after a given delay ( $d_A$ ).

$$d_{B(50)} = d_A \left[ \frac{1 + Q/q_A}{1 + Q/q_B} \right] + \left[ \frac{1}{K} \frac{1/(1 + Q/q_B) - 1/(1 + Q/q_A)}{1/(1 + Q/q_A)} \right]$$

In this linear relationship,  $K$ , the discounting parameter for delay, only effects the y intercept; increases in  $K$  decrease the intercept. However the sensitivity to reward magnitude,  $Q$ , effects both the slope and intercept; increases in  $Q$  cause increases in both the slope and intercept.

## Neurobiology of Delay Discounting

### Human Neuroimaging Research

The different mathematical models of DD have had a distinct influence on research into and our understanding of the neurobiology of DD. This influence is especially apparent in human neuroimaging studies using functional magnetic resonance imaging (fMRI) and psychometric-neurometric comparisons. In this literature, two system accounts are the most prominent interpretations of the neurobiology of DD, and they bear a striking parallel to Laibson's (1997) semi-normative, quasi-hyperbolic model proposing an impulsive  $\beta$  system and a rational  $\delta$  system. As such, these two system accounts posit the existence of two competing systems: a hot, impulsive system versus a cool, reflective, executive system. This concept is not new to the field of neuroscience and experimental psychology. Metcalfe and Mischel (1999) proposed that "a cool, cognitive 'know' system and a hot, emotional 'go' system" underlie performance on their delay of gratification paradigm. More specifically the balance and interaction of these two systems is critical. One system eventually wins out over the other, and as a result, behavior is either controlled or "impulsive."

These dual process approaches have been adopted to explain both addiction and DD (Bechara, 2005; Bickel et al., 2007). The competing neurobehavioral decisions systems theory (CNDS) is one recent conceptualization. This theory postulates the existence of two different neurobehavioral systems: an executive system consisting of the prefrontal cortex (dorsolateral & ventromedial; PFC), insula, and hippocampus and an impulsive system mainly comprised by limbic regions (Bechara 2005; Bickel et al., 2007). The relative activation of these two neural systems during a given decision determines the choice made by an individual. Extending to addiction, Bickel et al. (2007) make the argument that drugs of abuse alter executive system regions adversely explaining why addiction is associated with increased DD. Moreover, Bickel et al. (2007) suggest that increased DD and/or addiction may result from either an overactive impulsive system or an underactive executive system. Along similar lines, Bechara

(2005) proposed that drugs may trigger the bottom-up impulsive system and may bias or “hijack the goal-driven cognitive resources needed for the normal operation of the reflective system.”<sup>4</sup>

McClure, Laibson, Loewen, and Cohen (2004) looked for evidence of these two separate neural systems based on Laibson’s (1997) impulsive  $\beta$  and conservative  $\delta$  mathematical model of quasi-hyperbolic discounting. Using *fMRI* blood oxygenation level dependent signal (BOLD) as a measure of neural activity while participants performed a DD task, they examined neural activation across choices when either an immediate or delayed reward was chosen. The ventral striatum (VS), medial orbital frontal cortex (mOFC) and medial PFC (mPFC) were identified as showing greater activation during choices when an immediate reward was chosen and were ascribed to the impulsive  $\beta$  system. Conversely they identified  $\delta$  system areas by whether they were activated during choices regardless of the chosen outcome. Also,  $\delta$  system areas activity level had to be correlated with decision difficulty (to exclude sensory and motor processing areas). This  $\delta$  system included the intraparietal cortex, dorsolateral PFC (dlPFC), the ventrolateral PFC (vlPFC), and the lateral OFC (lOFC; McClure et al., 2004).

Next they tested whether these two systems might be competing with each other by determining if the relative activation of the two systems during a trial predicted the chosen option. Indeed when participants chose the delayed reward, the  $\delta$  system showed greater BOLD signal than the  $\beta$  system. Conversely, the  $\beta$  system showed greater activity compared to the  $\delta$  system when an immediate option was chosen (McClure et al., 2004). Individual choices tracking with the neural activation in these two systems has been taken as evidence that these two different neurobehavioral systems exist and are competing with each other (Bickel et al., 2007).

This differential pattern of activation has additional support from studies looking at the executive/ $\delta$  system. Increased BOLD signal in the left dlPFC has been independently replicated to be greater when a delayed reward is chosen (Hare et al., 2014). Also transcranial magnetic stimulation (TMS) used to disrupt activity in the dlPFC has been seen to increase the rate of DD. Although some lateralization exists as only

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<sup>4</sup> Bechara (2005) terms the cool, executive system the “reflective system.”

inhibition of the left, but not right, dlPFC increased selection of the immediate reward<sup>5</sup> (Figner et al., 2010). Transcranial direct current stimulation (DCS) of the dlPFC also affects DD. Left dlPFC stimulation/right dlPFC inhibition, but not the reverse, (i.e., anode over the left and cathode over the right) increases immediate reward selection as well (Hecht et al., 2013). Finally, increased DD is related to decreased activity in the dlPFC and IOFC after feedback in a card guessing task (Hariri et al., 2006).

There is also independent evidence supporting the hot, impulsive  $\beta$  system. In the above mentioned card guessing game, VS reactivity to feedback was positively correlated with DD rate. The activity in the mPFC, another  $\beta$  system area, displayed the same relationship with increased activity being related to increased DD (Hariri et al., 2006). Furthermore, choices that involve an immediate reward caused increase activation in the VS, anterior cingulate cortex (ACC), and mPFC. Furthermore this effect does not occur when the choice is being made for another person, suggesting the impulsive  $\beta$  system is not engaged when making choices for another individual (Albrecht et al., 2011). This is an interesting finding considering individuals tend to be less impulsive (decreased DD) when choosing for another person versus for themselves, and the rate of discounting decreases with increased social and genetic distance (Ziegler & Tunney, 2012). The  $\beta$  system not being sufficiently activated when making choices and/or valuing rewards for other individuals such that the  $\delta$  systems drives behavior could explain this phenomena.

DD has fairly clear and well replicated age dependent effects across species that can be explained by two system accounts as well. DD tends to decrease with increases in age (Eppinger et al., 2011; Green et al., 1994; Reimers et al., 2009; Simon et al., 2010). Adolescence, one particular high period for DD, is characterized by a dopaminergic overdrive in the mesolimbic dopamine (DA) system and incomplete prefrontal development (Spear, 2000). Therefore in adolescence key executive system areas may lack functionality, and impulsive  $\beta$  system areas may have increased influence on DD. In line with this hypothesis, the greater DD seen in younger individuals is correlated with increased VS activation in response to immediate rewards, and this increased VS activation was positively related to the rate of DD (Eppinger et al., 2011). This finding

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<sup>5</sup> TMS did not disrupt value judgments or affect decisions involving two different delayed rewards.

was replicated by another cross-sectional study that found age related decreases in DD are associated with decreased VS activation during a DD task (Christakou et al., 2011). Seemingly against this grain, another  $\beta$  system area, the vmPFC, showed increased activation was related to decreases in DD. However, the age related changes in DD were also associated with increased vmPFC activation coupling with  $\delta$  system areas (dlPFC, insula, and inferior parietal cortex) suggesting the development of top down control over the  $\beta$  system with age (Christakou et al., 2011). Also the increased BOLD signal seen in the vmPFC may actually correspond to neural inhibition and not activation. On a related behavior, risky decision making, adults have more activation in the vlPFC and dorsal ACC than adolescents supporting the notion of decreased executive system function during adolescence as well (Eshel et al., 2006). Combining these results suggest that age related differences in DD may be explained by an overactive hot, impulsive system and an impaired executive system in younger individuals, specifically adolescents.

In 2007, McClure et al. replicated their  $\beta$ - $\delta$  system findings and extended them. Previously participants made their choices based on hypothetical monetary rewards with a timescale of weeks (McClure et al., 2004). Now individuals chose between actually delivered primary rewards (fruit juice or water) delayed by minutes. In this new context, they again found the limbic  $\beta$  areas (NAcc, mOFC, PCC, precuneus, subgenual cingulate cortex) were preferentially activated for immediate rewards,  $\delta$  areas (PCC, posterior parietal cortex, anterior insula, dlPFC) for all choices, and the relative activation of these two systems predicted the choice an individual would make. However, when both options were delayed by an additional ten minutes, the  $\beta$  system did not appear to be recruited. Suggesting that in this case, only the  $\delta$  system weights rewards, but this is problematic to interpret via two system theories. As the two systems compete with each other and the  $\delta$  system is processing and valuing both, what is it competing with? Moreover, a direct neurometric-psychometric comparison did not yield the expected results. Specifically, the  $\beta$  term was fit to  $\beta$  system area's BOLD signal and the  $\delta$  term was fit to  $\delta$  areas BOLD signal. The fitted parameters were compared to their counterparts when the model was fit to the behavioral data. While the  $\delta$  terms from both models showed good correspondence, the  $\beta$  terms did not display such a quantitative match. Both of these findings can be interpreted away by proposing primary rewards are only given special weight via the  $\beta$

system when truly immediate and  $\beta$  system areas also perform other functions disrupting the psychometric-neurometric comparison. Never-the-less, these minor inconsistencies foreshadow a different trend in the literature.

NAcc/VS BOLD signal has been seen to track the subjective value of rewards, and not immediate reward choice. Ballard & Knutson (2009) used a DD task which presented individual components of each alternative in sequence. Combined with event related fMRI, separate BOLD signals were detected for reward delay and magnitude. Increases in delayed reward magnitude were related to increases in NAcc BOLD signal. According to the two system accounts, increases in NAcc activity should favor the immediate option. However, increases in delayed reward magnitude impart greater value, and increases in delayed reward magnitude cause discounting to occur at a slower rate (Baker et al., 2003). Furthermore, individuals who discounted at a greater rate should have greater NAcc activation. Rather these participants had the smallest increase in NAcc BOLD in relation to reward magnitude. While this makes intuitive sense that steeper discounters would have less of a neural response to a delayed reward, the two system explanations would predict this diminished response to occur in an executive system structure and not in the NAcc.

Consistent with Ballard & Knutson's (2009) findings, direct psychometric-neurometric comparisons show the NAcc/VS tracks the subjective value of the reward regardless of whether or not it is delayed. NAcc as well as left vmPFC neural activation in adolescents during a DD task is positively correlated to the subjective value of the delayed reward (Schneider et al., 2014). Interestingly, this measurement occurred in the context of a longitudinal experiment, and the same relationship was found at both time points. Moreover, same-sex parental reward inconsistency<sup>6</sup> assessed at the first timepoint not only predicted increased DD but decreased NAcc activation at the second time point. This finding suggests factors that increase the rate of DD also decrease this possible subjective value encoding of the delayed reward (Schneider et al., 2014). In adults, the VS, mPFC, and PCC have been seen to exhibit positive correlations with subjective value as well (Kable & Glimcher, 2007). Additionally, the  $k$  values obtained from fitting the

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<sup>6</sup> Parental reward inconsistency was measured by items such as "my father promises me a reward and then forgets about it" (Schneider et al., 2014).

hyperbolic equation to the behavior and the BOLD signal in each of these regions of interest (ROI), did not differ. However, when the  $\beta$ - $\delta$  model was used, none of the ROI's neural  $\beta$  or  $\delta$  terms matched with the behavioral data (Kable & Glimcher, 2007).

One critique of the above studies is that the immediate option was fixed in terms of both the time of presentation as well as its amount. Kable & Glimcher (2010) followed up and added variation in these domains, and the mPFC, VS, and PCC still showed correlations with subjective value. Specifically, BOLD signal in these areas was positively related to the absolute subjective value of both rewards, as opposed to the relative difference between the two options or only a single option (Kable & Glimcher, 2010). In separate correlations, the subjective value of both the immediate and the delayed reward was related to BOLD signal in these areas. The ROI analysis however could not determine which relationship was the strongest (i.e., BOLD with sum of absolute values, only the value of the larger-later reward, or only the value of the chosen reward; Kable & Glimcher, 2010). Finally they looked for direct evidence of separate  $\beta$  and  $\delta$  systems by seeing if the dlPFC and PPC, two  $\delta$  system areas, exhibited increased BOLD on choices where the delayed reward was chosen. They did not find the increased activity predicted by two system models, but rather activity in these regions increased with the difficulty<sup>7</sup> of the choices (Kable & Glimcher, 2010). Moreover the VS, mPFC, and PCC did not show increased levels of BOLD on immediate reward choices separate from subjective value.

These results led Kable & Glimcher (2010) to propose a single system interpretation of DD. In their "As soon as possible" single system model, they propose that rather than two competing systems there are several valuation areas that integrate different information about options. These areas then serve as a final common pathway which creates a value signal that is inversely related to delay (Kable & Glimcher; 2010). This value signal generated by a single system, then serves to guide choices.

Separate from single and two system accounts, diffusion tensor imaging (DTI) and functional connectivity studies also provide a unique insight into the biological basis of DD. Specifically they highlight that how strongly areas are interconnected may be important. Resting state functional connection intensity (rsFCI) between areas whose

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<sup>7</sup> Choice latency was used as an index of difficulty.

activation during a DD is positively correlated with reward magnitude (vmPFC, striatum, PPC, hippocampus, parahippocampus) has been seen to be directly related to DD rate. Moreover, the rsFCI between these magnitude areas and areas whose activation is negatively correlated with delay (aPFC, SFG, dlPFC, IFG, vmPFC, dmPFC, IPL) was negatively correlated with DD rates (Li et al., 2013). Also, functional coupling between the ACC and the hippocampus/amygdala predicts the degree to which episodic future event cues decrease DD (Peters & Büchel, 2010). Frontostriatal white matter integrity (WMI) has been shown to be negatively correlated with the rate of DD such that decreased WMI is related to increased DD (Peper et al., 2013). Similarly increased structural and functional connectivity between the dlPFC and the striatum, assessed via DTI and *f*MRI BOLD, is associated with decreased DD and increases in the connectivity are related to the decrease in DD with age (van den Bos et al., 2014; 2015). Hampton et al. (in press) looked to dissociate ventral versus dorsal frontostriatal white matter connectivity to DD. They found that the strength of the connection between the vmPFC and VS was related to increased DD, and the strength of the connection between the dorsal striatum and the dlPFC was similarly related to increased DD as well (Hampton et al., in press). Finally, longitudinally frontostriatal white matter development predicts future DD rate (Achterberg, 2016). Hence, the connectivity between regions, perhaps particularly the dlPFC to the striatum, has an influence on DD as well.

Two of the most commonly identified areas in both event related *f*MRI and connectivity studies are the VS/NAcc and the dlPFC. While the VS/NAcc may be implicated in encoding the subjective value of the delayed reward and a  $\beta$  system area in two system accounts, exactly how the dlPFC is been implicated has varied from study to study. It was identified as a  $\delta$  system area by McClure et al., (2004; 2007). Disruption by TMS and DCS has increased DD (Finger et al., 2010; Hecht et al., 2012). Ballard & Knutson (2009) founds its activity level to be inversely correlated with delay, and Kable & Glimcher, (2010) found its activity tended to increase with increases in choice difficulty. Its connectivity to the VS/NAcc is also related to DD (Li et al., 2013; Van den Bos, 2014; 2015; Achterberg, 2016). Never-the-less the precise role of the dlPFC in DD is not certain based on these studies beyond that it appears to be important for delaying gratification and is a “cooler” area involved in executive functions.

Additional insight into the role of the dlPFC may be provided by studies examining variation in catechol-O-methyltransferase (COMT) genotype in relation to DD. One common single nucleotide polymorphism, rs4680, is a G to A substitution that results in a valine (val) as opposed to a methionine (met) at codon 158 (val158met). The val allele is associated with increased metabolism of DA and higher protein levels of COMT (Chen et al., 2004). As COMT is responsible for the majority of DA clearance in the PFC, the val158met substitution results in large differences in the levels of PFC DA (Käenmäki et al., 2010). The val allele, which should result in a hypodopaminergic state in the PFC, and thus the dlPFC, is associated with increased DD. Boettiger et al. (2007) found that individuals homozygous for the val allele showed increased levels of DD, and moreover during the DD task these individuals showed increased BOLD signal in the dorsal PFC. This effect was later replicated with individuals heterozygous for the val allele displaying an intermediate phenotype (i.e., increased DD was associated with an increased val allele count; Gianotti et al., 2012). Furthermore, dorsal PFC (BA 9/10) resting state electroencephalogram (EEG) measurements (beta3 activity) were related to both DD and val allele count, and a mediation analysis found the resting state activity fully mediated the relationship between COMT genotype and DD. Taken together, these results suggest possession of the high activity val allele leads to a hypodopaminergic state in the dorsal PFC that causes increased DD.

However the exact opposite result has been observed as well; the met/met genotype has been associated with increased levels of DD (Paloyelis et al., 2010). Critically, this observation was in adolescent boys. Subsequent studies found that age moderated the effect of COMT genotype on DD (Smith and Boettiger, 2012). Adolescent val/val homozygotes show decreased DD compared to met/met homozygotes and val/met heterozygotes. In adulthood this pattern is reversed with the val/val homozygotes showing increased DD compared to the other genotypes. Smith and Boettiger (2012) interpreted these results in the context of an inverted U dose response function for DA in the PFC. The high activity val allele normalized the high levels of mesocortical DA seen in adolescence (Spear, 2000), but in adulthood the val/val genotype resulted in too little DA. Conversely, the met allele carriers had experienced an excess of dopamine in adolescence, but the optimal amount in adulthood.

Finally, administration of tolcapone, a COMT inhibitor, decreased DD having the greatest effect in impulsive individuals (Kayser et al., 2012). Tolcapone also increased BOLD signal in the dm- & dlPFC and decreased BOLD signal in default mode network cortical areas. The tolcapone mediated decrease in DD was inversely correlated with the change in BOLD signal in the striatum and anterior insula. Some caution is warranted as COMT is not solely expressed in the PFC. However, EEG and fMRI results support PFC, specifically dorsal PFC, involvement in COMT's relationship with DD coupled with downstream effects in areas such as the striatum (Gianotti et al., 2012; Boettiger et al., 2007; Kayser et al., 2012).

### **Preclinical Investigations**

Preclinical investigations of the neurobiology of DD have looked at several interconnected areas including the NAcc, OFC, mPFC, and the basolateral nucleus of the amygdala (BLA). Unlike human neuroimaging studies, the involvement of a single structure is typically examined in isolation. What is lost in being able to examine the entire human brain simultaneously is made up for by the use of experimental as opposed to correlational designs and more precise measures than *fMRI* BOLD.

Preclinically, DD is examined using various discrete choice<sup>8</sup> tasks (DCT) where subjects make individual distinct choices between two alternatives (as opposed to having two concurrently available schedules that one can switch back and forth between at will). They are typically set up on a trial by trial basis where subjects are presented with two different (free choice) or 1 (forced choice) response options that yield a specific outcome when completed on each trial. Completion of one response, precludes execution of the other. One key implication is that with the use of an inter-trial interval animals are not able to earn a greater relative rate of reinforcement by earning multiple sooner-smaller rewards in the span of time it would take to earn a single delayed reward.

In rodents there are five main DCT paradigms, although others exist, that are commonly used to measure DD. One of the first means to assess DD utilized a T-Maze. One arm of the "T" always contained a small reward available immediately and the other

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<sup>8</sup> In the current document a discrete choice is defined by when subjects are forced to choose between a finite set of alternatives in a trial by trial basis.

a large reward delivered after a delay. The delay to the large reward is typically implemented by detaining rats in the delayed arm using guillotine doors prior to reward delivery. Several trials are given per day. After several days the delay to the large reward is increased. The main dependent variable is percent choice of each arm. Greater choice of the small reward arm when delays are present on the larger reward indicate increased DD.

The adjusting delay (AD) paradigm was developed by James Mazur (1987). It is a psychometric titration procedure that derives an “indifference delay” for two rewards that differ in magnitude. On one lever (or response key as was the case for Mazur) a small reward is delivered immediately with no delay. On the other lever a large reward is delivered after a variable delay. The delay to the larger reward is adjusted based upon the subject’s choices. Forced choice trials, in which only one lever has any programmed consequences, are used to ensure subjects are acquainted with the changing delays. The sessions are divided into 4 trial blocks, 2 forced choice trials (one on each lever) followed by two free choice trials. If a subject chooses the larger delayed reward twice in a block, the delay is increased by one second in the next block of trials. If they choose the immediate reward twice it would decrease. Across one session to the next the delay is carried over. Once there is no change in the mean adjusted delay (MAD) across sessions, the MAD is inferred to be the delay to the larger reward necessary to equalize the value of the two rewards (i.e., the indifference delay)<sup>9</sup>. Longer MADs correspond, to decreased DD as longer delays are needed to sufficiently discount the value of the larger reward to achieve indifference.

In 1996, Evenden and Ryan described a different operant DD procedure wherein several delays would be utilized within a session. On each trial the rat responded on either a lever associated with one pellet delivered immediately or 3 or 5 pellets (pending experiment) delivered after a delay. The delay to the large reward started at 0 and it increased every 12 trials. In this fashion each session consisted of 5 blocks of 12 trials each with a different delay. The principle variable of interest was percent choice of the delayed reinforcer with decreased percent choice of the delayed option corresponding to

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<sup>9</sup> Note: for acute pharmacological investigations typically only a single session’s MAD score is used versus an average of several sessions.

increased DD. Also, an indifference delay can be derived via interpolation. One notable procedural variation of this paradigm is to decrease the delay across blocks versus increases them (i.e., start at a 60 second delay and work to 0). Later studies included 2 forced choice trials at the start of each block, one on each lever, to signal the changing delays. This task is often referred to as the delayed reward task (DRT).

A consideration with the DRT is within session carry over effects and the influence of increased behavioral perseveration. Increased behavioral perseveration would in theory cause the rat to continue to respond on the initially more reinforcing outcome in spite of the changing delay. These concerns and the effect they can have is demonstrated by Tanno et al. (2014) who found the effects of systemic amphetamine administration on a DRT depended on the order of delay presentation (ascending versus descending). Specifically, when ascending delays were used systemic amphetamine decreased DD. Conversely when descending delays were used, systemic amphetamine increased DD. Tanno et al. (2014) interpreted these results as amphetamine increasing behavioral perseveration on the initially more valued option (the delayed reward in ascending delays and the immediate reward with descending delays). However, the exact nature of the increase in behavioral flexibility needed to cause such a false positive is not clear. Lesions of the vHPC cause increased perseverative responding on a 5 choice serial reaction time task, but also increased DD on a DRT (Abela & Chudasama, 2013; Abela et al., 2012). Regardless, interpreting any decrease in DD or null result when only ascending delays are used is extremely difficult. An observed change in choice behavior may, or may not, actually be a result of underlying processes presumed to govern DD. Put another way, when only ascending delays are used (which is majority of cases) the DRT is susceptible to false positives for decreases in DD and false negatives for having any effect<sup>10</sup>.

Considering the converse situation, decreased behavioral perseveration, additional factors come into play. It could be argued that decreasing behavioral perseveration would cause increased DD in a DRT with ascending delays as subjects would be better able to track the changing reward values. However this argument assumes that under baseline

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<sup>10</sup> The vast majority of studies only use ascending delays. Unless it is specifically stated otherwise in this document, ascending delays are implied.

conditions without pharmacological manipulations, subjects are not already tracking the reward values optimally. While this situation is possible, it is not likely. The AD paradigm, in which perseveration is not a major concern, shows concurrent validity with the DRT suggesting that the DRT does provide valid measurement under baseline conditions. The concurrent validity of these two paradigms is evidenced by indifference delays derived from these two paradigms (derived via interpolation in a DRT and by MAD scores in an AD paradigm) inter-correlating very strongly (Spearman's  $\rho = .71$ ; Craig et al., 2014). Accordingly, at least under baseline conditions the DRT may not be confounded by behavioral perseveration.

The adjusting amount (AA) DD task was developed by Richards et al. (1997) and is akin to the AD procedure in that it utilizes a titration schedule. However as its name indicates the magnitude/amount of the immediate reinforcer is adjusted as opposed to the delay. On each trial the subject chooses between one response yielding a standard reward after a delay and an adjusting alternative reward delivered immediately. If the subject chooses the standard reward, the adjusting alternative reward's magnitude adjusts up on the next trial and vice versa if the adjusting alternative reward was chosen. The exact degree of titration varies from lab to lab, some utilizing a percent of the adjusting alternative reward and others using a fixed amount. The median adjusting amount of the last 20-30 trials is taken as a measure of indifference. These indifference points are taken as a measure of the subjective value of the delayed standard reward in units of the immediate reward. Lower indifference points correspond to increased DD as subjects are willing to settle for less immediate reward in lieu of the delayed reward.

The quantitative analysis (QA; Kheramin et al., 2002) method is not really a different paradigm. Rather it is a different approach to examining and analyzing DD data with a small, but meaningful procedural variation. The QA approach uses either a DRT or AD task to derive a series of indifference delays to the larger-later reward as a function of the multiple different delays to sooner-smaller reward. Then the QA paradigm applies the multiplicative hyperbolic model<sup>11</sup>. The relationship between the delay to the sooner-smaller reward and the indifference delay to the larger-later reward is linear with its slope affected by  $Q$  (magnitude sensitivity parameter) and its y intercept by both  $K$  and  $Q$ .  $K$  is

<sup>11</sup> Discussed above under Delay Discounting as Decision Making & Mathematical Models.

inversely related to only the intercept, and  $Q$  is directly related to both the slope and intercept. Differences in the slope and intercept of the indifference delays (in addition to fitting the model directly) are then used to make inferences about the rate of DD. In this fashion one is able to disentangle sensitivity to reward size from sensitivity to reward delay.

There are also variations within and across the different methods of assessing DD in rodents that are important to consider. The lengths of the delay to the larger reward varies wildly across (and within) the different paradigms from several seconds (Acheson et al., 2006) to over a hundred (Pardey et al., 2012; Bezzina et al., 2007). Different delay lengths may require different processes and recruit different neural structures to delay gratification. Also, there is variability in what cues are used. Some studies use explicit discriminative stimuli (DS) to signal the available options others do not.

Using a cued delay period (CDP) has also been shown to moderate the effect of manipulations on DD and affect the rate of DD in general (Zeeb et al., 2010). A CDP is when an explicit stimulus is presented after the selection of the delayed alternative and remains until the reward is delivered. For example, after pressing the delayed reward lever, a stimulus light comes on or flashes until the delayed reward is presented. Increasing the delay between response and reinforcement decreases the strength of the resulting response-reward association. Signaling the duration of the delay with a cue can facilitate learning and increase the effective value of the delayed reward, as the cue acquires some of the affective properties of the reward and may act as a conditioned reinforcer (Mazur 1997; Williams and Dunn 1991). Indeed it has been proposed that animals are actually choosing between the immediate reward and a conditioned reinforcer (Mazur, 1997).

Each of these methods and procedural variations has their own strengths and weakness. The DRT allows one to test all delays inside a single session easily allowing one to see if an effect only occurs at specific delays or causes animals to no longer value the larger reward when there is no delay. However this is also its major limitation as impairments in animals' ability to adapt to these within session shifts can lead to false positives for decreasing DD (Tanno et al., 2014). The AA and AD procedure do not have this drawback, but they only assess one delay for acute manipulations and make strong

assumptions about animals' ability to track changing reward values on a trial by trial basis. The QA method can provide additional information, but it takes longer to complete by orders of magnitude as one is essentially repeating the other paradigms multiple times. In lesion studies this can raise concern about the development of compensatory mechanisms, and it precludes the use of acute pharmacological manipulations.

However, given the differing limitations of each, converging evidence from multiple different paradigms is particularly valuable. Fortunately, studies looking into the concurrent validity of these tasks suggest that they are measuring the same construct. As was mentioned before, the indifference delays derived via an AD and DRT in the same rats correlate very strongly with each other (Craig et al., 2014). Also the AA and AD tasks show convergence on the same construct as well. Both the AA and AD procedures are equally well fit by Mazur's (1987) hyperbolic equation, and yield values of the discount parameter ( $k$ ) that are not different when derived in the same subjects using both paradigms (Green et al., 2007). As the different paradigms display concurrent validity with each other and have their own unique caveats, the ideal evidence of a change in DD is congruent evidence across multiple different paradigms. In this fashion, the caveats of an individual task can be excluded as an alternative interpretation.

The following sections review the brain site specific investigations of DD. The overall goal is to attempt to distil the structures and connections important for DD and what role they may subserve. While some of these studies examine the role of a neurotransmitter system in a specific structure in DD, the overall roles of a given neurotransmitter system will not be examined here. While they are undoubtedly important and influential, comprehensively reviewing the literature on systemic neurotransmitter manipulations would be a separate massive undertaking fraught with its own challenges. Accordingly, such a discussion has been deemed to be beyond the scope of the current document.

### *Nucleus Accumbens*

The NAcc has long been considered an interface between the limbic and motor system (Morgenson, 1980) in addition to proposed roles including reward processing and associative learning (Wise, 1980; 2004; 2005), incentive salience attribution (Berridge &

Robinson, 1998; Berridge, 2007), and invigorating and directing behavior (Cardinal et al., 2002). It has also been put forth that the NAcc represents different outcomes simultaneously and acts as a mechanism of action selection (Nicola, 2007; Floresco, 2015). The NAcc's limbic afferents and direct and indirect efferent connections to the basal ganglia and brain stem motor nuclei are central to the NAcc acting as a limbic motor interface involved in action selection (Basar et al., 2010; Floresco, 2015; Nicola, 2007).

The NAcc receives inputs from a number of different areas and is situated to serve as an integration site. The core subregion has afferent connections with the anterior end of the BLA, dorsal prelimbic cortex, cingulate cortex, parahippocampal cortex, as well as the caudal midline and intralaminar nuclei of the thalamus (Berendse et al., 1992; Berendse and Groenewegen, 1990; Brog et al., 1993; Wright et al., 1996). The shell of the NAcc is innervated by the posterior BLA, infralimbic cortex, ventral prelimbic cortex, hippocampus (subiculum & CA1), ventral tegmental area (VTA), and dorsal raphe (Berendse et al., 1992; Brog et al., 1993; Heidbreder and Groenewegen, 2003; Berendse and Groenewegen, 1990; Wright et al., 1996; Groenewegen et al., 1987; Ikemoto, 2007; Berridge et al., 1997).

In contrast to its inputs, the NAcc tends to project to the basal ganglia and other subcortical structures. The NAcc core's efferent connections include the ventral pallidum (VP), endopeduncular nucleus (human Globus pallidus interna), and the substantia nigra pars reticulata (SNpr; Deniau et al., 1994; Haver et al., 1990; Heimer et al., 1991; Groenewegen and Berendse, 1990; Alexander et al., 1990; Ferry et al., 2000; Groenewegen et al., 1993; Groenewegen et al., 1996; Zaham and Brog, 1992). The shell projects to the VP as well, but also the lateral hypothalamus, pedunclopontine nucleus, VTA, and SN pars compacta (SNpc; Groenewegen et al., 1993; Groenewegen et al., 1999a; Groenewegen et al., 1996; Zaham and Brog 1992; Gerfen, 2004).

Interestingly, the VP projects to some the same regions as the NAcc, namely the endopeduncular nucleus, VTA, SNpc, SNpr, and lateral hypothalamus (Nicola, 2007). As a result, activation of NAcc projections, which are GABAergic MSNs (Chang & Kitai, 1985; Ikemoto et al., 2015; Preston et al., 1980), can directly inhibit areas, or disinhibit them via inhibiting the VP's GABAergic projections (Ikemoto et al., 2015; Nicola, 2007;

Zahm, 1987; 1996). This potential parallel processing of information and inhibition/disinhibition mechanism is central to Nicola's (2007) conceptualization of NAcc's potential role in action selection. It is important to note that subserving this function is not necessarily incompatible with other proposed functions for the NAcc.

Looking at DD specifically, a number of studies report core lesions increase DD. One of the earliest of these reports was by Cardinal and colleagues (2001). Animals completed a DRT without DS or a CDP and with ascending delays. Excitotoxic lesions decreased selection of the large reward compared to sham lesioned animals (Cardinal et al., 2001). However the lesions did so at all delays, with the mean selection of the larger reward at just above 25% when no delay was present. While omitting or including all delays on alternate sessions did show NAcc core lesioned animals were still sensitive to delay, it did not restore a preference for the larger reinforcer with no delay. Consequently while an increased selection of the immediate reward was present, animals' ability to discriminate between rewards of different magnitudes may have been impaired by the NAcc core lesions.

Cardinal and Cheung (2005) followed up on whether NAcc core lesions affect sensitivity to reward magnitude. They tested whether excitotoxic NAcc core lesions would affect generalized matching behavior to two concurrent random interval 60 schedules. The schedules differed only in the magnitude of the reinforcer. While both groups undermatched, NAcc core lesioned rats more closely approximated perfect matching behavior compared to sham animals. Cardinal and Cheung (2005) inferred based upon this finding that NAcc core lesioned animals actually were more sensitive to reward magnitude. However, this finding of increased sensitivity to reinforcer magnitude conflicts with their earlier study (Cardinal et al., 2001) where animals displayed lack of preference, nearing an aversion, for a larger magnitude reward versus a short one when no delay was present. Their interpretation also downplays the possibility that core lesioned animals may process reward magnitude differently than sham animals.

Other studies have followed up on and attempted to account for this possible change in the sensitivity to reinforcer magnitude seen by Cardinal et al. (2001). These experiments have utilized the QA paradigm with either a shortened version of the DRT or an AD task to derive indifference delays. Bezzina et al., (2007) used an abbreviated DRT

with a CDP, and varied the delay to the receipt of the sooner-smaller reward. Excitotoxic lesions of the NAcc core decreased the intercept, but not the slope, of the indifference delays as a function of the delay to the sooner reward. This result indicated that the discount parameter for delay,  $K$ , but not magnitude,  $Q$ , was affected by the lesion. Specifically, the lesion increased the rate of DD. da Costa Araújo et al. (2009) used the QA method with an AD paradigm to derive indifference delays while they varied the magnitude of the sooner-smaller reward. They found that the ratio of the indifference delays between different magnitudes of the sooner-smaller reinforcer were unchanged between an excitotoxic lesion and sham lesion group, but the overall level of the indifference delays was lower in the lesioned animals. The multiplicative hyperbolic model predicts that this change occurs when the sensitivity to magnitude is unaffected but, DD is increased (da Costa Araújo et al., 2009). The same group of researchers also subsequently replicated<sup>12</sup> this effect (Valencia-Torres et al., 2012). As a whole, the research with the QA paradigm indicates that excitotoxic lesions of the NAcc core increase DD but do not affect sensitivity to magnitude.

Galtress & Kirkpatrick (2010) also found evidence that the NAcc core is involved in DD with a choice link procedure. In their choice link procedure, the first link had animals chose the schedule they would respond on with an FR1. The two options were a FI60 or a progressive interval (PI) schedule. The PI schedule started at 0s and increased by 15s with every selection of the PI schedule. Once the animal chose the FI60s schedule the PI interval reset to 0 seconds. The main dependent variable was the “changeover time,” the mean interval on the PI schedule at which animals chose the FI60 schedule. They found that when the FI60 schedule was reinforced with 4 pellets and the PI schedule with 1, animals with an excitotoxic lesion of the NAcc core had an increased changeover time. This result suggests increased DD because a longer delay to the 1 pellet reward is needed to equalize the value of the 4 pellet reward which has been discounted to a greater extent due to the longer delay<sup>13</sup>.

<sup>12</sup> Valencia-Torres et al. (2012) was an almost exact replication of da Costa Araújo et al. (2009). They changed the amounts of reward magnitudes such that there were specific proportional relationships between the sooner-smaller and larger-later rewards. This allowed them to simplify some of the null equations and test additional mathematical predictions that are beyond the scope of this document.

<sup>13</sup> Assuming the quasi-hyperbolic model of Mazur (1987),  $k=.1$ , a fixed delay of 60 seconds to a reward with a magnitude of 4, and an alternate reward magnitude of 1, a delay of 7.5 seconds to the

Galtress & Kirkpatrick's (2010) also used an incentive contrast procedure to examine if animals ability to process reward magnitude was disrupted. In this task, animals responded on two different levers each reinforced with a single sucrose pellet on a VI30s schedule. Only one lever was extended into the chamber at a time in a pseudo-random order and there were no explicit DSs. After animals exhibited a stable baseline and equal levels of responding for each lever, one lever, the induction lever, had its reinforcer increased to 4 pellets. The other lever's reinforcer stayed at 1 pellet (contrast lever). NAcc core sham lesioned animals showed the predicted increase in responding on the induction lever and a decrease on the contrast lever. However, core lesioned animals only showed the decreased responding on the contrast lever. These results suggest, at least to some degree, that core lesions disrupt normal processing and/or adaptation to reinforcer magnitude.

To avoid potential problems with magnitude perception, Pothuizen and colleagues (2005) used a choice procedure that had rewards that differed in terms of both delay and probability (as opposed to magnitude). One option provided continuous reinforcement that was associated with a delayed 1 pellet reward, and the other option yield partial reinforcement,  $p(\text{reward})=.25$ , for an immediate 1 pellet reward. The delay was changed across days (2-5 days at each delay) in a pseudorandom order with 0 second delay conditions in place before and between all other delays. Before training animals received excitotoxic NAcc core, shell, or sham lesions. Initially, no differences were seen under any-delay condition. However, after extended re-testing at the longest delay, core

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alternate reward is needed to equalize the value of the two rewards (to three digits to the left of the decimal place).

$$V_1 = \frac{4}{1 + .1(60)} = V_2 = \frac{1}{1 + .1(7.5)} = 0.571$$

Conversely when  $k=.4$ , a delay of 13.1 seconds to the alternate reward is needed to equalize the reward values.

$$V_3 = \frac{4}{1 + .4(60)} = V_4 = \frac{1}{1 + .4(13.1)} = 0.16$$

As a result, the relationship between  $k$  and the change over point in Galtress & Kirkpatrick's (2010) choice link task takes on a positive decelerating function. This pattern, at least mathematically, occurs because as  $k$  increases it discounts both the smaller and larger rewards faster hence while the larger-later reward is discounted more and needing more of a delay, smaller reward is discounted more and more needing less of a delay to equalize the values. Consequently, at higher levels of  $k$  the power to detect differences with this procedure is actually substantially reduced.

lesioned animals developed a greater preference for the immediate reward relative to sham lesioned animals. This result may suggest that core lesions increased DD<sup>14</sup>, and critically this was observed when the two rewards had equal magnitudes as core lesions may disrupt animals' ability to distinguish reward magnitudes.

Alternatively, Pothuizen and colleagues' (2005) core lesioned animals may have slowly extinguished delayed lever responding. This interpretation is supported by the deficit only being seen with extended testing, and Cardinal and Cheung (2005) found that NAcc core lesions impaired learning a response with a delayed, but not immediate, reinforcer. Specifically pretraining NAcc core excitotoxic lesions impaired acquisition of and rate of responding (ROR) for delayed rewards in a free operant schedule with no explicit cues. Post-training lesions disrupted responding for a delayed, but not immediate, reinforcer, and responding for a delayed 20 second reinforcer was no longer different from responding on the inactive lever.

While the majority of studies have found disrupting the NAcc causes an increase in DD, two have found the opposite effect. In a DRT with DS but no CDP, partial inactivation of the NAcc core with a low dose of microinjected baclophen and musimcol resulted in increased preference of the delayed reward (Moschak & Mitchell, 2014). Interestingly this effect was driven by individuals who were already low discounters at baseline. Steep discounters displayed no change. The second study to find an increase in DD characterized the effects of complete (core and shell) NAcc excitotoxic lesions. In an AA task with both DS and a CDP, post-training lesions increased indifference points at a delay of 8 seconds (but not 4), and increased k values (Mazur, 1987) indicative of decreased DD (Acheson et al., 2006).

Acheson et al.'s (2006) study also found that whole NAcc lesions decreased the model fit ( $R^2$ ) for Mazur's (1987) hyperbolic equation. Concerned that the lesion disrupted animals ability to adapt to changes in delay, they then retrained animals at a delay of 4 seconds and the switched them to a delay of 2 or 8 seconds. In this test, they

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<sup>14</sup> One potential caveat to this interpretation is whether core lesions alter probability discounting. Acheson et al. (2006) did not find whole NAcc lesions affected discounting. Cardinal & Howes (2005) found core lesioned animals to be risk averse. If the NAcc core is necessary to promote choice of uncertain rewards, it adds strength to Pothuizen et al.'s (2005) findings as lesioned animals developed a preference for the immediate reward based on delay aversion despite an increase in probability discounting.

found that lesioned animals had greater indifference points at the 8 second delay. Acheson et al., (2006) argue that this was evidence of lesioned animals being less sensitive to changes in delay. However, they did not observe the corresponding result with a delay of two seconds (decreased indifference points). Indeed, this test truly only replicated their prior results of no difference at the 4 second delay and increased indifference points at the 8 second delay. Consequently, there may not be sufficient evidence to assuredly make the inference that animals were less sensitive to changes in delay as a decrease in DD is still a plausible explanation for their results.

Two other studies have also attempted to assess if lesioning the NAcc core makes animals less able to adapt to changing delays. da Costa Araújo et al. (2009) and Valencia-Torres et al. (2012; discussed above) used a Fourier transform on the adjusting delays in an AD task to conduct a power spectrum analysis. If animals ability to perceive and adapt to the changing delays was impaired, then they would continually “over and under shoot” during their titration of the adjusting delay. In theory, this in turn would lead to more trial blocks per oscillation in adjusting delays and a greater peak to trough difference in oscillations of the adjusting delay. Thus if animals’ ability to adapt to and perceive changing delays was impaired, then the both dominate frequency band and its power would be expected to be greater. However, they found that the dominate frequency band, and the power in said band, did not differ as a function of the lesion group suggesting animals ability to adapt to the changing delays was not impaired.

Dopamine neurotransmission in the NAcc appears to be involved in DD as well. Day et al. (2010) performed fast scan voltammetry in the NAcc core and shell while animals completed a DCT where stimulus lights cued the available rewards for 5 seconds before lever extension. Then animals chose between two equal magnitude rewards, only one of which was delayed. On all trials, a phasic dopamine signal was observed in the core that occurred with cue onset. On forced choice trials where only one alternative was cued and available, the high value (no delay) cue was associated with a greater phasic dopamine release than the low value cue. On free choice trials when both cues were presented concurrently, there was no difference in the phasic DA signal between trials

where the low or high value alternative was selected. In the NAcc shell, a phasic dopamine signal was observed with cue onset, but it did not differ based on the type of forced choice trial or free trial choice.

Day et al.,(2010) interpreted this as indicating that phasic dopamine release in the NAcc core, but not shell, encodes the value of rewards, and on free choice trials it encoded the value of the greater value reward regardless of the eventually chosen option. While this is a plausible explanation, especially as the DA signal occurred before animals reach the point of no return for their choice (the lever press), they did not parametrically vary either of the reward values and correlate the DA signal with the options (and their combination) on each free choice trial. Consequently, alternative explanations, such as the DA signal encoding a combination of the two rewards values, cannot be ruled out.

Manipulation of this dopamine signal affects DD as well. Adrianai et al. (2009) transfected the NAcc of male wistar rats with a lenti-virus causing overexpression of DAT, silencing of DAT, or expression of green fluorescent protein (GFP). Animals completed a DCT where either an immediate, low magnitude (1 pellet) reward was pitted against a delayed, larger magnitude (5 pellets) reward. The delay increased across sessions and was constant within given session, and a CDP was used. Compared to GFP controls, both silencing and overexpression of DAT in the NAcc caused increased selection of an immediate, low magnitude reward (Adriani et al., 2009).

However, 6-OHDA lesions, which selectively target dopamine innervation, have not been demonstrated to affect DD. Winstanley et al. (2005) found that such lesions of the entire NAcc did not affect choice behavior on a DRT with ascending delays and no CDP or DS. A conference abstract by Richards et al. (2002) reports a similar null effect with an AA procedure (based on the group the procedure would have had DS and a CDP). Never-the-less, both of these reports do not differentiate between the core and shell and did not completely ablate dopamine levels (~70% reduction reported by both).

Additional light may be shed by microinjections of d-amphetamine into the nucleus accumbens. In DRT without DS or a CDP, d-amphetamine in the NAcc (core & shell) caused increased selection of the delayed reward when ascending delays were used and the opposite pattern when descending delays were used (Orsini et al., 2017). This finding is the same pattern seen by Tanno et al., (2014) and is indicative of a behavioral

flexibility deficit which confounds the measurement of DD. One key caveat that Orisini et al. (2017) point out is that there is evidence suggesting acute changes in DA release may play a role, and not a change in overall DA levels as would have resulted from amphetamine microinjection.

The activity of the NAcc during inter-temporal choice tasks has also been the subject of multiple investigations. Increased c-fos expression has been seen in the NAcc core after completing a DCT where animals are required to process different delay lengths to reward receipt but not after a magnitude based task (da Costa Araújo et al., 2010). In the delay based task one option yield either a sucrose solution reward after a delay of 2 or 18 seconds (equal probability of each) and the other an equal magnitude reward after a delay that adjusted pending the subjects choices. The magnitude based task had one option that yielded either a 20ul or 180ul sucrose solution reward while the other option an adjusting magnitude reward. Compared to a control task which had two options that yield rewards with no delay and equal magnitudes, an increase in c-fos positive cells was seen in the Nacc core after the delay, but not the magnitude, based task suggesting the NAcc core is involved in processing delayed rewards.

The NAcc core appears to exhibit neural activity that tracks with reward value. Gutman & Taha (2016) had animals complete a delay or a magnitude based DCT. In both tasks, stimulus lights acted as DS, and there was no CDP. The delay task had animals chose between two equal magnitude rewards one of which was delivered after a delay. In the magnitude task, the two rewards were both delivered immediately and had different magnitudes. They only recorded from forced choice trials and found increased firing time locked to both DS, and the lever press. For firing associated with DS, there was a greater response with the more valuable reward. However firing that occurred just before the lever press was unrelated to reward value. Moreover, much of this firing was directionally selective (only occurring when only the right or left lever was pressed; rewards were repeatedly changed between the two levers in both tasks). This pattern held for both tasks, suggesting that the NAcc core has neural activity related to reward value derived from both reward delay and magnitude in response to predictive cues (Gutman & Taha, 2016).

Another study found that neural activity immediately preceding response selection in the VS tracked reward value. This activity was selective for the chosen alternative on free choice trials and was directionally selective (Roesch et al., 2009). Single unit recordings were taken while animals completed a DCT where different odors signaled a forced choice on either the right or left nosepoke or a free choice. Across blocks of trials the delay and magnitude of the reinforcers for each option were varied. Animals chose between an equal magnitude reward that was delayed or given immediately and between two rewards of unequal magnitude both of which were provided immediately. Inside each session, both the left and right option were associated with each outcome. On forced choice trials, neurons displayed a greater firing rate just before the response for high (immediate & large magnitude) versus low (delayed and low magnitude) value rewards, but only when the option was in the neurons' preferred direction (left versus right). On free choice trials a similar pattern was observed, but the activity was selective for the eventually chosen option. Further indicating that these signals encoded outcome value, the level of activity was also negatively correlated with response latency. It is important to note that free choice trials were signaled by a third odor. Consequently, directional specific firing cannot be attributed to the DS, but rather to the direction of the movement/spatial location of the subsequent nosepoke. Roesch et al. (2009), also found that after selection of a delayed reward there was continued firing after the response. Consequently, the NAcc may encode and represent the value of a selected choice immediately before its execution, and it may subservise another function as well based upon the activity during the delay period.

In summary, lesion, microinjection, electrochemical, and electrophysiological studies all indicate the NAcc, primarily the core, is involved in DD. Lesions of the core tend to increase DD and disrupt learning with delayed reinforcers (Cardinal et al., 2001; Pothuizen et al., 2005; Cardinal & Cheung, 2005; Bezzina et al., 2007; da Costa Araújo et al., 2009; Valencia-Torres et al., 2012; Galtress & Kirkpatrick, 2010). However, partial inactivation's of the core (Moschak & Mitchell) and lesions of the entire NAcc have been seen to decrease DD (Acheson et al., 2006). DA release to DS for choices tracks reward value (Day et al., 2010), and NAcc single unit activity scales with reward value as a

function of both delay and magnitude (Gutman & Taha, 2016; Roesch et al., 2009). All of these converging lines of evidence indicate NAcc, specifically core, is involved in DD.

**Table 1: Preclinical Nucleus Accumbens DD Studies.**

Study	Site	Technique	Paradigm	Cues			Finding
				DS	Delay	DL(s)	
Cardinal et al., 2001	Core	Post-T. ETL	DRT			0, 10, 20, 40, 60	↑DD; MD
Pothuizen et al., 2005	Core	Pre-T ETL	DCT		Yes	0, 10, 15, 20	↑DD
	Shell	Pre-T ETL	DCT		Yes	0, 10, 15, 20	No effect
Cardinal & Cheung, 2005	Core	Post-T. ETL	FO			0, 10, 20	↓ROR w/ D
	Core	Pre-T. ETL	FO			0, 10, 20	↓ ROR w/ D
Bezzina et al., 2007	Core	Pre-T. ETL	QA w/ DRT	Yes	Yes	da (.5-12) db(0-128)	↑DD
da Costa Araujo et al., 2009	Core	Pre-T. ETL	QA w/ AD	Yes	Yes	0-60	↑DD
Valencia-Torres et al., 2012	Core	Pre-T. ETL	QA w/ AD	Yes	Yes	0-60	↑DD
Acheson et al., 2006	Whole	Post-T. ETL	AA	Yes	Yes	0, 2, 4, 8	↓DD
da Costa Araujo et al., 2010	Core	c-fos	AD		Yes	2-18	↑ c-fos
	Core	c-fos	AM		Yes	NA	No effect
Galtress & Kirkpatrick., 2010	Core	Post-T. ETL	CC			60s vs 0-∞	↑DD
Moschak & Mitchell, 2014	Core	LDBM	DRT	Yes		0, 2.5, 5, 10, 20	↓DD
Winstanley et al., 2005	Whole	Post-T. OHDA	DRT			0, 10, 20, 40, 60	No Effect
Richards et al., 2002	Whole	Post-T. OHDA	AA	Yes	Yes	1, 3, 9	No Effect
Orsini et al., 2017	Whole	d-amph	DRT			0, 4, 8, 16, 32	↓DD
	Whole	d-amph	DRT			32, 16, 8, 4, 0	↑DD
Adriani et al., 2016	Whole	DAT ↑	DCT		Yes	0, 5, 10, 20, 30, 45, 60, 75	↑DD
	Whole	DAT ↓	DCT		Yes	0, 5, 10, 20, 30, 45, 60, 75	↑DD
Day et al., 2010	Core	FSCV	DCT	Yes		5s	↑DA at cue
Roesch et al., 2009	Whole	Single Unit Recordings				3-7	Directionally selective activity that tracks value and chosen option
Gutman & Taha, 2016	Core	Single Unit Recordings				10	↑firing at cue onset that tracks value & ↑firing before response

\*Note: Abbreviations: DL(s)= delay length seconds; Post-T=Post-training; Pre-T=Pre-training; FO=Free operant; MD=magnitude discrimination disruption; CC=concurrent choice; AM=adjusting magnitude; FSCV=fast scan cyclic voltammetry; LDBM=low dose baclophen mucimol partial inactivation; d-amph=d-amphetamine microinjections.

There is some discordance about whether disrupting the core causes disruptions in animals' ability to accurately perceive reward magnitude, potentially complicating interpretations of any DD effects. One study indicates a severe disruption (Cardinal et al., 2001). Several others suggest the sensitivity to reward magnitude remains unaffected (da Costa Araújo et al., 2009; Valencia-Torres et al., 2012). Examinations of whether animals conform to the generalized matching law suggest core lesioned animals are actually better at discriminating based on magnitude (Cardinal & Cheung, 2005), but core lesioned animals have a mild deficit in an incentive contrast procedure (Galtress & Kirkpatrick, 2010). Electrophysiological studies indicated that NAcc activity is sensitive to both delay and magnitude (Roesch et al., 2009). Interestingly studies which show a change in magnitude sensitivity with lesions do not utilize any predictive cues (Cardinal et al., 2001; Cardinal & Cheung, 2005; Galtress & Kirkpatrick, 2010), and studies that show magnitude sensitivity is unaffected do use such cues (da Costa Araújo et al., 2009; Valencia-Torres et al., 2012). It is possible that while the NAcc core is involved in processing magnitude, the use of predictive cues engages a secondary mechanism which is able to compensate for any loss of function with core disruption. Critically for interpretations of DD, Pothuizen et al. (2005) found increased DD with two choices with equal magnitudes, only one of which was delayed.

It is possible that the NAcc, specifically the core, serves as a final common pathway where value information from several different sources summates. Eventually, the chosen option's representation wins out and the NAcc serves to help initiate the motor pattern necessary for execution of this response. This is evidenced by multiple different areas projecting to the NAcc that have been implicated in DD (Berendse et al., 1992; Brinley-Reed et al, 1995; Brog et al., 1993; Hoover et al., 2011), the NAcc core's role in learning/maintaining operant responding for delayed reinforcers (Cardinal & Cheung, 2005), response linked neural activity that is directionally selective and appears to encode the value of rewards associated with specific actions (Roesch et al., 2009; Gutman & Taha, 2016), and DA neurotransmission directly related to reward value that is temporally congruent with predictive cues (Day et al., 2010).

The lesion studies are a little bit more difficult to fit in with this interpretation. If the NAcc core is a general valuation and action selection center, a lesion or inactivation

might be expected to produce a general disruption of behavior that is not specific to either delayed or immediate options. However, the NAcc is not necessary to maintain operant responding (Cardinal & Cheung, 2005), and the NAcc may be specifically necessary for action selection when rewards and reinforcers are unpredictable either due to being probabilistic or delayed (Nicola, 2007; Floresco, 2015). By contrast when the reward/reinforcer is certain, the dorsal striatum is able to facilitate selection and action initiation. Hence, the lesion studies may have had the functional effect of disrupting the main mechanism by which delayed rewards are selected, but not immediate ones.

### ***Orbital Frontal Cortex***

The OFC has been implicated in multiple different decision making tasks (Floresco et al., 2008a). It has been shown to encode reward value (Padoa-Schioppa & Assad, 2006) as well as be involved in stimulus-outcome representations and associations (Ostlund & Balleine, 2007). More specifically, the OFC may signal the desirability of expected outcomes (Schoenbaum et al., 2006) and generates a common currency value signal of those expected outcomes (Kringelback, 2005; Montague and Berns, 2002). Coincidentally (or perhaps not), the OFC is heavily implicated in addictive disorders (Koob & Volkow, 2010; Goldstein & Volkow, 2002; Volkow & Fowler, 2000) for which DD is a risk factor (MacKillop et al., 2011; Mitchell, 2011).

Anatomically, the OFC projects to other areas which likely affect DD. It is reciprocally connected with the BLA (Krettek and Price, 1997; McDonald, 1991; Cassell & Wright 1986) as well as the mPFC (Sesack et al., 1986), and the hippocampus (Burwell & Amaral 1989; Jay & Witter 1991). The OFC sends projections to the NAcc (Hoover et al., 2011). It also receives projections from the VTA (Dunnett and Robbins 1992; Oades and Halliday, 1987). In the rat the OFC is typically divided into medial (mOFC), ventral (vOFC), lateral (lOFC) and dorsolateral (dlOFC) regions. These different regions have been seen to have a different efferent connections, particularly the mOFC (Schilman et al., 2008). The mOFC is heavily interconnected with the mPFC, and its projections have been reported to be more akin to the rat mPFC. In part, this differential connection pattern has led to the suggestion that the mOFC may be a functional link between the mPFC and the more lateral areas of the OFC (Hoover &

Vertes, 2011; Hoover et al., 2011). In sum, among the OFC's many connections are areas implicated in DD in preclinical studies, and it can be divided into several distinct anatomical subregions.

There is evidence indicating the OFC is critical for valuing the delayed rewards. One of the first lesion studies of OFC involvement in DD used a DCT with a CDP but no DS (Mobini et al., 2002). Delays to a larger reward progressed across multiple sessions (20-30 sessions) in ascending order. A pre-training excitotoxic lesion of the whole OFC (wOFC) comprising medial (mOFC), ventral (vOFC), and lateral OFC (lOFC) subregions, caused an increased preference for the sooner-smaller reward as the delays increased but not a difference when there was no or minimal delays to the larger reward. OFC lesions causing increased DD was later replicated. In a T-maze task vIOFC lesioned rats displayed increased choice of a sooner-smaller reward compared to sham animals (Rudebeck et al., 2006). Hence, there is evidence that lesioning the OFC increases DD.

However, not all lesion studies show an increase in DD. Using a DRT without DS or a CDP Winstanley et al. (2004) found post-training excitotoxic lesions of the vIOFC caused a decrease in DD. However, given the OFC lesioned subjects tend exhibit perseverative behaviors (Chudasama & Robbins, 2003), a confounding increase in behavioral perseveration underling these results is a concern (Tanno et al., 2014).

A number of other studies have also found no effect of OFC manipulation on DD. In a cued, non-spatially dependent T-maze task the sooner-smaller and larger-later rewards alternated locations but were always associated with either grey or black and white painted walls, floor, and guillotine doors. vIOFC lesions did not affect DD relative to sham animals (Mariano et al. 2009). Male Long Evans rats in a touchscreen version of the DRT with DS and ascending delays did not exhibit a change in DD after vIOFC pre-training excitotoxic lesions (Abela & Chudasama, 2013). While the behavioral perseveration confound is a concern with this study as well, probability discounting was also performed with the same paradigm, and it did not prevent a decreased selection of a larger uncertain reward in later trial blocks. Using a six arm maze with each arm associated with either a small immediate reward or larger delayed one, wOFC lesions also caused no change in preference for immediate over delayed arms (Jo et al., 2013). Another study found mOFC reversible inactivations with baclofen/musimol

microinjections had no effect on DD in a DRT with ascending delays, no DS, and no CDP (Stopper et al., 2014). Finally in a T-maze task in which animals had to wait outside the goal arm for 15 seconds while not collecting the immediate reward, IOFC inactivation with baclophen/musimol did not cause an increase in DD (Churchwell et al., 2009). In sum, a number of studies show no effect of OFC lesions on DD.

Several studies have tried to resolve the discordant literature on OFC involvement in DD. They have tested whether anatomical location or the use of a CDP are moderators, and if OFC lesions alter sensitivity to reward magnitude. Hypothesizing that the specific OFC subregion is a moderator, Mar and colleagues (2011) performed excitotoxic lesions in either the wOFC, mOFC, or IOFC in animals performing a DRT without DS or a CDP and ascending delays (primarily). wOFC lesions transiently “flattened” percent choice of the delayed lever by decreasing it at the 0 second delay and increasing it at later delays. After several more sessions, normal DD behavior was restored and no difference in preference was seen between the sham controls, even when they removed the delay to the larger reward. However, reversing the levers, such that the delayed lever now became the immediate and vice versa, showed wOFC lesioned rats were slower to adapt to the change than shams. Finally, Mar et al., (2011) reversed the order of delay presentation, such that delays were presented in descending order, and found no difference from shams. mOFC lesions initially displayed no effect on DD, but after several more sessions showed decreased DD. Removing the delays, no difference was observed with sham animals. Upon restoring the delays, the decreased DD in mOFC animals was observed again. When the levers were reversed, mOFC animals actually adapted more quickly than sham animals. By contrast, IOFC lesions displayed increased DD paired with a slower adaptation to a lever reversal. To summarize, anatomical subregion appears to moderate the effects of OFC lesions. wOFC lesions transiently disrupted choice behavior overall but did not change DD. mOFC lesions caused a decrease in DD that developed over several sessions, and IOFC increased DD.

These findings are particularly interesting as one would expect the opposite pattern based on the results of Tanno et al. (2014). Animals that show inflexible behavior and adapt more slowly to the lever reversal, might be expected to also be impaired when there are within sessions shifts in reinforcer contingencies. This would be represented by

decreased DD on a DRT with ascending delays as was seen by Tanno et al. (2014). Yet Mar et al. (2011) found the opposite pattern: increased DD with a slower adaptation to the lever reversal and decreased DD with a faster adaptation to the lever reversal<sup>15</sup>. One potential implication is that not all forms of behavioral inflexibility confound the DRT and may not necessarily obscure effects on choice behavior. Regardless, it highlights that using both ascending and descending orders of delay presentation in a DRT is strongly advisable. Doing so constitutes a best practice because without both orders of delay, presentation of a null effect or effect congruent with an increase in behavioral perseveration is very difficult to clearly interpret.

Whether the OFC is also involved in the processing of sensitivity to reward magnitude in addition to DD has also been examined. In a QA paradigm with a DRT using a CDP to derive indifference delays, pre-training, excitotoxic lesions of the vOFC increased the slope, but not the intercept, of the indifference delays as a function of the delay to the sooner-smaller option (Kheramin et al., 2002). This result suggests the sensitivity to reinforcer size and DD are both increased. The sensitivity to reinforcer size,  $Q$ , increases both the slope and intercept of indifference delays. The DD parameter,  $K$ , decreases the intercept. Therefore an increase in slope has to be due to an increase in  $Q$ , but  $Q$  should also increase the intercept unless an increase in  $K$  drives it back down (Ho et al., 1999). One implication of this finding is that, pending the delay, overall size of reinforcers used, and the ratio of the two reward sizes, one could observe either increased or decreased preference for delayed rewards (Kheramin et al., 2002). The same group repeated this experiment using a sooner-uncertain reward of equal magnitude versus a delayed certain reward (i.e., used probability to reduce the value of the immediate reward versus magnitude). Again an increase in the DD parameter,  $K$ , was found indicating increased DD along with an increase in the probability discounting parameter,  $H$  (Kheramin et al., 2003). Accordingly, discordance in OFC lesion studies may also be due to other processes in addition to DD, such as magnitude sensitivity being altered.

Specific aspects of DD tasks, namely the use of a CDP, also appear to alter the effects of OFC manipulations. Zeeb et al. (2010) had Long Evans rats complete two

<sup>15</sup> Mar et al. (2011) only performed a “delay reversal” where delays are presented in descending order on wOFC lesioned and sham animals, not mOFC or IOFC lesioned animals and shams.

different versions of a DRT with ascending delays. The only difference between the two tasks was whether or not a CDP was used. When there was no CDP, baclophen/musimol microinjections into the IOFC caused a decrease in DD in high discounting subjects (but not low). However when a CDP was used, the opposite pattern was seen; inactivation increased DD in subjects with low levels of DD. Accordingly, the effects of OFC disruptions on DD are also moderated by the use of a CDP as well as showing some rate dependent effects.

Microinjections of DA antagonists also demonstrated that a CDP is critical moderator of IOFC involvement in DD. When no cue was present, microinjections of eticlopride (D2 antagonist) and SCH 23390 (D1 antagonist) had no effect, but when a CDP was used, eticlopride increased DD and SCH 23390 trended towards the same effect. Zeeb et al. (2010) note that these effects appeared to be rate dependent based on the level of DD (low discounters increasing DD), but the effect was not significant (or shown).

The results of these DA manipulation have been replicated. In another DRT with ascending delays and a CDP, SCH 23390 and raclopride microinjections into the IOFC trended towards and increased DD respectively (Pardey et al., 2013). In a QA paradigm with CDPs, 6-OHDA lesions of dopaminergic innervation in the vIOFC caused both increased DD behavior as well as increased sensitivity to large magnitude rewards (Kheramin et al., 2004). Against this grain, mRNA transcript levels of DA receptors (D1-D5) and DA related signaling molecules in the OFC, quantified via real-time polymerase chain reaction (RT-PCR), are not related to DD assessed using a DRT with DS and a CDP (Loos et al., 2013), but mRNA transcript levels do not necessarily relate to protein levels nor availability of the ligand.

The lack of OFC DA effects when no CDP is used have also been replicated. Neither OFC microinjections of reuptake inhibitors with actions on the dopamine transporter (amphetamine; methylphenidate; atomoxetine), the D1 agonist SKF81297, the D1 antagonist SCH 23390, the D2 agonist quinpirole, or the D2 antagonist eticlopride had any effect on DD behavior in an AD paradigm without a CDP (Yates et al., 2014). Similarly, Simon et al. (2013) used in situ hybridization to examine D2 receptor mRNA levels in the OFC and found no relationship to DD behavior on a DRT without DS or a

CDP. Although the quantitative as opposed to qualitative utility of in situ hybridization is questionable. Rats performing a DRT without DS or a CDP exhibit increases in DA's main metabolite, DOPAC, in the OFC<sup>16</sup>, but DA levels were below the threshold for detection by microdialysis (Winstanley et al., 2006).

Exactly why a CDP is a moderator for OFC DA manipulations has not been directly examined, but it may be related the activation of the meso-cortical DA system to conditioned cues and reinforcers. Studies examining moderation of systemic dopaminergic drugs' effects on DD by the use of a CDP have not been consistent (Cardinal et al., 2000; Slezak & Anderson, 2009; van Gaalen et al., 2006). One possible reason a CDP matters specifically for OFC microinjections of dopaminergic drugs centers around the cue predicting reward and becoming a conditioned reinforcer. Cues paired with rewards have been shown to activate the mesolimbic and mesocortico-limbic DA system (Schultz et al., 1997). This activation would cause an increase in DA in the OFC. Without the increased levels of DA from activation of the meso-cortical DA system, there may not be sufficient DA neurotransmission for the antagonists to block. Winstanley et al.'s (2006) finding that OFC DA levels were too low to detect via microdialysis and HPLC is in line with this possibility as they did not use a CDP or DS. Hence, DA antagonists microinjected into the OFC may only have an effect when a CDP activates the meso-cortical DA system.

However the above rationale does not account for why DA agonists do not have any effect when microinjected into the OFC. The reason for this pattern of results may be due to dopamine's role as a neuromodulator. DA release in the OFC during the presence of a cue could be facilitating glutamatergic neurotransmission encoding a representation of a cue by various mechanisms (Seamans & Yang, 2004). In this instance, simply adding DA or a DA agonist to the OFC when no cue is being encoded by fast synaptic transmission is not likely to have any overt effect as there is no encoded representation to facilitate. With this interpretation in mind, an important line of future research will be to investigate what will happen when a DA agonist is applied to the OFC when a cue is

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<sup>16</sup> No increase in OFC DOPAC was seen for rats in two different yoked control groups: one controlling simply for reward presentation (in chambers and presented with the same rewards earned by the master rat), and one controlling for operant responding for rewards (in chambers completing the task, but only forced choice trials defined by the choices of the master rat).

present. DA agonists could further facilitate the encoding of the cue, functionally decreasing impulsive choice, or have the opposite effect by disrupting the representation of the cue and functionally increasing impulsive choice.

With regards to the weak and inconsistent effects of D1 antagonists microinjected into the OFC when a cue is present, Kheramin et al.'s (2004) results offer a possible explanation. Kheramin et al. (2004) found that in addition to 6-OHDA lesions of the OFC increasing DD, the lesions also increased the sensitivity to large magnitude rewards. If D1 antagonism in the OFC is having a similar effect both increasing DD and increasing the sensitivity to large magnitude rewards, then in DD paradigms not designed to disentangle these two factors one could see a weak or null effect. This pattern would be seen because the value of the larger-later reward starts out at a higher point, and it would have to be discounted to a greater degree to even result in the same pattern of choice behavior prior to the manipulation, let alone increased "impulsive choice."

Serotonin function in the OFC also appears to be involved in modulating cue processing, but there is limited evidence. In an AD paradigm with DS but no CDP, the 5-HT<sub>1A</sub> agonist 8-OH-DPAT decreased DD (Yates et al., 2014). As the 5-HT<sub>1A</sub> receptor is thought of as an autoreceptor (Albert et al., 1996), 8-OH-DPAT may actually decrease 5-HT neurotransmission, suggesting 5-HT in the OFC promotes impulsive choice. However, DOI (5-HT<sub>2A/2C</sub> agonist), Way-100635 (5-HT<sub>1A</sub> antagonist) and Ketanserin (5-HT<sub>2A</sub> antagonist) had no effects (Yates et al., 2014). Moreover, Winstanley et al., 2006 found that there were no task specific increases in 5-HT or its metabolite 5-HIAA during DRT without DS or a CDP. However, in a T-maze task, DOI infusions increased DD, and this increase was blocked when LY379268 (mGlu2/3 agonist) was coadministered systemically despite LY379268 having no independent effect (Wischhof et al., 2011). Given the limited evidence, it is tough to decipher exactly what role serotonin may be playing with regards to the OFC and DD. As neither Yates et al., (2014) nor Winstanley et al., (2006) used a CDP and enclosing an animal in the goal arm in a T-maze could be argued to be a CDP, 5-HT may be important for modulating glutamate neurotransmission in the OFC involved in processing the CDP.

In a DCT with DS but not a CDP in which rewards differed by either magnitude or delay, two different populations of neurons sensitive to reward delay and a third

associated with magnitude were found (Roesch et al., 2006). In the first population, neurons responded more to an immediate reward choice. Their activity increased briefly after responding for an immediate or a delayed reward, and then a second increase in activity was seen at the delivery of the delayed reward. This immediate response activity was greater than the combined activity for delayed reward. Furthermore, the strength of this response was correlated with choice behavior on free choice trials. The second population of neurons in the OFC fired more in response to a delayed reward choice (Roesch et al., 2006). For both rewards, the increase in activity began immediately following the response, but the delayed reward activity continued to increase throughout the delay, peaking just prior to reward delivery. Activity in these first two populations of neurons did not show differential activity to large versus small magnitude rewards. Rather a third population of neurons showed differential activity based on magnitude upon reward delivery. The activity in the reward magnitude encoding population was unrelated to reward delay.

A “dual role” for the OFC has been proposed where it both discounts delayed rewards and supports their learning and thus their selection (Schoenbaum & Roesch, 2005; Roesch et al., 2006). Examining all of the literature reviewed here, a ternary if not quaternary role may be also be the case. There is evidence that the OFC is involved in discounting the delayed reward, encodes an expectancy signal for the delayed reward, and processes cues associated with the delayed reward. Disrupting the OFC causes a decrease in DD when no CDP is used (Winstanley et al., 2004b; Zeeb et al., 2010). In this situation the “discounting” process may be the most prominent and outweigh the influence of reward magnitude and a possible outcome expectancy signal. By contrast when a CDP is used, the addition of a “common currency” value signal for a conditioned reinforcer may tip the balance of OFC involvement towards favoring delayed rewards. Accordingly when a CDP is used, OFC disruptions tend to increase DD (Mobini et al., 2002; Zeeb et al., 2010; Kheramin et al., 2002; 2003).

**Table 2: Preclinical Orbital Frontal Cortex DD Studies**

Study	Site	Technique	Paradigm	Cues		DL(s)	Finding
				DS	CDP		
Mobini et al., 2002	wOFC	Pre-T. ETL	DCT		Y	1, 2, 4, 8, 12, 16, 20, 30, 1	↑DD
Rubeck et al., 2006	vIOFC	Post-T. ETL	T-maze		Y	15	↑DD
Winstanley et al., 2004b	wOFC	Post-T. ETL	DRT			0, 10, 20, 40, 60	↓DD
Mariano et al., 2009	vIOFC	Post-T. ETL	Cued T-Maze			15	No Change
Abela & Chudasama, 2013	wOFC	Pre-T. ETL	DRT	Y		0, 8, 16, 32	No Change
Jo et al., 2013	wOFC	Post-T. ETL	6 arm Maze			0, 3, 6, 9, 12	No Change
Stopper et al., 2014	mOFC	Baclofen/Musimol	DRT		Y	0,15, 30, 45	No Change
Churchwell et al., 2009		Musimol	T-Maze			15	No Change
Mar et al., 2011	wOFC	Post-T. ETL	DRT			0, 20, 40, 60	No Change
	wOFC					60, 40, 20, 0	No Change
	mOFC					0, 20, 40, 60	↓DD
	vIOFC					0, 20, 40, 60	↑DD
Kheramin et al., 2002	vIOFC	Pre-T. ETL	QA w/DRT		Y	0-75	↑DD
Kheramin et al., 2003	vIOFC	Pre-T. ETL	QA w/DRT		Y	0--100	↑DD
Zeeb et al., 2010	IOFC	Baclofen/Musimol	DRT		Y	0, 15, 30, 45	↑DD
		SCH 23390			Y		↓DD
		Electropride			Y		↑DD
							No Change
Yates et al., 2014	vIOFC	MPH	AD	Y		0-45	No Change
		AMPH					No Change
		ATO					No Change
		SKF81297					No Change
		SCH 23390					No Change
		Quinpirole					No Change
		Eticlopride					No Change
		8-OH-DPAT					↓DD
		Way-100635					No Change
		Ketanserin					No Change

Table 2 continued

Study	Site	Technique	Paradigm	Cues			Finding
				DS	CDP	DL(s)	
Pardey et al., 2012	IOFC	SCH23390	DRT	Y	0-400	↑DD	
		Raclopride				↑DD	
		Phenylephrine				No Change	
		Guanfacine				No Change	
Kheramin et al., 2004	vIOFC	6-OHDA	QA w/ DRT	Y	0-75	↑DD	
Yates et al., 2014	vIOFC	8-OH DPAT	AD	Y	0-45	↓DD	
		Way-100635				No Change	
		DOI				No Change	
		Ketanserin				No Change	
Wischhof et al., 2012	vIOFC	DOI	T-Maze	Y	10	↑DD	
		DOI+LY379268				No Change	

\*Note: Abbreviations: DL(s)= delay length seconds; Post-T=Post-training; Pre-T=Pre-training; FO=Free operant.

Two additional layers influence matters as well. The differential effects of a CDP appear to be dependent on the rate of DD (Zeeb et al., 2010). When no cue is used, the involvement of the OFC is only tipped towards the immediate reward in steep discounters and vice versa when a CDP is used. This possibility explains the numerous null results (Mariano et al., 2009; Abela & Chudasama, 2013; Jo et al., 2013; Stopper et al., 2014; Churchwell et al., 2009) as well as Zeeb et al., (2010) directly observing this pattern. Roesch et al. (2007) also proposed the outcome expectancy signal maybe critical for learning about delayed rewards, hence why pre-training lesions tend increase DD (Kheramin et al., 2002; 2003; Mobini et al., 2002), and post training lesions, which may only disrupt the discounting process, decrease DD (Winstanley et al., 2004b). Furthermore all of these different processes may be subserved by different neuronal populations (Roesch et al., 2006). These populations may not be anatomically localized explaining why subregion is a moderator as well (Mar et al., 2011). In sum, it appears the OFC subserves multiple potential roles in DD. Moreover, these roles may differ across anatomical subregion.

### ***Basolateral Amygdala***

The basolateral amygdala (BLA) is interconnected with other areas implicated in DD by preclinical studies. It is reciprocally connected to the OFC (Krettek and Price, 1997, McDonald, 1991; Cassell & Wright, 1986) and has glutamatergic projections to the NAcc and prefrontal cortex (McDonald, 1996; Brinley-Reed et al., 1995). Specifically, it projects to both the NAcc core and shell (Wright et al., 1996; Brog et al., 1993), and some of these projections bifurcate and send collaterals to the mPFC (Shinonaga et al., 1994). However it also has independent projections to the mPFC (Shinonaga et al., 1994; Gabbott et al., 2006), and in turn the mPFC projects back to the BLA's primary projection neurons (Brinley-Reed et al., 1995; Cassell & Wright, 1986; Gabbott et al., 2005). The BLA also receives projections from the HPC (Ishikawa & Nakamura, 2006; Pitkänen et al., 2000) as well as projects back to the HPC (Pikkarainen et al., 1999; Pitkänen et al., 2000; Krettek & Price, 1997).

Compared to investigations into other structures, there are fewer studies that have looked at the BLA. However, these studies have consistently found the BLA is needed for DD. In a DRT with ascending delays and no CDP, excitotoxic BLA lesions caused an increase in DD (Winstanley et al., 2004b). Similarly in a T-maze task where subjects had to wait outside a goal arm for 15 seconds while not collecting an immediately available reward, microinjected muscimol caused increased collection of the immediate reward (Churchwell et al., 2009). Electrophysiological recordings indicate the BLA contains neurons that encode predicted outcomes and display sustained activity during the delay to the reward that increases over the course of the delay (Roesch & Bryden, 2010; Roesch et al., 2010). One of several possible explanations put forth by Roesch & Bryden (2010) was that this sustained activity may serve to maintain an expectancy of the delayed reward until it is received. This interpretation is in keeping with a broader role of the BLA in signaling the outcomes associated with various actions (Hatfield et al., 1996; Balleine et al., 2003). The BLA has also been implicated in conditioned reinforcement via representing value of conditioned stimuli (Cador et al., 1989; Burns et al., 1993; Gallagher, 2000). However, the use of a CDP does not appear to be a moderating variable for the BLA. Consequently the BLA's possible role in conditioned reinforcement

may not play a large role in DD. Rather its ability to generate an expected value signal/outcome representation may be more critical.

**Table 3: Preclinical Basolateral Amygdala DD Studies**

Study	Site	Technique	Paradigm	Cues			Finding
				DS	CDP	DL(s)	
Winstanley et al., 2004b	BLA	Post-T. ETL	DRT			0, 10, 20, 40, 60	↑DD
Churchwell et al., 2009	BLA	Muscimol	T-Maze			15	↑DD

\*Note: Abbreviations: DL(s)= delay length seconds; Post-T=Post-training; Pre-T=Pre-training; FO=Free operant.

### *Hippocampus*

The hippocampus (HPC) is involved in a variety of functions some of which may be important for DD. There is strong evidence that the HPC is involved in working memory (Floresco et al., 1997; Seamans et al., 1998; Rawlins and Tsaltas et al., 1983), spatial learning and navigation (Moser et al., 1993; Eichenbaum et al., 1999), and contextual conditioning (Holland & Bouton, 1999; Anagnostaras et al., 2001). A dorsal (dHPC) versus ventral (vHPC) functional and anatomical division has been proposed as well (Bannerman et al., 2004). Potentially relevant to DD, the HPC has also been suggested to be important for the sequential ordering of events (Fortin et al., 2002) and contribute to decision making via representing future outcomes (Johnson et al., 2007; Schacter et al., 2007). Hence, HPC function may be important for DD.

Anatomically, the HPC is interconnected with other areas implicated in DD such as the mPFC, OFC, BLA, and NAcc. The HPC projects to the mPFC, specifically the prelimbic (PL), and infralimbic (IL) cortices (Jay et al., 1991; Cenquizca & Swanson, 2007). These cortical efferent connections appear to be glutamatergic (Jay et al., 1992). Some evidence indicates that these HPC efferents arise primarily from the vHPC and temporal HPC as opposed to its dorsal subdivision based on retrograde tracer studies (Verwer et al., 1997). Some of these mPFC projections appear to have collaterals to the BLA (Ishikawa & Nakamura, 2006). These collaterals may, or may not, correspond to other known HPC projections to the BLA and other amygdala nuclei (Pitkänen et al., 2000). The BLA in return projects back to the HPC (Pikkarainen et al., 1999; Pitkänen et al., 2000; Krettek and Price, 1977). For the OFC, there is evidence indicating the mOFC

receives HPC projections, similar to the mPFC, but the more lateral areas of the OFC (IOFC, vOFC, dIOFC) may not be innervated very strongly if at all (Cenquizca & Swanson, 2007; Jay et al., 1989). The vHPC also projects to the NAcc (Groenewegen et al., 1987; Brog et al., 1993). Kelley & Domesick (1982) found with anterograde tracing of fornix fibers that the projections were limited to the medial NAcc being particularly dense in the caudal dorsomedial area of the NAcc (inside the shell). However, tracing studies using the subiculum as a point of origin indicate projections cover the whole of the NAcc (Groenewegen et al., 1987), and retrograde tracer studies from the central NAcc identify the subiculum as a glutamatergic afferent connection to the NAcc (Christie et al., 1987). In sum, the HPC is strongly interconnected with other areas implicated in DD by preclinical studies.

Lesioning or inactivating the HPC almost always increases measures of DD. In a Y-maze where entry into one arm was associated with delayed continuous reinforcement and the other with immediate partial reinforcement<sup>17</sup> [ $p(\text{reward})=.25$ ], HPC aspiration lesioned rats showed increased selection of the partially reinforced arm versus sham lesioned animals (Rawlins et al., 1985). Moreover, both medial and dorsolateral septal lesions increased selection of the immediately available partial reinforcement arm (Rawlins et al., 1985).

McHugh and colleagues (2008) looked into if there was a subregion specificity to HPC lesions in addition to using a lesion technique that would spare the fibers of passage. In a T-maze DD task, they found complete HPC, vHPC, and dHPC lesioned animals all showed greater preference for a low reward option that was not delayed versus a high reward, delayed option. Moreover, when equal 10-second delays were placed on each reward, all animals displayed a clear preference for the high reward arm suggesting magnitude discrimination and spatial navigation were not confounding factors (McHugh et al., 2008). The same group then followed up this result with a non-spatial, cued version of the T-maze task in which the sooner-smaller and larger-later reward alternated location but were always associated with either grey or black and white painted walls, floors, and guillotine doors. This method rules out a possible impairment in animals ability to

<sup>17</sup> Abela and Chudasama (2013) found vHPC lesions did not affect probability discounting.

navigate the maze as a confound. In this alternate T-maze task<sup>18</sup>, increased DD was still observed in HPC lesioned animals versus shams (Mariano et al., 2009).

**Table 4: Preclinical Hippocampus DD Studies**

Study	Site	Technique	Paradigm	Cues		DL(s)	Finding
				DS	CDP		
Mariano et al., 2009	cHPC	Post-T. ETL	Cued T-maze	Y		10-15, 30s, 60s	↑DD
Rawlins et al., 1985	cHPC	Pre-T. AL	Y-Maze			10	↑DD
	M. Septum	Pre-T. AL	Y-Maze			10	↑DD
	DL. Septum	Pre-T. AL	Y-Maze			10	↑DD
McHugh et al., 2008	dHPC	Post-T. ETL	T-Maze			10	↑DD
	vHPC	Post-T. ETL	T-Maze			10	↑DD
	cHPC	Post-T. ETL	T-Maze			10	↑DD
Cheung & Cardinal, 2005	cHPC	Post-T. ETL	DRT			0, 10, 20, 40, 60	↑DD
Abela & Chudasama, 2013	vHPC	Pre-T. ETL	DRT	Y		0, 8, 16, 32	↑DD
Abela & Chudasama, 2012	vHPC	Muscimol/baclofen	DRT	Y		0, 8, 16, 32	↑DD
	vHPC	Guanfacine	DRT	Y		0, 8, 16, 32	↓DD
	vHPC	SCH 23390	DRT	Y		0, 8, 16, 32	-DD

\*Note: Abbreviations: DL(s)= delay length seconds; Post-T=Post-training; Pre-T=Pre-training; FO=Free operant.

Operant based tasks also indicate the HPC is necessary for normal DD behavior. In a DRT without DS or a CDP, HPC lesions caused increased DD (Cheung & Cardinal, 2005). This lesion also caused a decrease in selection of the delayed reward under 0 second delay conditions, but when the 0 second delay condition was applied to the entire session, no difference was observed between HPC lesioned animals and shams (Cheung & Cardinal, 2005). Pretraining excitotoxic lesions of the vHPC as well as inactivation with a muscimol/baclofen cocktail also caused increased DD on a touchscreen DRT with DS but not a CDP (Abela & Chudasama, 2012; 2013). Microinjection of the norephinerine  $\alpha_{2A}$  receptor agonist guanfacine in to the vHPC, but not the DA D1 antagonist SCH 23390, decreased DD (Abela & Chudasama, 2012). However caution is warranted in interpreting this sole study looking into neurotransmitter specific effects in the vHPC on DD. Only ascending delays were used, and lesions of the exact same area, vHPC, cause increased premature and perseverative responding on the 5CSRTT (Abela et

<sup>18</sup> Described in greater detail in the OFC section.

al., 2012). A “false positive/negative” cannot be excluded. Never-the-less, lesion and inactivation studies using operant methods show that the vHPC is important for DD.

### ***Medial Prefrontal Cortex***

The rodent mPFC can be divided into three (or four if the mOFC is considered part of the mPFC vs OFC) different sub-regions. However these different regions have often been lumped together by researchers. These three regions are the prelimbic (PL), infralimbic (IL), and cingulate cortices (Cg). There has been some debate as to what these areas translationally correspond to in a human brain. Uylings et al. (2003) argued that the rodent medial PFC (mPFC) corresponds to the primate dlPFC based on these areas sharing similar subcortical afferent and efferent connections and subserving the same “common class functions.” Specifically, both the primate dlPFC and rodent mPFC both are involved in working memory as well as monitoring and planning behavior, (Uylings et al., 2003). Seamans, Lapish, & Durstewitz (2008) agree with this conclusion in part, but focusing on electrophysiological evidence they argue the rat mPFC is more of a combination of the primate dlPFC and ACC.

In addition to numerous other areas of the brain, the mPFC is interconnected with areas whose contribution to DD has been investigated in preclinical studies. The PL and IL receive a glutamatergic afferents from the vHPC (Jay et al., 1991; 1992; Verwer et al., 1997; Cenquizca & Swanson, 2007), and recently a monosynaptic projection to the HPC from the cingulate region in mice has been identified (Rajasethupathy et al., 2015). Additional reciprocal connections are present with the OFC (Sesack et al., 1989). The PL, IL, and Cg all receive dense projections from both the mOFC and vOFC with the PL area receiving the most innervation (Hoover & Vertes, 2007; 2011). In turn the mPFC projects heavily to the mOFC and has some efferent connections with more lateral areas of the OFC (Sesack et al., 1989). Closely associated with the OFC, the BLA is interconnected with the mPFC as well. mPFC projections to the BLA form asymmetric synapses onto spiny, but not aspiny, neurons (Brinley-Reed et al., 1994). Some of these mPFC-to-BLA projections also have collaterals that go to the NAcc (Gabbot et al., 2005). These spiny cells are the BLA’s projection neurons (McDonald, 1992). The BLA projections to the mPFC synapse onto both pyramidal cell spines as well as basket and chandelier

GABAergic interneurons (Gabbot et al., 2006). Finally, the NAcc receives input, but does not appear to have a monosynaptic return. The core subregion is innervated by the dorsal areas of the PL cortex, and the shell receives inputs from the IL and ventral PL areas (Berendse et al., 1992; Brog et al., 1993; Heidbreder & Groenewegen, 2003). While this is not an exhaustive list of the mPFC's afferents and efferents, the highlighted connections show how the mPFC, particularly the PL area, is interconnected with other structures hypothesized to be involved in DD.

Lesions and pharmacological inactivations of the mPFC during DD have been limited, yielded mixed results, and have alternative explanations for their findings due to potential response inhibition and behavioral perseveration confounds. In a T-maze DD that included a response inhibition component, muscimol microinjections into the mPFC<sup>19</sup> caused increased selection of the sooner-smaller reward (Churchwell et al., 2009). Interestingly, functionally disconnecting the BLA from the mPFC by unilaterally microinjecting muscimol into both structures on contralateral sides caused increased selection of the smaller immediate reward as well. However, in this task rats had to wait outside an arm for 15 seconds while not collecting an immediately available small reward in order to receive a larger reward. If the muscimol microinjections caused a response inhibition deficit, subjects might not have been able to refrain from collecting the immediate reward, skewing the choice behavior without affecting DD per se. In a DRT with no DS, CDP, and only ascending delays, neither Cg1 nor combined PL and IL lesions increased DD (Cardinal et al., 2001). However, the PL/IL lesion group appeared to no longer adjust their choices as a function of delay. Compared to sham controls, PL/IL lesioned animals had decreased preference for the larger reward when there was no delay, but increased preference for the larger reward when delays were present. A second study looking at PL/IL involvement found reversible inactivation with muscimol microinjections failed to effect DD, but they also used a DRT with only ascending delays (Feja & Koch, 2014). The DRT with ascending delays used by both studies with null results is susceptible to a behavioral perseveration confound which can cause a false negative result (Tanno et al., 2014). Given potential response inhibition and behavioral perseveration confounds, it is important to note that the mPFC is involved in behavioral

<sup>19</sup> Coordinates and representative image suggest PL cortex, but no hit map was provided.

flexibility and response inhibition (Ragozzino, 2007, Chudasama & Robbins, 2003; Narayanan et al., 2006). Hence, the current lesion and inactivation literature is limited, discordant, suffers from plausible alternative explanations, and would benefit from additional investigations utilizing DD tasks that do not possess response inhibition and behavioral perseveration elements.

DA neurotransmission in the rodent mPFC appears to play a similar role to the hypothesized role for DA in the human dlPFC. As discussed above,<sup>20</sup> studies investigating the role of allelic variation in COMT suggest that too little or too much dopamine in the dlPFC causes increased DD (Boettiger et al., 2007; Paloyelis et al., 2010; Smith & Boettiger 2012; Gianotti et al. 2012). This parallels the inverted “U” model of prefrontal function where an optimal level of DA neuromodulation is needed (Arnsten, 2011, Cools and D’Esposito, 2011; Floresco et al., 2013). Rodent preclinical investigations show a similar pattern with DA antagonists and agonists microinjected into the mPFC (both prelimbic and infralimbic areas; PL & IL) both causing increases in DD.

Specifically, DA D1 receptor signaling in the mPFC has been shown to be implicated in DD. In a DRT without DS or CDP and using ascending delays, microinjections of the D1 agonist SKF38393 and the D1 antagonist SCH 23390 into both the PL and IL increased DD (Loos et al., 2010). Moreover, D1, D5 and Caly expression was positively associated with increased DD (Loos et al., 2010). When a CDP was used in a separate study with similar methods, SCH 23390 mPFC<sup>21</sup> microinjection caused increased DD as well (Pardey et al., 2013). Sonntag et al. (2014) utilized a T-maze version of a DD task coupled with a lentiviral vector to increase D1 expression in PL glutamatergic neurons. This overexpression of D1 receptors led to an increase in selection of the arm containing a small immediate reward as opposed the arm with the large delayed reward corresponding to increased DD. However, Yates et al. (2014) found in an AD paradigm that microinjecting a D1 agonist and antagonist (SKF81297; SCH23390) as well as amphetamine, methylphenidate, and atomoxetine into the mPFC had no effect. However some caution is warranted as all three of these reuptake inhibitors are not specific to the dopamine transporter (DAT; nor is the DAT even specific to DA). Also,

<sup>20</sup> Human Neuroimaging Section

<sup>21</sup> Hit map and coordinates suggest PL but the authors did not specify.

examining D1 expression via in situ hybridization with densitometric analysis was unrelated to DD on a DRT without DS or a CDP and ascending delays (Simon et al., 2013), but in situ hybridization is a qualitative technique being used quantitatively, and increased mRNA does not necessarily correspond to increased protein levels.

Collectively, the above studies indicate that increases or decreases in D1 signaling, pharmacologically or in terms of receptor expression, cause increases in DD although there is a conflicting result.

DA D2 receptor signaling in the mPFC has also been studied in the context of DD. Pardey and colleagues' (2012) DRT with ascending delays and a CDP found microinjected raclopride caused increased levels of DD. Quinpirole and eticlopride, DA D2 agonist and antagonist respectively, also caused increased DD when microinjected into the mPFC during an AD task (Yates et al., 2014). Finally, D2 mRNA levels, quantified via in situ hybridization, are negatively correlated with DD on a DRT without DS or a CDP (Simon et al., 2013; see above concerns with this methodology).

Further evidence for mPFC DA involvement was provided by a well-controlled microdiagnosis study. Rats that completed a DRT without DS or a CDP showed increases in mPFC DA and DOPAC from baseline. However the increase in DA was not different from a similar increase seen in rats who completed yoked forced choice only and reward presentation only versions of the task (Winstanley et al., 2006). Nevertheless that does not rule out that DA has no influence on reward valuation or other processes that affect DD (Floresco et al., 2008a).

Given that the majority of studies examining the effects of DA manipulations of the mPFC on DD use a DRT, consideration must be given to any possible role that increased behavioral perseveration may be playing. As was demonstrated by Tanno et al. (2014), in DRT with ascending delays, increases in behavioral perseveration can cause a decrease in impulsive choice without necessarily causing decreased DD. This change in choice behavior can be observed without a change in the underlying process presumed to govern it because the DRT is susceptible to within session carryover effects due to the use of multiple delays in a non-random order. Increased behavioral perseveration would, in theory, cause the rat to continue to respond on the initially more reinforcing outcome in spite of the changing delay. As both Pardey et al. (2012) and Loos et al. (2010) used

ascending delays (i.e., the delay to the larger reward became longer as the session progressed) if D1 and D2 $\rightarrow$  agonists/antagonists were causing increased behavioral perseveration, then one would expect to see decreased DD based on the findings of Tanno et al. (2014). However the opposite pattern, increased DD, was observed in both studies.

Considering the converse situation, that mPFC DA drug microinjections caused decreased behavioral perseveration, additional factors come into play. It could be argued that decreasing behavioral perseveration would cause increased DD in a DRT with ascending delays as they would be better able to track the changing reward values. However this argument assumes that under baseline conditions without pharmacological manipulations, subjects are not already tracking the reward values optimally. While this situation is possible, it is not likely. The AD paradigm, in which perseveration is not a major concern, shows concurrent validity with the DRT suggesting that the DRT does provide valid measurement under baseline conditions (Craig et al., 2014). Therefore, the increased DD seen in Loos et al. (2010) and Pardey et al. (2012) is not likely to be due solely to decreased behavioral perseveration.

To date, there are no published reports of electrophysiological recordings of the rat mPFC during a DD task. Burton and colleagues (2009) saw a potential anticipatory signal in a spatial foraging task. Once animals navigated to a goal zone (changed daily), a food pellet was released in one of three separate locations after two seconds. Once animals reached the goal zone, the activity in the PL cortex increased in an event related manner until delivery of the reward. Interestingly, lesioning of the HPC abolished the PL anticipatory activity and instead a short burst of activity was seen upon reward delivery. Burton et al., (2009) tested if the PL activity could be explained as “place field” however the activity was only weakly related to spatial location. Another study used an alternating reward task where rats have to nosepoke for alternating food and water rewards on opposite sides of an open field. In this task mPFC<sup>22</sup> neurons showed increased activity during a delay between nosepoking and reward presentation, but not when the reward was presented immediately. Moreover, approximately half of these neurons only

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<sup>22</sup> Recording electrode was set-up on a Microdrive assembly and with initial coordinates suggesting PL; no hit map was provided.

displayed increased firing for a specific reward type independent of spatial location; the others were indiscriminate based on reinforcer commodity (Miyazaki et al., 2003). One possible interpretation of these studies is that the mPFC, specifically the PL, is involved in generating an anticipatory signal or outcome representation when the reward is delayed.

**Table 5: Preclinical Medial Prefrontal Cortex DD Studies**

Study	Site	Technique	Paradigm	Cues		DL(s)	Finding
				DS	CDP		
Cardinal et al., 2001	PL/IL	Post-T. ELT.	DRT			0, 10, 20, 40, 60	MD
	Cg	Post-T. ELT.	DRT			0, 10, 20, 40, 60	No Change
Feja & Koch, 2014	PL/IL	Muscimol	DRT	Y		0, 10, 20, 40, 60	No Change
Churchwell et al., 2009	PL	Muscimol	T-Maze			15	↑DD
Loos et al., 2010	PL	SKF38393	DRT	Y	Y	0, 5, 20, 40	↑DD
	IL	SCH 23390					↑DD
Sonntag et al., 2014	PL	Lenti-viral ↑D1R	T-Maze			5, 10, 15	↑DD
Pardey et al., 2013	PL	SCH 23390	DRT		Y	0-400	↑DD
		Raclopride					↑DD
		Phenylephrine					No Change
		Guanfacine					No Change
Yates et al., 2014	PL	MPH	AD	Y		0-45	No Change
		AMPH					No Change
		ATO					No Change
		SKF81297					No Change
		SCH 23390					No Change
		Quinpirole					↑DD
		Eticlopride					↑DD
		8-OH-DPAT					No Change
		Way-100635					No Change
Ketanserin					No Change		

\*Note: Abbreviations: DL(s)= delay length seconds; Post-T=Post-training; Pre-T=Pre-training; FO=Free operant; MD=Magnitude discrimination disruption.

The mPFC, in particular the PL region, being involved in generating an outcome representation of the delayed reward is consistent with studies looking at outcome devaluation and contingency degradation. The PL region has been suggested to be necessary for goal directed learning (Balleine & O'Doherty, 2010). Rats with PL lesions no longer show a devaluation effect after contingency degradation even with limited

training when sham animals have yet to develop habitual behavior. However, IL lesions prevented the development of habitual behavior after extended training (Killcross & Coutureau, 2003).

### **Potential Interpretation of Delay Discounting Neurobiology Studies**

The human neuroimaging literature's dominant interpretation is a two system account where one cool, executive system competes with a hot, impulsive one. In the case of the human dlPFC, there is particularly good evidence to support this interpretation. The dlPFC exhibits greater BOLD activity when delayed choices are made (Hare et al., 2007; McClure et al., 2004; 2007). Furthermore disrupting the dlPFC with either TMS or DCS increases the rate of DD (Figner et al., 2010; Hect et al., 2013), and COMT genotypes linked to either an excess dlPFC DA in adolescence or deficit in adulthood are associated with increased DD statistically mediated by altered dlPFC activity (Boettiger et al., 2007; Gianotti et al., 2012; Smith & Boettiger, 2012; Kayser et al., 2012).

Evidence pertaining to delayed reward subjective value representations are difficult to reconcile with two system accounts. The most commonly identified impulsive system area is the NAcc/VS. Many studies have seen greater NAcc/VS activation when individuals make immediate reward choices (Albrecht et al., 2011; Hariri et al., 2006; McClure et al., 2004; 2007). Greater activity in the NAcc/VS has been shown to be related to increased DD, and decreases in DD with age are accompanied by decreases in NAcc/VS activity (Christakou et al., 2011). However, it is precisely this structure, in addition to several other areas, in which subjective value representations are seen (Ballard & Knutson, 2009; Kable & Glimcher, 2007; 2010; Schneider et al., 2014). These subjective value representations are problematic because increases in delayed reward value, which favor selection of the delayed reward, are related to corresponding increases in NAcc/VS BOLD signal. According to two system accounts this increase in NAcc/VS activity should cause an increased propensity to choose the immediate reward (Bickel et al., 2007). However, this increase in activity is inversely correlated with that outcome in this instance.

Structural and functional connection studies point out how important the strength of connections are to DD, and also raise questions about the existence of two distinct and competing neurobehavioral systems. Multiple studies have shown that the functional and structural connection between brain regions is associated with the rate of DD both cross sectionally and longitudinally (Achterberg, 2016; Christakou et al., 2011; Hampton et al., in press; Li et al., 2013; Peper et al., 2013; Peters & Büchel, 2010; van den Bos et al., 2014; 2015). In the context of two system accounts, one could see how increased connection strength between the areas of given neurobehavioral system could be related to DD. For example, increased connection strength in between the areas of the impulsive system could result in a feed forward mechanism resulting in increased impulsive system activity. This increased activity would in turn lead to increased selection of the immediate reward. While some of the connections whose strength is associated with DD would correspond to intra-system connections (Li et al., 2013), many are inter-system connections. Increased structural and functional connectivity between the dlPFC and the striatum is associated with decreased DD and increases in the connectivity are related to the decrease in DD with age (van den Bos et al., 2014; 2015). Also increased functional coupling between the vmPFC and the dlPFC with increases in age is associated with decreases in DD (Christakou et al., 2011). One implication is that if these two systems exist, they do not operate independently of one another.

Whether or not two different executive and impulsive neurobehavioral systems exist does not mean that there are not “cooler” and “hotter” areas in an interconnected network with the NAcc/Vs as a final common output pathway. Indeed the animal literature supports this idea. The areas implicated in DD in rodent studies (BLA, OFC, mPFC, HPC) are all interconnected and all have a common projection to the NAcc without a monosynaptic return from the NAcc which projects to other areas including the SNpr/pc, VP, and other basal ganglia structures<sup>23</sup>. As such, the NAcc is well positioned to serve as a comparator of different value signals and be a final common output that interfaces with the motor system.

The NAcc/Vs being the final common output of this DD network is consistent with a number of pieces of evidence. First, the NAcc has long been thought of as an

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<sup>23</sup> Connections and their citations reviewed at the start of all the prior sections.

interface of the limbic and motor systems (Morgenson, 1980). More recently it has been proposed to be involved in action selection when outcomes are delayed or uncertain in some fashion (Nicola, 2007; Floresco, 2015). Neural activation in the VS on during a DD task is directly related to the subjective value of both rewards (Kable & Glimcher; 2010). Single unit recordings also indicate that both outcome values are represented in the core. NAcc core neurons exhibit increased activity in response to DS that tracks with reward value, and show activity immediately before a response that is directionally sensitive (Gutman & Taha, 2016; Roesch et al., 2009). Lesions and inactivation of the NAcc core also caused disruptions in magnitude discrimination (Cardinal et al., 2001) and variable effects on processing reward magnitude (Cardinal & Cheung, 2005; Galtress & Kirkpatrick, 2010). This may be the result of a global disruption in animals' ability to adaptively compare and select an outcome. However, not all studies show this deficit with NAcc lesions. Nicola (2007) and Floresco (2015) both suggest that the NAcc action selection function may be geared towards outcomes with a degree of uncertainty or delay. By contrast the dorsal striatum may facilitate action selection when outcomes are more predictable (Nicola, 2007). Hence lesioning the NAcc may disrupt selection of actions with delayed outcomes to a greater degree.

The BLA may be involved, along with the OFC, in the generation of an expected value signal for the delayed reward. It exhibits sustained activity during the delay period, and lesions and inactivations of the BLA increase DD (Winstanley et al., 2004b; Churchwell et al., 2009). Such a role is consistent with a broader role of the BLA in signaling the outcomes associated with various actions (Hatfield et al., 1996; Balleine et al., 2003), and representing value of conditioned stimuli (Cador et al., 1989; Burns et al., 1993; Gallagher, 2000). However ultimately very few studies have examined the BLA in DD.

The OFC is clearly intimately involved in DD based on the proclivity with which it is identified by human neuroimaging studies and the numerous preclinical results. It is also very clear that there are multiple different functions that the OFC is fulfilling. Based on the literature reviewed, there is evidence that the OFC is involved in discounting the delayed reward, encoding an expectancy signal for the delayed reward, and processes cues associated with the delayed reward. All of this is consistent with a more general

proposed role for the OFC in signaling the desirability of expected outcomes and a generating a common currency value signal of those expected outcomes (Schoenbaum et al., 2006; Kringelback, 2005; Montague and Berns, 2002).

The literature on HPC involvement is anomalous. It is not commonly found in human neuroimaging studies unlike the OFC, NAcc, and dIPFC (though some do find it), but it has perhaps the most consistent preclinical effects. Across methodological variations and paradigms, disrupting the HPC almost always causes an increase in DD without disrupting overall task behavior (Abela & Chudasama, 2012; 2013; Cheung & Cardinal, 2005; Mariano et al., 2009; Rawlins et al., 1985).

The other functions the HPC is involved in may provide a clue to its role in DD. There is strong evidence that the HPC is involved in working memory (Floresco et al., 1997; Seamans et al., 1998; Rawlins and Tsaltas et al, 1983). Working memory is negativity associated with DD (Shamosh et al., 2008), and increasing working memory load increases DD (Hinson et al., 2003). Also the HPC has been proposed to contribute to decision making via representing future outcomes which are converted to expected value signals by other downstream areas such as the OFC (Johnson et al., 2007; Schacter et al., 2007). Providing individuals with future episodic tags, to help them generate a representation of the delayed reward, decreases DD (Peters & Büchel, 2010). Moreover this behavioral manipulation is dependent upon the degree of functional coupling between the ACC and the hippocampus/amygdala (Peters & Büchel, 2010). Furthermore, these two different functions may be interconnected. Working memory resources may be necessary to substantiate a representation of the delayed outcome.

This role in the generation of outcome representations and working memory may be shared by the mPFC. Working memory has been shown to have an inverted U shaped dose response function in the rodent mPFC/primate dorsolateral PFC with regard to D1 receptor stimulation (Goldman-Rakic et al., 2000; Zahrt et al., 1997). This mirrors the effects of DA agonists and antagonists microinjected into the mPFC causing increased DD (Loos et al., 2010; Pardey et al., 2013; Sonntag et al., 2014; Yates et al., 2014), and the hypothesized effect of DA levels on DD in the mPFC's potential human analog the dIPFC (Boettiger et al., 2007; Gianotti et al., 2012; Smith & Boettiger, 2012; Kayser et al., 2012). Critically there is direct evidence in the mPFC for this outcome representation.

The PL cortex exhibits outcome specific delay period activity (Burton et al., 2009; Miyazaki et al., 2003). Also PL lesions block the effect of outcome devaluation suggesting they are necessary for outcome directed responding (Balleine and Dickinson, 1998; Balleine & O'Doherty, 2010; Killcross & Coutureau, 2003). Interestingly, it is also the PL that has the strongest connections to the BLA, OFC, NAcc core, and the HPC (Berendse et al., 1992; Brinley-Reed et al., 1995; Cassell & Wright, 1986; Hoover & Vertes, 2007; 2011; Gabbott et al., 2006; Shinonaga et al., 1994).

In sum, it may be the case that a network of areas contributes specific elements to delayed reward valuation and decision making. The mPFC, specifically the PL cortex, may be involved in generating an outcome representation of the delayed reward. Along with a representation generated by the HPC, these potential outcome representations are then converted to an expected value signal by the OFC, and potentially the BLA, such that different rewards can be compared in a common currency in the NAcc core. The OFC and BLA may also process any conditioned reinforcers and cues, and the OFC may also account for reward delay by lowering the expected value signal. The NAcc core then serves as a mechanism for reward selection and initiation of the motor moment necessary to choose a reward. It is also possible that the mPFC and HPC send information directly to the NAcc core in addition to any expected value signals the core may receive. Across this network there is built in redundancy.

## EXPERIMENT 1

### Experiment 1: Introduction

The goal at the outset of experiment 1 was to test the necessity of the rat mPFC for DD by inactivating it using designer receptors exclusively activated by designer drugs (DREADDs). It was hypothesized that inactivation of the mPFC would result in increased DD. Preclinical lesion and inactivation studies investigating the mPFC have been discordant indicating both an increase in DD or no change, but the paradigms used have response inhibition and behavioral perseveration methodological concerns (Cardinal et al., 2001; Feja & Koch 2014; Churchwell et al., 2009). However, DA D1 and D2 agonists and antagonists microinjected into the mPFC as well as viral mediated changes in D1 receptor expression have shown increased DD (Loos et al., 2010; Pardey et al., 2012; Yates et al., 2014; Sonntag et al., 2014). Electrophysiological recordings where a delay is imposed between a response and reward presentation show increased activity during the delay period which can be specific to the reward type (Burton et al., 2009; Miyazaki et al., 2003). One possible interpretation of these findings is that the mPFC maintains a representation of the delayed reward, which is then sent to “downstream” areas such as the BLA and OFC to be integrated into a common currency value signal. This value signal would then be summated and compared in the NAcc core with a corresponding value signal for the immediate reward. If this representation is necessary for the generation of the common currency value signal, then disrupting it via decreasing the activity of or inactivating the mPFC should in turn disrupt the value signal for the delayed reward only. Consequently, the main hypothesis of experiment 1 was that inactivation of the mPFC will result in increased DD.

The same hypothesis can be derived by looking at the literature translationally. The studies of DA involvement in DD in the rat mPFC suggest that an optimal level of DA is needed following the model of an inverted “U” for prefrontal function. A similar body of evidence exists for the primate dlPFC (Boettiger et al., 2007; Gianotti et al., 2012; Smith & Boettiger, 2012; Kayser et al., 2012). This parallel pattern between the human and rat literature suggests the rat mPFC could be used to model the primate

dIPFC. Obviously, the rat mPFC does not correspond one-to-one with the human dIPFC. However, it is hypothesized that they are related, possibility being divergent evolutionary paths from the same ancestral structure (Uylings et al., 2003; Seamans et al., 2008). Indeed the functions performed by the primate dIPFC, along with other cortical areas, appears to be mediated on a more rudimentary level by the rat mPFC. If these functions, whatever they may be, are needed for adaptively delaying gratification, then impairing them should increase the rate of DD.

Using the rat mPFC to model the primate dIPFC, the two system models of DD predict that inactivating the rat mPFC will lead to an increase in DD. This derivation allows for more “wobble-room” in what the rat mPFC may actually correspond to in the primate brain. Bechara (2005) lists the vmPFC, ACC and dIPFC as executive system structures. It has been argued that the rat mPFC actually displays functionality and physiology corresponding to the primate ACC and vmPFC in addition to the dIPFC (Seamans et al., 2008). Hence, the rat mPFC can be considered an executive system structure. Two system accounts postulate that the activity of the executive system is pitted against the activity in the impulsive system, and the system with the greater level of activity determines whether a delayed or immediate option is chosen. Decreasing the activity in the executive system via inactivating a structure should then shift a subjects’ pattern of choices to the immediate option. Therefore once again, inactivating the rat mPFC should result in increased DD.

DREADDs are a new tool available to further investigate the neurobiology underlying DD (See Urban & Roth, 2015, for an in depth discussion). DREADDs were created via mutagenesis of the human muscarinic (hM) receptor, and these novel receptors now lack any functional affinity for their endogenous ligand, acetylcholine (Armbruster et al., 2007). Rather, they respond to clozapine-N-oxide (CNO), a pharmacologically inert and bioavailable clozapine analog and metabolite (Krashes et al., 2011; Armbruster et al., 2007; Bender et al., 1994; Chang et al., 1998)<sup>24</sup>. Using a recombinant adeno associated virus (rAAV) delivery system, cells can be transfected

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<sup>24</sup> After the completion of this experiment MacLaren et al., (2016) published a paper indicating that CNO and its metabolites are not completely biologically inert. Specifically it is likely that CNO back metabolizes into clozapine and N-desmethylclozapine (N-Des). This caveat is discussed in the discussion of experiment 1.

with a DNA sequence encoding a DREADD receptor. The host cell then expresses the DREADD receptor, and if the rAAV is applied locally, CNO can be administered systemically to affect changes in only those cells transfected with and expressing the DREADD receptor. This allows for brain site specific manipulations with a systemic route of administration. Multiple studies have utilized this strategy combined with tying expression of the DREADD receptor to a specific promoter, such as Synapsin (Syn), to result in neuronal specific expression (Mahler et al., 2014).

There are multiple different DREADD receptors that have been created via mutating different muscarinic receptor subtypes, allowing for selective manipulations of different G-protein coupled signaling cascades. Mutagenesis of hM3 resulted in a DREADD receptor coupled to  $G_q$  (hM3Dq; Urban & Roth, 2015). When hM3Dq is expressed in neurons, application of CNO results in depolarization and increased excitability, but simply expressing the DREADD receptor did not change basal activity levels (Alexander et al., 2009). The DREADD receptor created from the hM4 receptor (hM4Di) is  $g_i$  linked and associated with a G-protein inward rectifying potassium current (GIRK) which induces hyperpolarization (Armbuster et al., 2007).

Studies of CNO's pharmacokinetics indicate that it rapidly crosses the blood brain barrier, and is quickly eliminated from CNS tissue and the circulatory system. Bender et al. (1994) injected NMRI mice (~30g) with 3-4nmol of radiolabeled clozapine or CNO into their tail vein. Animals were sacrificed at 2, 5, 10, 20 and 60 minutes post-injection, and the radioactivity of specific organs/structures was used as an index of CNO biodistribution. Maximum CNS signal was seen at 2 and 5 minutes after injection, and baseline levels were reached by 10 minutes. In the periphery, blood CNO levels peaked at 2 minutes and reached baseline levels by 20 minutes. The highest concentrations were seen in the kidneys 2 minutes post injection followed by the liver at 5, 10, and 20 minutes. In summary, CNO reaches the CNS within 2 minutes and is at pre-injection levels by 20 minutes in both CNS and blood.

In contrast to the rapid elimination of CNO, several studies have demonstrated prolonged behavioral and electrophysiological effects. In a transgenic mouse line expressing the hM3Dq DREADD receptor under the control of the CaMKII $\alpha$  promoter in the cortex, hippocampus, and other areas, administration of 0.3mg/kg CNO resulted in

increased motor activity versus controls for a 9 hour period and increased local field potential gamma power for 9 hours as well (Alexander et al., 2009). Utilizing an hM4Di DREADD and 3mg/kg CNO to inactivate the ventral tegmental area decreased NAcc DA release measured by fast scan cyclic voltammetry for over an hour post injection (Ferguson et al., 2011). Using dual-recombinase genetic techniques to knock in and selectively express to express the hM4Di in serotonergic neurons, administration of 10mg/kg CNO decreases body temperature within 10 minutes reaching peak effects at approximately 1-2 hours (Ray et al., 2011). Finally, activation of an hM4Di DREADD in the motor cortex via systemic CNO injection blocks seizure related activity caused by microinjection of the convulsant picrotoxin. Specifically, increased immediate frequency and power in the 4-14hz range caused by pilocarpine were reversed within 20 minutes of CNO injection, and the reversal lasted through the final timepoint at 70 minutes (Kätzel et al., 2014). Hence DREADD receptor activation, via hM3Dq or hM4Di, onsets within 20 minutes and lasts on a timescale measured in hours.

Repeated activation of DREADDs also appears to result in continued effects on behavior and neural activity. After a 0.3mg/kg dose of CNO combined with an hM3Dq DREADD increased local field potential gamma power for 9 hours, a second administration 24 hours later and did not see any decrease in effect (Alexander et al., 2009). Chronically stimulating parabrachial nucleus calcitonin gene –related peptide (PBelo CGRP) neurons with an hM3Dq DREADD receptor and CNO (1mg/kg injection every 12 hr for 8 days) caused large and repeated decreases in food intake across days as well as a corresponding decreases in body weight. Conversely, chronically inhibiting PBelo CGRP neurons after administration of anorexogenic compounds using an hM4Di DREADD receptor and the same CNO regime partially rescued the decrease in intake and blocked the starvation observed in control animals (Carter et al., 2013). Selectively inhibiting serotonergic neurons with hM4Di and a 10mg/kg CNO injection repeatedly did show a reduction in effect size with multiple administrations, with regards to a decrease in body temperature (Ray et al., 2011). However, body regulation temperature is a critical homeostatic function subserved by multiple mechanisms. Thus is difficult to infer whether this decreasing effect size is due to a loss of function of the hM4Di receptor with repeated administrations or a learned conditioned response to mitigate the severe

hypothermia which would follow an injection of CNO. Regardless, using DREADDs to manipulate neural activity and thus behavior multiple times is a viable experimental methodology.

Experiment 1 sought to test the hypothesis that the mPFC is involved in maintaining a representation of the delayed reward that is necessary for DD. In animals completing an AA DD task, an hM4Di inhibitory DREADD with a mCherry tag was transfected and expressed in the rat mPFC. A dose response of CNO was tested. It was hypothesized that activation of the hM4Di DREADD would result in a disruption of the potential representation of the delayed reward. This, in turn, would prevent subjects from appropriately valuing the delayed reward and cause an increase in DD represented by a decrease in indifference points.

## Experiment 1: Methods

### Animals

Twenty-five male Long Evans rats obtained from Harlan (Indianapolis, IN) served as research subjects. All animals were age matched at approximately 50 days at the start of the experiment and weighed between 310g and 277g ( $M=294.52$ ,  $SD=8.37$ ). Throughout the experiment animals had ad libitum access to water and were individually housed in polypropylene shoebox cages and were maintained on a 12 hour reverse light/dark cycle. Animals were run during dark period in four separate cohorts one time per day, 5 consecutive days per week. For all behavioral testing, animals were food restricted to 85% of their free feeding weight.

### Apparatus

Behavioral testing was conducted in modular operant chambers with electrical inputs and outputs controlled by an IBM compatible PC (Med-Associates, St. Albans, VT; 30 x 30 x 24.4cm). All chambers had a house light, stainless steel bar floor, and were enclosed in sound attenuating boxes with exhaust fans for ventilation and masking external noise. Chambers were equipped with a nosepoke recess with an internal stimulus light and a photocell to record beam breaks. The nosepoke was centered on the front wall

2 cm above the floor. A 4,500Hz tone generator was located 18cm above the nosepoke. On both sides of the nosepoke recess were retractable levers with stimulus lights 4cm above each lever. Opposite the nosepoke was a retractable graduated cylinder tube equipped with a lickometer, stainless steel spout containing double ball bearings, and rubber stopper.

### **Delay Discounting Task**

An AA DD procedure based on Beckwith and Czachowski (2014) was used. Training was conducted in a series of stages each with a criterion for advancement. After being handled for 4 days, animals received 200 free licks of the sipper tube containing a 10% sucrose solution (w/v) and then were hand shaped to press either lever for 20 seconds of access to the sipper tube. During the session the levers remained extended and the stimulus lights were illuminated above both levers signaling the availability of reinforcement for pressing the lever. Once animals completed 10 trials in less than or equal to 20 minutes they moved onto the second stage of training. Here subjects no longer received any free licks and responded on an fixed ratio (FR) 1 on either lever for 10 seconds of access to the sipper tube. To advance subjects were required to complete 20 trials in less than or equal to 60 minutes.

In the third stage of training, the levers were retracted to start the session, the stimulus lights were extinguished, and the nosepoke's internal stimulus light was turned on. Animals were hand shaped to nosepoke on an FR1 for extension of the levers, turning on the stimulus lights, and extinguishing of the nosepoke's internal stimulus light. Upon pressing either lever all the stimulus lights extinguished, levers retracted, and the sipper tube extended into the chamber for 10 seconds. After the sipper tube retracted, a 5 second inter-trial interval (ITI) was imposed. The nosepokes internal stimulus light turned on after the ITI elapsed signaling the start of the next trial. Forced choice trials were also introduced in stage 3 of training. If an animal pressed the same lever two consecutive times, on the following trial only the opposite lever and stimulus light were activated. After animals completed at least 30 trials in 60 minutes the sipper access was reduced to 5 seconds and the ITI increased to 25 seconds, and a new a criterion of 40 trials in 60 minutes was imposed. In the fifth and final stage of training, the sipper access

was further reduced to 2 seconds and a 28 second ITI was used. Animals had to have at least 3 sessions and at least 40 trials in 60 minutes to advance. During the last 3 sessions training, lever preference was assessed for each subject. The stages of training are summarized in table 6.

**Table 6: Experiment 1 Delay Discounting Training Stages**

Stage	Nosepoke	Forced Trials	Sipper Access (s)	ITI (s)	Criteria
1	No	No	200 free licks & 20	0	$\geq 10$ trials in 20min
2	No	No	10	0	$\geq 20$ trials in 60min
3	Yes	Yes	10	5	$\geq 30$ trials in 60min
4	Yes	Yes	5	25	$\geq 40$ trials in 60min
5	Yes	Yes	2	28	$\geq 40$ trials in 60min & $\geq 3$ sessions

\*Note: s=seconds; min=min.

Next the 0 second delay (0sD) condition began. The same pattern of chained responding occurred. However, a response on the nonpreferred lever from stage 5 of training yielded a standard reward of 2 seconds of access to the sipper tube, and a response on the preferred lever resulted in the delivery of an adjusting, alternative reward. The adjusting, alternative reward started at 1 second of access to the sipper tube, but then titrated by .2 second increments based on the subjects choices ranging between 0 and 3 seconds. Selection of the standard reward caused an increase, and selection of the alternative reward caused a decrease on the next trial. The median of the last 20 alternative reward amounts was taken as an indifference point (IDP). Moreover, a variable ITI was implemented ensuring 30 seconds always elapsed between when the animal made a selection and when the next trial began preventing subjects from earning several alternative rewards in the time it would take to earn one standard reward. To advance, animals had to demonstrate magnitude discrimination and complete  $\geq 20$  free choice trials. Magnitude discrimination was operationally defined as exhibiting an average indifference point of 1.5 seconds or greater across 4 days.

Once all animals met criteria for magnitude discrimination, viral microinjection surgeries occurred. After surgeries the 0sD was in place for another two weeks to reassess magnitude discrimination. A four second delay (4sD) to the standard reward was then implemented for 5 sessions. During the delay to the standard reward, the stimulus

light above the delayed lever remained on until the standard reward was delivered (i.e., a CDP was used). Next an eight second delay (8sD) was implemented for another 5 sessions, and then the delay decreased back to 4 seconds for another 5 sessions before the CNO injections.

### **Viral Microinjection Surgeries**

Rats were anesthetized with isoflurane and placed in a stereotaxic apparatus (Benchmark Digital Stereotaxic; myNeurolab, St. Louis, MO), and given 5mg/ml/kg ketoprofen (Zoetis Inc., Kalamazoo, MI) via subcutaneous (s.c.) injection, 2.5mg/kg Maricane (Hospira Inc., Lake Forest, IL) via s.c. injection at the site of the incision in a concentration of 5mg/ml, and Cefazolin (West-Ward Pharmaceutical Corp., Eatontown, NJ) 30mg/ml/kg via intraperitoneal (i.p.) injection. Then stainless steel guide cannulae (26 gauge) with removable wire obturators (33 gauge) extending 1mm beyond the cannulae were lowered to into the mPFC (A.P. +3.2mm, M.L.  $\pm$ .7mm, and D.V. - 3.3mm). The obturators were then removed and stainless steel microinjectors extending 1mm beyond the cannulae were used to inject 1 $\mu$ l/side of rAAV8-hSyn-hM4Di-mCherry ( $8.3 \times 10^{12}$  viral molecules/mL; University of North Carolina Vector Core) at a rate of .1 $\mu$ l per minute. An additional 10 minutes post microinjection was provided to ensure diffusion into the surrounding tissue before removing the microinjectors. Sterile bone wax (Surgical Specialties Corp., Reading, PA) was used to repair the skull after injections, and the incision was sutured closed. Post-surgery, animals were held in isolation for one week and were given 10ml/kg of sterile saline (s.c.) and wet food to aid in recovery.

### **Clozapine-N-Oxide Administration**

Clozapine-N-Oxide (CNO; Tocris Bioscience, Bristol, UK) was administered at doses 0, 3, and 9mg/kg/ml (i.p.) in 1% Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and sterile water with a 30 minute pretreat. Each dose was given for 5 consecutive days, and was followed by a washout period of 2 days (where no behavioral testing occurred). The order of the different doses was administered via a latin square

design. All doses were tested at a delay of 4 seconds to the standard reward. This delay was selected to ensure sufficient signal window to detect a decrease in DD.

### **Immunohistochemistry**

After behavioral experiments, all animals were transcardially perfused with phosphate buffered saline (1xPBS) and 4% formaldehyde made up from paraformaldehyde in 1xPBS no more than 24 hours before perfusions. Brains were postfixed in 4% formaldehyde for 24 hours. Brains were then cryoprotected by soaking in 20% sucrose in 1xPBS for 48-72 hours followed by 30% sucrose in 1xPBS for another 48-72 hours. Brains were then SNAP frozen in isopentane chilled via dry ice and sectioned at 40 $\mu$ m on a cryostat at -20°C.

Sections were blocked in 5% normal goat serum in 1xPBS (5% NGS) for 1 hour. Next sections were incubated for 18 hours in rabbit anti-mCherry (ABCAM ab167453; 1:500 in 5% NGS) at 4°C on a plate shaker followed by 3x5min washes in 1xPBS. Then goat anti-rabbit IgG AlexaFluor594 (ABCAM ab150080; 1:1000 in 5% NGS) was added and incubated for 1 hour at room temperature on a plate shaker. Finally sections were washed in 1xPBS (3x5min) and rinsed in Milli-Q water before mounting on subbed slides and coverslipped with Dako fluorescent mounting medium (Agilent Technologies, Santa Clara, CA). Images were subsequently acquired with a Leica LMD 6500 system (Leica Microsystems, Buffalo Grove, IL). Hits were dichotomously coded based upon bilateral expression of hM4Di-mCherry in the PL cortex (Paxinos & Watson, 1998) when experimenters were blind to behavioral results. Animals were not deemed misses if the expression of the hM4Di-mCherry extended beyond the bounds of the PL region.

### **Electrophysiological Recordings**

Prior to perfusion, a subset of animals underwent electrophysiological recording under urethane anesthesia. 64-channel silicon probes (Cambridge Neurotech, Newmarket, Suffolk, U.K.) and custom multi-tetrode probes made in house and gold electroplated to an impedance of approximately 300k $\Omega$  were lowered into the mPFC. Signals were amplified and digitized by 64 and 32 channel headstages (Intan, Los Angeles, CA) respectively. Raw data (30 kHz) was acquired using an Open Ephys recording system

comprised of a multi-channel electrophysiology acquisition board (Open Ephys) and a PC computer running Linux with Open Ephys GUI. After lowering the probes, a baseline period of 30 minutes was recorded then animals received a vehicle injection (0mg/kg; i.p.). 9mg/kg CNO was administered at least 40 minutes after the vehicle injection, and recordings continued for another 120 minutes. Single units were identified, automatically spike sorted, and manually refined in phy (Rossant et al., 2016). Finally, spike timestamps were exported using Matlab, binned in 5 minute increments, and z-score transformed to limit unit to unit variability.

### **Data Analysis**

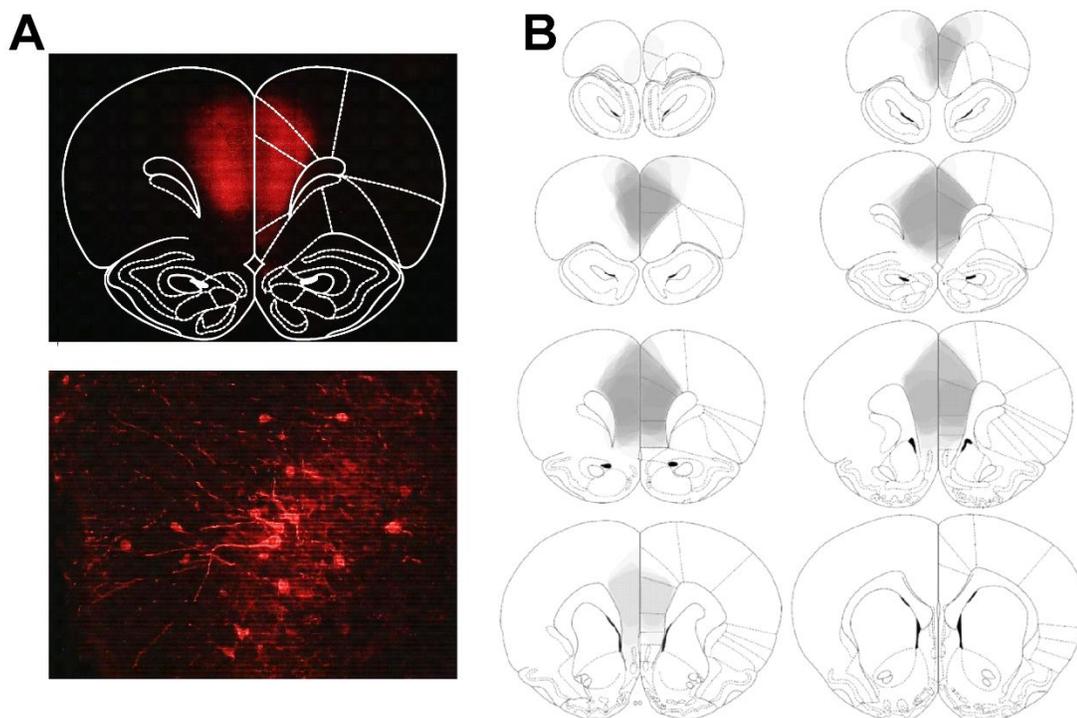
For behavioral data, normality assumptions were tested via visual inspection of histograms and q-q plots as well as Shapiro-Wilks tests. If normality and homogeneity of variance assumptions were violated, log10 transformations were used for right skewed data, and for left skews the data were reflected, anchored at 1, and log10 transformed. If data transformations were unable to normalize the data, non-parametric tests were used.

Primary data analysis was conducted using repeated measures analysis of variance (ANVOA) with factors of dose (0-, 3-, & 9mg/kg) and day (1-5). Main effects of dose were followed up with paired samples t-tests with Dunnett's corrected alpha levels ( $\alpha=.05/2$  comparisons=.025) with 0mg/kg serving as the control condition. Main effects of day were followed up with polynomial trend analysis. Curve fitting started with centered, 0 order functions and worked up until a more complex function no longer provided a significantly better fit. Dose by day interactions were followed up by examining the effect of day inside of each dose with polynomial trend analysis and by examining the effect of dose inside each day with Dunnett's corrected paired sample t-tests inside of each day ( $\alpha=.05/(2 \times 5)$  comparisons)=.005).

### **Experiment 1: Results**

The final n after all sources of attrition was 11 animals who exhibited bilateral expression of the hM4Di-mCherry in the PL cortex (hits). For sources of attrition, 1 animal failed to learn how to lever press, 6 animals died as a result of surgical complications, and 7 animals did not express hM4Di-mCherry bilaterally in the PL

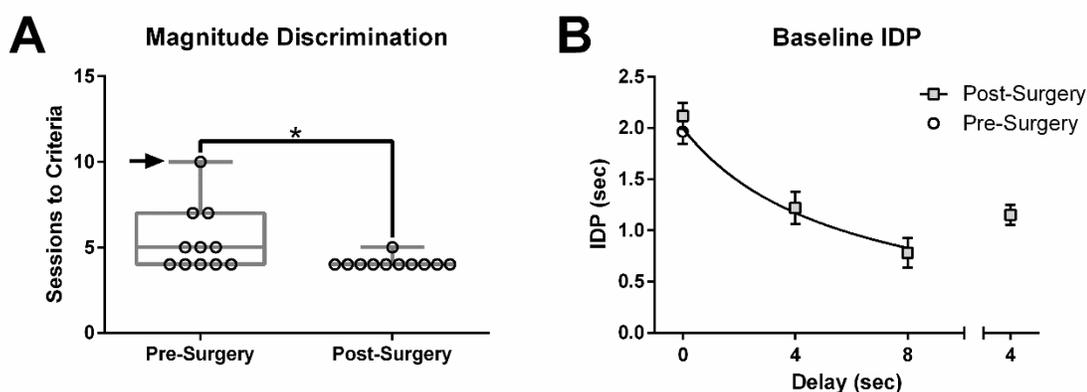
Cortex (misses). Figure 1B displays the range of expression in hits which included the mOFC (8/11), IL (2/11), and Cg1 (7/11) areas. Inside the 7 animals classified as misses, animals had bilateral expression limited to the MO cortex (5/7), unilateral expression in the PL cortex (1/7), and unilateral expression in the VO cortex (1/7).



**Figure 1:** Experiment 1 hM4Di Expression **A)** Representative images of hM4Di expression. **B)** Combined expression map across all animals classified as hits. The area of expression was visual drawn for each subject, made partially transparent and then overlaid. Diagrams are adapted from Paxinos and Watson (1998).

Among animals classified as hits, both before ( $Mdn=5$ ,  $Mode=4$ ,  $Range=6$ ) and after ( $Mdn=4$ ,  $Mode=4$ ,  $Range=1$ ) surgeries the majority of subjects took 4-5 sessions to reach magnitude discrimination criteria. Pre-surgery the distribution was right skewed with one extreme outlier two  $SD$  beyond the mean and post-surgery only one subject took more than the minimum number of sessions giving rise to a near uniform distribution. A Wilcoxon signed rank's test indicated that after surgery subjects took fewer sessions to reach magnitude discrimination criteria,  $Z=-1.983$ ,  $p=.047$ . Excluding the extreme outlier reduced this effect to a trend,  $Z=-1.730$ ,  $p=.084$ . IDPs when animals met magnitude discrimination criteria did not differ before ( $M=1.96$ ,  $SD=.39$ ) or after surgery ( $M=2.13$ ,  $SD=.47$ ),  $t(10)=-.845$ ,  $p=.418$ . However post-surgery increasing the delay to the standard

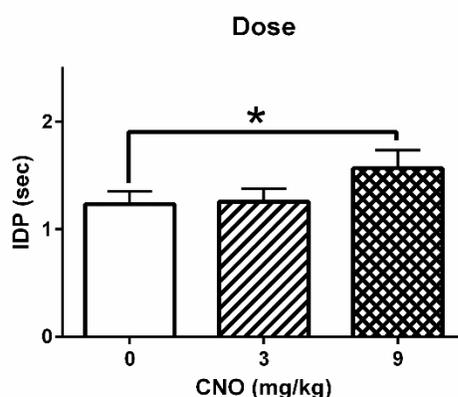
reward decreased IDP,  $F(2,20)=44.345$ ,  $p<.001$ . Specifically Bonferroni corrected paired sample t-test revealed that increasing the delay to 4,  $t(10)=7.2$ ,  $p<.001$ , as well as 8 seconds,  $t(10)=7.954$ ,  $p<.001$ , decreased indifference points relative to the 0sD condition. Moreover the 8sD had lower indifference points compared to the 4sD,  $t(10)=3.187$ ,  $p=.010$ . In a specific a priori planned comparison to determine if IDPs were stable across time, the IDPs at the 4sD both before and after a week of the 8sD condition were compared with a uncorrected paired sample t-test and no difference was found,  $t(10)=.360$ ,  $p=.727$ . A hyperbolic equation (Mazur, 1987) was fit to the mean IDPs through the determination of the 8sD. The best fitting  $k$  value was .1768 with a standard error of 0.02494. The model fit the data extremely well with an  $R^2$  value of 0.98.



**Figure 2:** Experiment 1 Magnitude Discrimination and Baseline Indifference Points (IDP). **A)** Boxplots of the number of sessions animals required to reach magnitude discrimination criteria. The arrow indicates the outlier that was excluded in the second analysis where the indicated significant difference dropped to a trend. \* $p<.05$  on Wilcoxon signed rank test. **B)** Mean ( $\pm$ SEM) IDP plotted as a function of pre- vs. post-surgery and delay. The break in the x-axis is used to separately represent the second determination of a 4 second delay to the standard reward.

Indifference points (IDP), the main dependent variable, were normally distributed. A main effect of dose,  $F(2,20)=5.5$ ,  $p=.018$ , was observed, but effects of day,  $F(4,40)=0.4$ ,  $p=.797$ , and the interaction of dose by day,  $F(8,80)=1.3$ ,  $p=.278$ , were not significant. Dunnett's corrected paired samples t-test revealed that only the 9mg/kg dose,  $t(10)=2.8$ ,  $p=.018$ , was significantly different from the 0mg/kg dose. The same repeated measures ANOVA was conducted in animals classified as misses ( $n=7$ ) as well. In

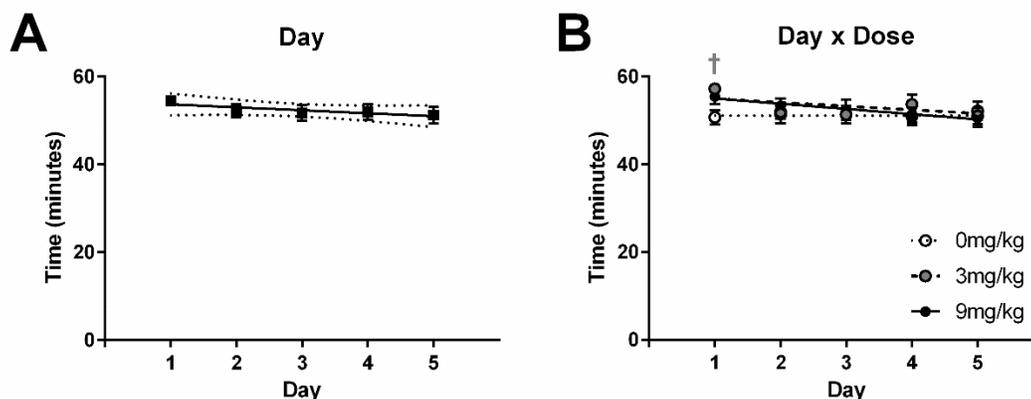
misses, no significant effects of dose,  $F(2,12)=0.5$ ,  $p=.594$ , day,  $F(4,24)=0.3$ ,  $p=.897$ , or an interaction of dose by day,  $F(8,48)=0.6$ ,  $p=.775$ , were observed.



**Figure 3:** Experiment 1 Indifference points (IDP) in animals classified as hits. Mean ( $\pm$ SEM) IDP in seconds graphed by dose. \* $p<.05$  on Dunnett's test.

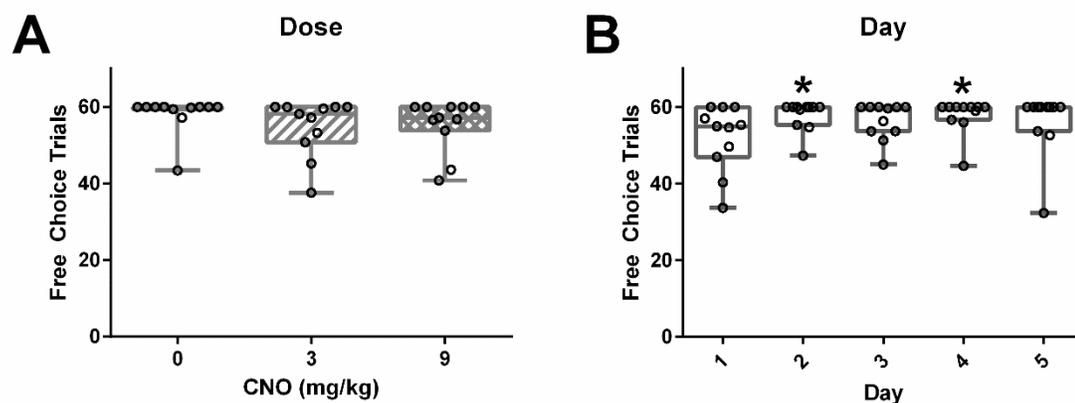
The delayed lever preference inside the last 20 free choice trials, termed the indifference point choice ratio (IDP CR), was normally distributed. A 2 way repeated measures ANOVA found no effect of dose,  $F(2,20)=1.7$ ,  $p=.205$ , day,  $F(4,40)=0.4$ ,  $p=.829$ , or dose by day interaction,  $F(8,80)=1.1$ ,  $p=.352$ . Additionally, one-sample t-tests were conducted inside each dose using 0.5 as a test value. IDP CR was not significantly different from a hypothetical population value of 0.5 at either the 0mg/kg,  $t(10)=0.3$ ,  $p=.744$ , 3mg/kg,  $t(10)=0.8$ ,  $p=.430$ , or 9mg/kg dose,  $t(10)=1.9$ ,  $p=.085$ . Mean values are reported in table 7.

The time it took to complete a session in minutes was not affected by dose,  $F(2,20)=2.3$ ,  $p=.131$ , but was by day,  $F(4,40)=2.6$ ,  $p=.049$ . There was a trend towards a day by dose interaction,  $F(8,80)=2.0$ ,  $p=.054$ . Trend analysis following up the effect of day revealed, that there was a negative linear trend across all days that failed to reach significance,  $F(1,53)=1.8$ ,  $p=.188$ , nor did Dunnett's follow up tests find any significant effects. Following up the trending interaction, there were no significant linear trends inside any dose. Only, inside the first day was the 3mg/kg dose trending towards increased time after corrections for multiple comparisons,  $t(10)=3.5$ ,  $p=.005$ .



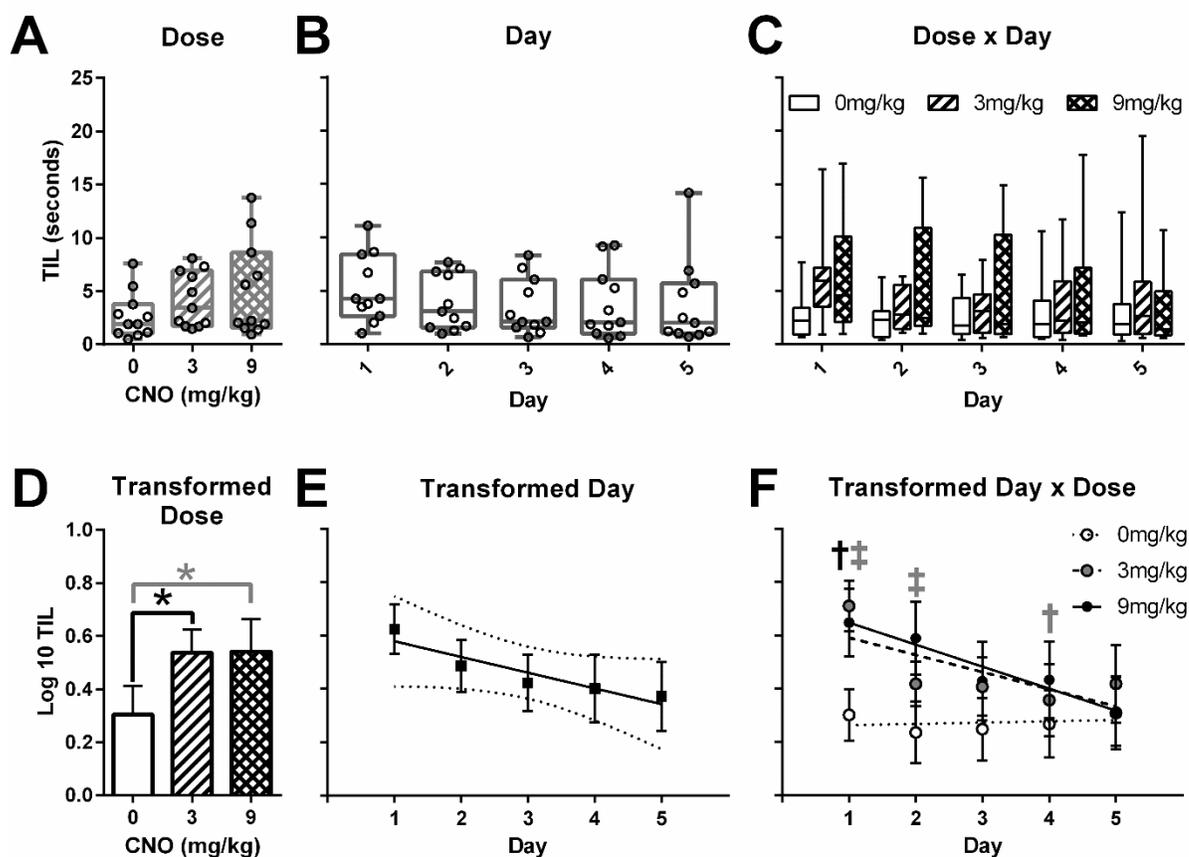
**Figure 4:** Experiment 1 Session Time. **A)** Mean ( $\pm$ SEM) session time in minutes collapsed across dose and plotted as a function of day along with the regression line and 95% confidence band. **B)** Mean ( $\pm$ SEM) session time in minutes plotted separately by dose as a function of day with regression lines. †(grey) trend for 3mg/kg vs. 0mg/kg inside day.

The number of free choice trials completed were left skewed, and the median number of trials for all doses approached 60 trials (0mg/kg: Mdn=60, Range =16.60; 3mg/kg: Mdn=58.2, Range=22.40; 9mg/kg: Mdn=57.2, Range=19.20). Data transformations failed to correct for the deviation from normality. Friedman's tests showed a trend towards an effect of dose,  $\chi^2(2)=5.25$ ,  $p=.072$ , and an effect of day,  $\chi^2(4)=16.794$ ,  $p=.002$ . Dunnett's corrected Wilcoxin signed rank tests using day 1 as the control condition showed days 2,  $Z=-2.521$ ,  $p=.012$ , and 4,  $Z=-2.521$ ,  $p=.012$ , had a greater number of trials completed. Forced choice trials were normally distributed and centered around 18 trials (Table 7). No effect of dose was observed,  $F(2,20)=0.5$ ,  $p=.64$ . There was no significant effect of day,  $F(4,40)=0.8$ ,  $p=.53$ . Finally, there was no interaction of dose and day,  $F(8,80)=0.5$ ,  $p=.84$ , for forced choice trials.



**Figure 5:** Experiment 1 Free Choice Trials. **A)** Boxplots of free choice trials as a function of dose. **B)** Boxplots of free choice trials as a function of day. \*significantly different from day 1 on Wilcoxin signed ranks with a Dunnett's corrected alpha.

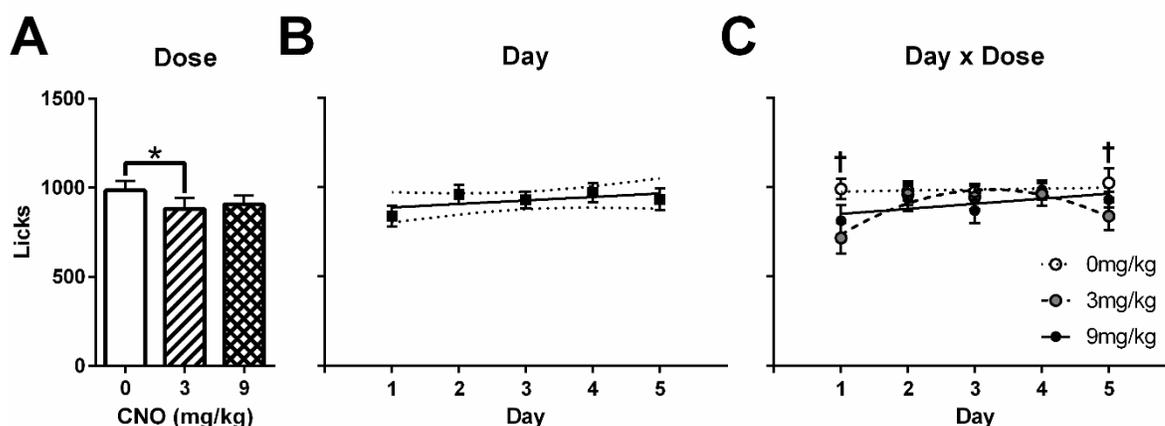
Median trial initiation latencies from each session (TIL) were right skewed and were normalized by log10 transforming them. The log10 TIL exhibited an effect of dose,  $F(2,20)=4.8$ ,  $p=0.019$ , an effect of day,  $F(4,40)=3.7$ ,  $p=.012$ , and a dose by day interaction,  $F(8,80)=2.4$ ,  $p=.025$ . After correcting for multiple comparisons, significantly greater log10 TIL were seen in the 3mg/kg dose,  $t(10)=4.5$ ,  $p=.001$ , and a trend towards greater log10 TIL in the 9mg/kg dose,  $t(10)=2.4$ ,  $p=.036$ , versus 0mg/kg. Trend analysis following up the effect of day revealed a linear decrease across days that approached significance,  $F(1,53)=2.9$ ,  $p=.09$ . Following up the day by dose interaction, inside each dose, a linear function best described the change across days. However, only the 3mg/kg,  $F(1,53)=3.1$ ,  $p=.09$ , and 9mg/kg,  $F(1,53)=3.8$ ,  $p=.056$ , approached and trended toward a significant negative change across days. Compared to 0mg/kg, Dunnett's testing inside of each day revealed the 3mg/kg dose induced significantly increased log10 TIL on day 1,  $t(10)=4.5$ ,  $p=.001$ , as well as a trend on day 4,  $t(10)=3.0$ ,  $p=.014$ . Meanwhile the 9mg/kg dose trended towards increased log10 TIL on day 1,  $t(10)=3.0$ ,  $p=.013$ , and day 2,  $t(10)=2.5$ ,  $p=.033$ .



**Figure 6:** Experiment 1 Trial Initiation Latencies (TIL). **A)** Boxplots of untransformed TIL by dose. **B)** Boxplots of untransformed TIL by day. **C)** Boxplots of untransformed TIL by both day and dose. **D)** Mean ( $\pm$ SEM) log<sub>10</sub> TIL by dose. \*Significantly different versus 0mg/kg on Dunnett's corrected paired sample t-tests; \*(grey) trend vs. 0mg/kg. **E)** Mean ( $\pm$ SEM) log<sub>10</sub> TIL plotted as function of day with the best-fitting regression line and 95% confidence band. **F)** Mean ( $\pm$ SEM) log<sub>10</sub> TIL plotted separately by dose as a function of day. † Significantly difference between 0mg/kg and 3mg/kg on Dunnett's corrected paired sample t-test †(grey) trend for 3mg/kg vs. 0mg/kg. ‡(grey) trend 9mg/kg vs. 0mg/kg.

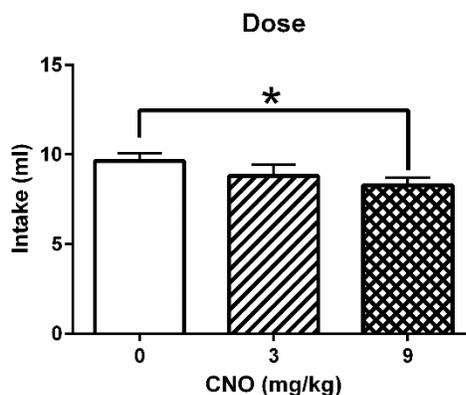
Free choice latencies from each session (CL) exhibited a mild right skew. Median CL inside each dose were 1.08 (Range=.94), 1.00(Range=1.41), and 1.11(Range=0.85) for the 0-, 3-, and 9mg/kg doses respectively. Log<sub>10</sub> transforming CL corrected the deviation from normality. However log<sub>10</sub> CL did not show effects of dose,  $F(2,20)=0.3$ ,  $p=.73$ , day,  $F(4,40)=1.7$ ,  $p=.16$ , and dose by day,  $F(8,80)=1.4$ ,  $p=.22$ . Untransformed mean values are reported in table 7.

The number of licks animals earned was distributed normally. Main effects of dose,  $F(2,20)=3.8$ ,  $p=.039$ , and day,  $F(4,40)=3.7$ ,  $p=.012$ , as well as a dose by day interaction,  $F(8,80)=2.2$ ,  $p=.033$ , were significant. Follow-up paired samples t-test revealed that there were fewer licks at the 3mg/kg dose versus the 0mg/kg dose,  $t(10)=4.5$ ,  $p=.001$ , but not the 9mg/kg dose,  $t(10)=1.7$ ,  $p=.12$ . Across all doses, the effect of day was best characterized by a linear function,  $a=924.7$ ,  $b_1=19.45$ . However, the slope was not significantly different from zero,  $F(1,53)=1.3$ ,  $p=.27$ . Inside each day, there were significant effects of the 3mg/kg dose at days 1,  $t(10)=3.8$ ,  $p=.003$ , and 5,  $t(10)=4.3$ ,  $p=.002$ . Polynomial trend analysis found that the 3mg/kg dose's effect across days was best described by a quadratic function,  $a=982.5$ ,  $b_1=24.58$ ,  $b_2=50.04$ . The 0mg/kg,  $F(1,53)=0.1$ ,  $p=.78$ , and 9mg/kg,  $F(1,53)=1.8$ ,  $p=.19$ , doses were best described by a linear function across days, but neither reached significance.



**Figure 7:** Experiment 1 Licks. **A)** Mean ( $\pm$ SEM) licks graphed as a function of dose. **B)** Mean ( $\pm$ SEM) licks collapsed across dose and plotted by day along with a regression line and 95% confidence band. **C)** Mean ( $\pm$ SEM) licks separately plotted by dose as a function of day. Regression lines correspond to the best fitting polynomial. † $p<.05$  3mg/kg vs. 0mg/kg Dunnett's test within each day.

Intake of 10S in milliliters (ml) was normally distributed. A main effect of dose,  $F(2,20)=5.9$ ,  $p=.009$ , was observed. Effects of day,  $F(4,40)=2.0$ ,  $p=.114$ , and dose by day,  $F(8,80)=1.8$ ,  $p=.097$ , were not significant. Dunnett's test revealed 9mg/kg dose,  $t(10)=4.3$ ,  $p=.002$ , decreased intake relative to the 0mg/kg dose. Mean values are graphed in figure 8.



**Figure 8:** Experiment 1 Intake of 10% sucrose (10S) in milliliters. Mean ( $\pm$ SEM) intake in ml as a function of dose. \* $p < .05$  vs. 0mg/kg dose on Dunnetts corrected paired sample t-test.

**Table 7:** Experiment 1 Secondary Variables. Mean ( $\pm$ SEM)

Variable	0mg/kg	3mg/kg	9mg/kg
IDP CR	0.49(0.02)	0.48(0.03)	0.54(0.02)
Session Time (minutes)	51.14(1.64)	53.32(1.51)	52.68(1.65)
Forced Choice Trials	18.62(0.75)	18.06(0.83)	17.98(0.90)
Choice Latencies	1.13(0.08)	1.16(0.13)	1.17(0.09)

For the electrophysiological recordings, 30 individual units were identified. Firing rates were assessed in 5 minute bins and the spikes per bin was z-score transformed inside each unit to account for disparate baseline firing rates (zSpikes). To grossly examine if CNO administration affected zSpikes, a repeated measures ANOVA was conducted collapsing across bin on condition (vehicle, baseline, 9mg/kg CNO). This analysis found a main effect of condition,  $F(2,58)=8.419$ ,  $p < .001$ . Paired sample t-tests with Bonferroni corrected alpha levels ( $\alpha = .017$ ) found that the CNO condition differed from both the vehicle,  $t(29)=3.628$ ,  $p = .001$ , and baseline conditions,  $t(29)=2.722$ ,  $p = .011$ , which did not differ from each other,  $t(29) = -.607$ ,  $p = .548$ .

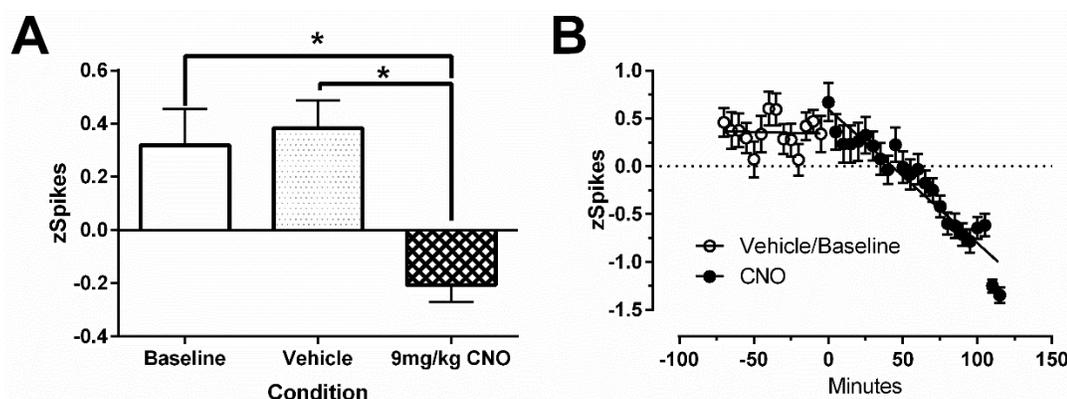
A hierarchical multiple regression was then conducted with z-score transformed firing rates across all bins as the outcome variable. Steps 1-3 progressively included CNO (CNO vs. baseline/vehicle), bin (centered), and a CNO by bin interaction in that order. Congruent with the results of the ANOVA, dummy coded CNO predicted a significant amount of variance in firing rates across all bins,  $\beta = -.275$ ,  $p < .001$ , in the first step, such that firing rates were lower with CNO on board. In the second step, there was a

significant increase in  $R^2$  up from .076 to .204,  $F(1, 1137)=183.767$ ,  $p<.001$ . CNO remained significant predictor,  $\beta=.271$ ,  $p<.001$ , and bin also significantly predicted a unique amount of variance,  $\beta=-.653$ ,  $p<.001$ . Including the interaction term in the third step, again resulted in a significant increase in the amount of variance explained,  $F(1,1136)=35.187$ ,  $p<.001$ . However neither CNO,  $\beta=-.025$ ,  $p=.718$ , nor bin,  $\beta=-.020$ ,  $p=.864$ , predicted unique variance from the interaction term,  $\beta=-.446$ ,  $p<.001$ .

To follow up the interaction, bin was regressed onto zSpikes separately after CNO administration and during vehicle and baseline. For the baseline/vehicle regression, the model failed to significantly predict variation in zSpikes,  $F(1,418)=.026$ ,  $p=.872$ . However, the CNO model did explain a significant amount of variance,  $F(1,718)=243.084$ ,  $p<.001$ , with bin showing a negative relationship with zSpikes,  $\beta=-.503$ ,  $p<.001$ . In sum, when CNO was on board zSpikes were lower compared to baseline and vehicle. Under baseline/vehicle conditions zSpikes displayed no significant linear trend, but under CNO zSpikes tended to decrease across bins. Both model and predictor level statistics from the hierarchical multiple regression are reported in table 8, and mean zSpikes as a function of condition and condition by time are graphed in figure 9.

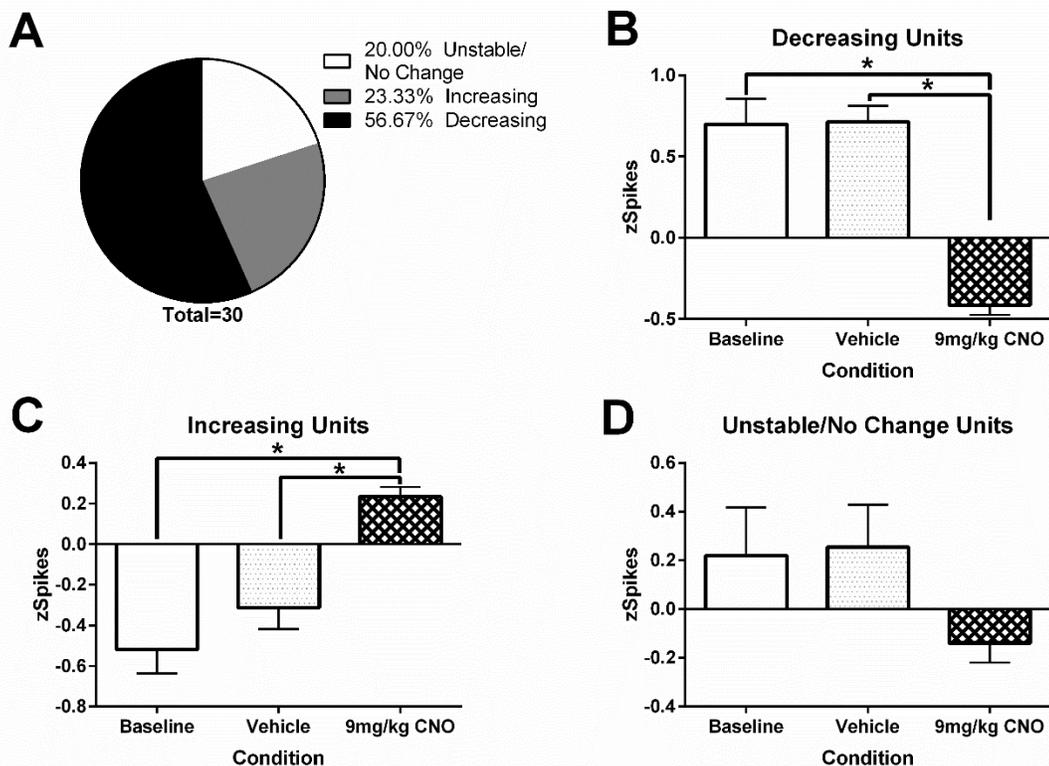
**Table 8: Experiment 1 All Units Regression Analysis.**

Step	Model Statistics			Predictor	Predictor Statistics		
	<i>R</i>	<i>F</i>	<i>p</i>		$\beta$	<i>b</i>	<i>p</i>
1	.275	93.185	<.001	Constant	--	.355	<.001
				CNO	-.275	-.563	<.001
2	.452	183.767	<.001	Constant	--	-.350	<.001
				CNO	.271	.554	<.001
				Bin	-.653	-.059	<.001
3	.478	35.187	<.001	Constant	--	.334	.012
				CNO	-.025	-.051	.718
				Bin	-.020	-.002	.864
				CNO x Bin	-.446	-.068	<.001



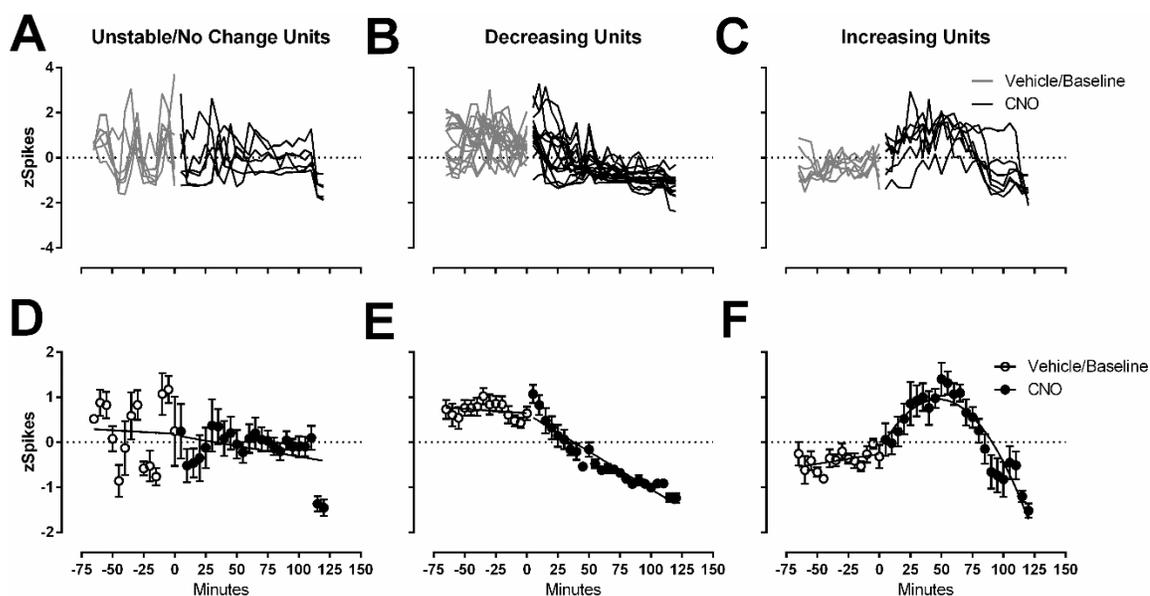
**Figure 9:** Experiment 1 All units z-score transformed firing rates. **A)** Mean ( $\pm$ SEM) z-score transformed spikes per 5 minute bin. \*significantly different on paired sample t-tests with Bonferroni corrected  $\alpha$  for multiple comparisons.

Visual inspection individual unit activity revealed that some neurons actually increased their firing rate in response to CNO administration. Visually neurons were then categorized into increasing (7), decreasing (17), and unstable/no change categories (6). Inside unstable/no change units the RMANOVA collapsing on bin found no effect of condition,  $F(2,10)=1.518$ ,  $p=.266$ , but for increasing units there was an effect of condition,  $F(1,6)=13.231$ ,  $p<.001$ . Bonferroni corrected paired samples t-tests found that under CNO, firing rates were higher than during vehicle,  $t(6)=3.804$ ,  $p=.009$ , and baseline,  $t(6)=4.848$ ,  $p=.003$ , conditions which were not different from each other,  $t(6)=1.325$ ,  $p=.233$ . Finally, inside decreasing units the RMANVOA found an effect of condition,  $F(2,32)=27.663$ ,  $p<.001$ . Post hoc testing with Bonferroni corrected paired sample t-tests revealed that z score transformed firing rates were significantly lower with CNO on board compared to both vehicle,  $t(16)=7.503$ ,  $p<.001$ , and baseline,  $t(16)=5.275$ , periods which were not different from each other,  $t(16)=-0.101$ ,  $p=.921$ .



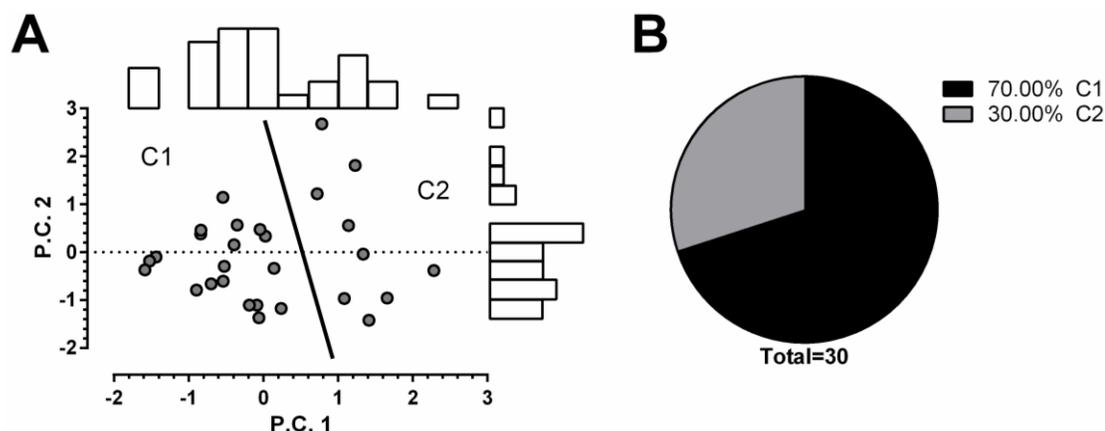
**Figure 10:** Experiment 1 Visual Classification Means **A)** Pie chart of the proportion of units classified in each subgroup. **B)** Mean ( $\pm$ SEM) z-score transformed firing rates (zSpikes) based on condition inside decreasing units. **C)** Mean ( $\pm$ SEM) zSpikes inside increasing units. **D)** Mean ( $\pm$ SEM) zSpikes inside unstable no change units. \*Significant different on Bonferroni corrected paired sample t-test.

Linear regressions were carried out for each visual classification under separately under both CNO and vehicle/baseline (collapsed together) conditions. For unstable/no change units there were no significant linear trend under vehicle/baseline conditions,  $F(1,12)=0.02494$ ,  $p=.8771$ , nor under CNO conditions,  $F(1,22)=3.481$ ,  $p=.0755$ . For decreasing neurons there was no linear for vehicle/baseline,  $F(1,12)=.7542$ ,  $p=.4022$ , but there was a significant linear decrease across bin after CNO administration,  $F(1,22)=181.0$ ,  $p<.001$ . For increasing units, there was no vehicle/baseline trend,  $F(1,12)=3.228$ ,  $p=.0976$ , and under CNO conditions there was a clear non-linear pattern. A centered second order polynomial which predicted an initial increase followed by a decrease provided a significantly better fit than a linear function,  $F(1,21)=67.28$ ,  $p<.001$ .



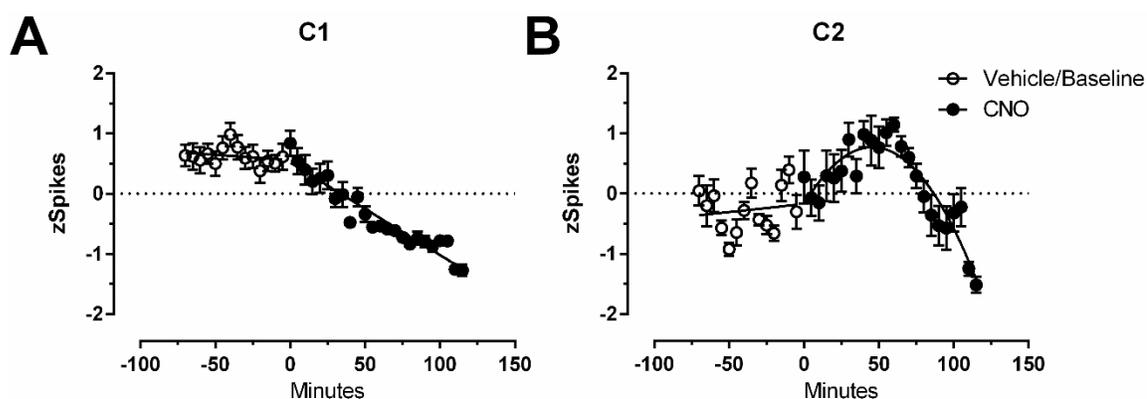
**Figure 11:** Experiment 1 Visual Classification Time Course **A-C)** Individual units z-score transformed firing rates (zSpikes) plotted as a function of 5 minute bin for units classified as unstable/no change (A), decreasing (B), and increasing (C). **D-F)** Mean ( $\pm$ SEM) zSpikes plotted as a function of 5 minute bin for units classified as unstable/no change (D), decreasing (E), and increasing (F). Regression lines correspond to the best fitting linear function except for increasing units under CNO conditions where the best fitting quadratic function is graphed.

To verify that multiple different responses to CNO were present, a principal component analysis was conducted with the z-score transformed firing rates in each bin serving as the input variables. The first seven components were retained explaining 86.8% of the overall variance. The first (P.C.1), second (P.C.2), and third principal components explained 33.3%, 23.2%, and 10.2% of the variance individually. For the first component, the five largest loadings were the z-score transformed firing rates at 75-80 (.909), 60-65(.867), 55-60(.854), 65-70(.847), and 70-75 (.847) minutes post CNO injection. Two different groups of units were identified by plotting P.C.1 versus P.C.2 and using a single straight line was used to create two different groupings of units termed C1 and C2. C1 was comprised of 21 units. Previously C1 units had classified as decreasing (17), increasing (1), and unstable/no change (3). C2 was comprised of 9 units previously classified as increasing (6) and unstable/no change (3).



**Figure 12:** Experiment 1 PCA classification of single unit firing rates. **A)** Principal component 2 (P.C.2) plotted as a function of principal component 1 (P.C.1) with marginal histograms. The drawn classification line divides C1 units from C2 units. **B)** Pie chart showing the breakdown of the number of units classified as C1 versus C2.

C1 units show the same pattern as the overall data set and the visually identified decreasing units. Inside the vehicle/baseline period there was not a significant linear trend,  $F(1,292)=.4411$ ,  $p=.507$ , and inside the CNO period there was a significant negative linear trend,  $F(1,502)=308.7$ ,  $p<.001$ . For C2 units, there was no significant linear trend in the vehicle/baseline period,  $F(1,124)=.7192$ ,  $p=.398$ , and similar to the visually identified increasing neurons a clear non-linear pattern was observed after CNO administration. A centered second order polynomial provided a better fit than a linear function for CNO bins,  $F(1,21)=59.90$ ,  $p<.001$ . The nonlinear function predicted an increase in firing rates followed by a decrease similar to visually identified increasing units.



**Figure 13:** Experiment 1 PCA Classification Z-score transformed firing rates. **A)** Mean ( $\pm$ SEM) z-score transformed spikes inside 5 minute bins for C1 units. **B)** Mean ( $\pm$ SEM) z-score transformed spikes inside 5 minute bins for C2 units.

## Experiment 1: Discussion

Prior literature implicated the mPFC in being involved in DD and necessary in valuation of delayed rewards. Based on this, rats were transfected with the inhibitory DREADD construct AAV8-hSyn-hM4Di mCherry in the mPFC and CNO was administered to subjects while they completed an AA DD task with DS and a CDP. It was hypothesized that this would result in a decrease in IDPs corresponding to an increase in DD. However, the opposite result was observed with CNO administration causing an increased in IDP (i.e., decreased DD). Subsequent electrophysiological confirmation of the hM4Di-mCherry's ability to decrease neural activity revealed that while the overall effect and the majority of neurons firing rates decreased, a subgroup of neurons actually increased firing rates after CNO administration. This pattern raises the possibility that a disinhibition effect occurred on pyramidal cell neurons, and the functional output of the mPFC to other structures actually increased. This possible increase in the functional output of the mPFC may be what resulted in a decrease in DD.

To determine if the surgery alone and transfection of the virus would disrupt behavior on the task, several factors were examined. First if the viral microinjection globally disrupted behavior on the task, it may take animals longer to complete magnitude discrimination post-surgery. However, this was not the case, and in fact the opposite pattern was true with animals trending towards taking fewer sessions to attain magnitude discrimination criteria post-surgery. Moreover, once delays were added to the standard reward animals exhibited the expected delay dependent decrease in IDPs. This decrease fitted the hyperbolic model of DD (Mazur, 1987) very well, and the obtained  $k$  values ( $M=0.177$ ) were approximate to ones obtained and previously published for Long Evans rats in the same paradigm with minor procedural differences ( $M=.169$ ; Beckwith & Czackowski, 2014). Finally after animals completed the 8sD a second week of the 4sD was run before testing CNO and compared to the first week at the 4sD that began two weeks prior. No difference was found. All in all, this evidence suggests that neither the viral microinjection surgery, nor any possible constitutive activity of the hM4Di-mCherry construct affected animals behavior on the task.

IDPs dose-dependently increased in response to CNO administration in a manner that was anatomically specific. In animals classified as hits, injection of 9mg/kg CNO

caused an increase in IDP. However in animals classified as misses, no effect was present indicating that bilateral transfection of the mPFC with hM4Di-mCherry is necessary for the effect. Furthermore, IDP CR, the choice ratio in the last 20 trials, was not affected by any factor, and it did not significantly differ from 0.5 under any dose. The IDP CR findings suggest that animals were not prevented from reaching indifference, ruling out a possible alternative explanation. As a result, the increase in IDPs only in hits indicates activation of the hM4Di-mCherry construct causes a decrease in DD, presumably via inactivating/disrupting the mPFC.

To verify the action of the hM4Di-mCherry construct to decrease neural activity, recordings of mPFC activity were carried out in rats under urethane anesthesia. Across all identified units, the mean level of activity decreased sharply after CNO administration, but did not vary during baseline or after a vehicle injection. Further examination of the data revealed individual unit differences in the response to CNO. While the majority of neurons showed a decrease in firing post CNO, a subgroup showed the opposite effect. Specifically a subgroup of neurons, identified both visually and via PCA, showed an increase in neural activity which lasted for over 75 minutes before returning to and ultimately dropping below baseline levels.

This increase in firing in a subset of neurons may be explainable by the mPFC's cytoarchitecture and the promoter used for the hM4Di-mCherry construct. Using a hSyn promoter would result in the expression of the hM4Di-mCherry in both the mPFC's pyramidal cells and its GABAergic interneurons (Gabbot et al., 1997). Activating the hM4Di-mCherry with CNO would cause a hyperpolarization in both cell types. As the GABAergic interneurons inhibit the pyramidal cells (Gabbot et al., 2006), inhibition of the GABAergic cells could result in a disinhibition of pyramidal cells. The increase in firing only by a subset of neurons may represent a disinhibition of the pyramidal cells. The eventual decrease in firing seen with extended time may be due to direct inhibition on the pyramidal cells via CNO-hM4di eventually overcoming this disinhibition.

Pyramidal cell disinhibition may explain why IDPs changed in the opposite direction versus what was expected. One potential implication of these two possible opposing forces on pyramidal cell activity (inhibition and disinhibition) is that they may "tune" pyramidal cell activity to favor strong inputs. If an input has enough strength to

overcome the direct inhibition of the hM4Di-mCherry, then it may then be favored due to disinhibition of the pyramidal cell. The mPFC exhibits delay period activity (Burton et al., 2009; Miyazaki et al., 2003), and it is hypothesized that this activity encodes a representation of the delayed reward which is then sent to other structures for valuation. The principal projection neurons of the mPFC are the glutamatergic pyramidal cells (Gabbott et al., 2005). Hence disinhibition of these cells would be predicted to actually increase the strength of this representation sent to downstream regions.

It is not unreasonable that a stronger representation of the delayed reward would drive behavior to a greater degree and have more value attributed to the delayed reward. Classically, increasing the representation of a future reward in preschool children by showing them an image of it increased their ability to abstain from collecting a mutually exclusive less desirable reward immediately during a waiting period (Mischel & Moore, 1973). Having individuals complete a DD task where delayed reward amounts were presented alongside an episodic tag describing their planned activities on the day of its receipt also causes a decrease in DD (Peters & Büchel, 2010). Increasing working memory/cognitive load would be expect to impair one's ability to represent the delayed reward. Accordingly, participants who have to remember a series of digits or have consider more options in a DD task also exhibit increased DD (Hinson et al., 2003). Hence, activation of the hM4Di DREADD receptor expressed in the mPFC may have disinhibited the pyramidal cells and strengthened a representation of the delayed reward which in turn caused a decrease DD.

Alternatively, activation of the hM4Di-mCherry may have caused animals to switch to a simpler decision making strategy. Animals may have no longer factored delay into the valuation of the standard reward. Indeed inactivation and lesioning of the mPFC disrupts interval timing behavior (Dietrich & Allen 1998; Kim et al., 2009). If animals could no longer accurately perceive and thus factor the delay to the standard reward into their decision, choices would be based upon the magnitude of the rewards because no other factor systematically varied between the two choices. Focusing solely on the magnitude of the rewards would result in animals favoring the standard alternative. However adoption of this simpler strategy, should decrease the difficulty of the decision.

As less difficult decisions should take less time, a decrease in choice latency should accompany the adoption of the simpler choice strategy. Such a decrease in choice latency was not observed.

The largest caveat to the main results is the now known back metabolism of CNO into clozapine with the implication being that systemic CNO injections are not completely biologically inert. MacLaren et al. (2016) tested a dose response of CNO in several behavioral and neurochemical assays. In a pre-pulse inhibition task, 1mg/kg CNO was found to decrease the initial startle magnitude at higher decibels, but had no effect on percent inhibition. Also, CNO at 2 and 5mg/kg were unable to affect percent inhibition nor block PCP and scopolamine induced decreases in percent inhibition. On locomotor behavior, 1, 2, and 5mg/kg CNO had no effects in isolation, but 5mg/kg CNO was able to reduce, but not abolish, the increase in distance travelled due to 1.5mg/kg amphetamine administration. NAcc fast-scan cyclic voltammetry under conditions of urethane anesthesia also revealed that neither 2- nor 5mg/kg CNO was unable to alter DA release caused by electrical stimulation of the VTA, but it reduced the DA release caused by such stimulation when d-amphetamine (1.5mg/kg) was on board. Finally HPLC was used to quantify blood plasma levels of CNO, clozapine, and N-Des after CNO administration. After a 5mg/kg injection of CNO clozapine and N-Des were detectable at 30, 90, 180, and 360 minutes post injection (MacLaren et al., 2016). As a frame of reference for MacLaren et al.'s (2016) effects, clozapine robustly decreases startle magnitude and increases percent inhibition (7.5 & 10mg/kg; Feifel et al., 2011), blocks PCP induced decreases in prepulse inhibition, (12mg/kg; Leng et al., 2003; 5mg/kg; Bakshi et al., 1994) decreases locomotor behavior (0.5mg/kg; Pinar et al., 2015), and completely blocks the increase in locomotor behavior caused by 2.5mg/kg amphetamine injections (all doses 0.31-2.5mg/kg clozapine; O'Neill & Shaw, 1999).

Critically this raises the implication that CNO, either directly or via back metabolizing into clozapine, caused the decrease in DD and not activation of the hM4Di-mCherry construct by CNO. Clozapine is a second generation, atypical antipsychotic medication, and is one of the few effective treatments for treatment-resistant schizophrenia (Remington et al., 2016; Wenthur & Lindsley, 2013). There is dispute over exactly why clozapine works in treatment resistant schizophrenia probably because of

clozapine's multiple mechanisms of action (Wenthur & Lindsley, 2013). Like typical antipsychotic medications, it acts as an antagonist at the D2 receptor, though it has a lower affinity than typical antipsychotics (Lako et al., 2013; Naheed & Green, 2001; Fakkra & Azorin, 2012). Clozapine also acts at the D1, D4, 5-HT<sub>2A</sub>, 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>,  $\alpha_1$ -adrenergic, histamine H<sub>1</sub> and muscarinic m<sub>1</sub> receptors (Meltzer, 1989; Meltzer, 1992, 2002). In spite of its multiple mechanisms of actions, its antagonistic effects on D2 and 5-HT<sub>2A</sub> receptors are often discussed, and clozapine's antagonism of these two receptor systems is believed to underlie its therapeutic effects and decreased extrapyramidal side effects (Iversen et al., 2008).

To the best of my knowledge there are no *published* studies examining clozapine's effects in a DD task, human, rodent, or otherwise. However there have been studies examining the role of DA and 5-HT receptor systems in DD. Examining this literature may provide some insight into what effects clozapine might have on DD. Notably, much of this work has been completed in the DRT task. This task is susceptible to false negatives for increases in DD and false positives for decreases in DD; nevertheless, an increase in DD can still be interpretable in this task<sup>25</sup>.

Global serotonin depletion tends to increase DD, at least in animals. Systemic injection of para-chlorophenyl-alanine methyl ester (pCPA), a 5-HT synthesis inhibitor, decreases selection of the delayed reward on a T-maze task (Denk et al., 2005; Bizot et al., 1999). Similarly, 5,7-DHT infused into the median raphe increased DD on an AD task with a CDP as well in a T-maze paradigm (Wogar et al., 1993; Bizot et al., 1999). In contrast with these findings, a pair of studies using a DRT with ascending delays failed to find an effect of intracerebroventricular 5,7-DHT (Winstanley et al., 2003; 2004a). Given that only DRTs with ascending delays show no effect, and 5-HT depletion also tends to increase premature responding on the 5-CSRTT, though not perseverative responding (Dalley & Roiser, 2012), it is probable these null results for global 5-HT depletion may be due to the DRT behavioral perseveration confound (Tanno et al., 2014), and global serotonin depletions actually increase DD.

Non-selectively increasing 5-HT signaling systemically appears to decrease DD on T-maze tasks but not on DRTs. The selective serotonin reuptake inhibitors zimelidine,

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<sup>25</sup> See discussion of this topic in the introduction.

citalopram, and indalpine (Bizot et al., 1988) as well as fluoxetine and fluvoxamine decrease DD on a T-maze task (Bizot et al., 1999). On a DRT, citalopram fails to change DD (Evenden & Ryan, 1996). Also chronic fluoxetine had no effect on an AD with DS and a CDP (Logue et al., 1992). Tricyclic antidepressants, which among other actions also block serotonin reuptake, follow this same pattern where on T-mazes they decrease DD (clomipramine, desipramine; Bizot et al., 1988) but have no effect on DRTs (imipramine; Evenden and Ryan, 1996). Finally the monoamine oxidase inhibitor nialamide (Bizot et al., 1988) and the serotonin releaser dexfenfluramine (Poulos et al., 1996) have both been seen to decrease DD on a T-maze.

Looking at the 5-HT<sub>1</sub> receptor family, differential, sometimes opposite, effects on DD pending their specific target and the dosage used exist. 8-OH-DPAT, a 5-HT<sub>1A</sub> agonist, at a low dose (.1mg/kg) caused a reduction in immediate reward selection but a higher dose (.3mg/kg) respectively caused decreased and increased selection of the delayed reward under no delay and maximum delay conditions on a DRT (i.e., disrupted behavior globally; Evenden & Ryan 1999). In T-maze DD tasks, 8-OH-DPAT caused decreased DD in one study (0.015-.5mg/kg; Bizot et al., 1999). Another group found opposite dose dependent effects with the low doses (0.006 & 0.031mg/kg) causing increased DD and the high dose causing decreased DD (0.062mg/kg) in a T-maze task (Poulos et al., 1996). However, an AD task with DS but no CDP found the opposite effect with 8-OH-DPAT causing increased DD for a highly palatable delayed solution (supersaccharin) versus an immediately available sucrose solution (.3 & 1mg/kg; Blasio et al., 2012). In a DRT, Flesionoxan, a 5-HT<sub>1A</sub> agonist, increased choice of the immediate reward including when the larger reward wasn't delayed, and Eltoprazine, 5-HT<sub>1A/1B</sub> agonist, has no effects (van den Berg et al., 2006). Thus activating 5-HT<sub>1A</sub> receptors has discordant effects across paradigms, opposite dose dependent effects inside the same paradigm, and has been shown to disrupt magnitude discrimination.

Partial agonists and antagonists for the 5-HT<sub>1A</sub> receptor tend to increase DD on T-maze tasks but antagonists fail to do so on DRTs. The 5-HT<sub>1A</sub> partial agonists buspirone, ipsapirone, and MDL-73005EF all increase DD in a T-maze DD task (Bizot et al., 1999) and buspirone also increases DD on a DRT with no DS or CDP (Liu et al., 2004). WAY-100635, a 5-HT<sub>1A</sub> antagonist also causes an increase in DD in a T-maze (Bizot et al.,

1999). Though WAY-100635 does not affect DD on a DRT with ascending delays (Evenden & Ryan, 1999; Liu et al., 2004), but this task has concerns about potential false negatives for increasing DD perhaps explaining why results differ based on task (Tanno et al., 2014). Finally, the 5-HT<sub>1/2</sub> antagonist metergoline caused a reduction in DD in a DRT (Evenden & Ryan, 1996). However, its effects are not limited to the 5-HT<sub>1</sub> receptor but also include effects on the 5-HT<sub>2</sub> receptor.

For the 5-HT<sub>2</sub> receptor family, agonists selective for the 5-HT<sub>2A</sub> receptor increase DD, but antagonists selective for this receptor do not have any effects. Also, blocking the 5-HT<sub>2c</sub> receptor may decrease DD, but the only evidence for this comes from DRTs with ascending delays. The 5-HT<sub>2A/2C</sub> agonist DOI<sup>26</sup> has been seen to modestly increase DD in a DRT with ascending delays (Evenden & Ryan, 1999), an AD paradigm (Blasio et al., 2012), and a T-maze task (Hadamitzky et al., 2009). The increase seen in the T-maze task appears to be dependent on signaling via the 5-HT<sub>2A</sub> receptor as coadministration of ketanserin, a selective 5-HT<sub>2A</sub> antagonist blocked the increase in DD, but ketanserin alone had no effect on DD in the T-maze (Hadamitzky et al., 2009). Independently ketanserin has also been seen not to affect DD on a DRT with ascending delays and no CDP (Paterson et al., 2012; Talpos et al., 2006). For the 5-HT<sub>2C</sub> receptor, SER-082, a 5-HT<sub>2c/2b</sub> antagonist, causes a decreased DD in DRTs (Paterson et al., 2012; Talpos et al., 2006). Finally the non-subtype selective 5-HT<sub>2</sub> antagonist Ritanserin did not affect DD on a DRT (Evenden & Ryan 1999). In sum, it appears activating 5-HT<sub>2A</sub> receptors can increase DD, but antagonizing this receptor has no effects on DD. 5-HT<sub>2c/2b</sub> antagonism may either decrease DD or cause increase behavioral perseveration.

Clozapine's other main mechanism of action is via antagonizing the DA D2 receptor, though it still has some effects on D1 receptors (Meltzer, 1989; Meltzer, 1992, 2002). The DA system has long been implicated in impulsivity and DD, in large part because the frontline treatment for disruptive behavior disorders characterized by impulsivity such as ADHD are semi-selective mono-amine reuptake inhibitors such as amphetamine (Castle et al., 2007). Moreover, DA agonist therapy for Parkinson's disease

<sup>26</sup> (+)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropan

may increase DD (Milenkova et al., 2011; Foerde et al., 2016; Antonelli et al., 2014). Fortunately, multiple preclinical studies have examined DA specific manipulations on a systemic level<sup>27</sup>.

Non-specifically increasing DA has been seen to decrease DD or disrupt behavior globally, but all findings have come from a single paradigm, the DRT. The selective DAT inhibitor GBR-12909 in a DRT with DS and no CDP has been seen to either not effect (Koffarnus et al., 2011) or decrease DD (van Gaalen et al., 2006). In this same paradigm apomorphine, a non-selective DA agonist, tends to disrupt choice at the 0sD indicating impaired magnitude discrimination and a general disruption of choice behavior (Koffarnus et al., 2011).

Non-specific DA antagonists tend to increase DD; though, a discordant finding exists. The D1/D2 antagonist flupenthixol has been seen to cause an increased in DD in both an AA paradigm with DS and a CDP (Wade et al., 2000), and DRTs both with and without a CDP (Floresco et al., 2008b; Cardinal et al., 2000). Interestingly, flupenthixol's effect had a significantly larger effect when a CDP was used versus when it was not (Cardinal et al., 2000). In an AD paradigm with DS and no CDP, Fluphenazine caused a decrease in DD in spontaneously hypertensive rats, but not in Wistar-Kyoto or Sprague-Dawleys (Wooters & Bardo, 2011).

Looking at D1 specific agonists and antagonists, manipulations tend to increase DD but also disrupt magnitude discrimination, especially at higher doses. Administration of SKF 81297 in a DRT with DS but no CDP increases selection of the immediate reward, even when the larger reward is not delayed (Koffarnus et al., 2011). The D1 antagonist SCH 23390 has been more widely studied than SKF 81297. In DRTs with DS but no CDPs, it tends to increase DD at lower doses, but at higher doses it also decreases choice of the larger reward when it isn't delayed (Koffarnus et al., 2011; van Gaalen et al., 2006; Tian et al., 2006). In an AA paradigm SCH 23390 was found not to affect indifference points (Wade et al., 2000).

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<sup>27</sup> The effects of amphetamine, and other non/semi-selective monoamine reuptake inhibitors will not be reviewed here for multiple reasons including their lack of specificity for the DA reuptake transporter. This is not due to a belief that their effects do not exist or lack importance. Amphetamine's effects on DD in rodent studies are highly discordant. There is a strong possibility multiple moderating factors exist (Slezak et al., 2009; Tanno et al., 2010; Cardinal et al., 2000). Such an undertaking would be an extensive review paper in and of itself and is deemed beyond the current scope.

Agonists of D2-like receptors also tend to disrupt performance globally or have no effect. 7-OH-DPAT, a D3 agonist, increases selection of the immediate reward at all delays including when the larger-later reward is not delayed (DRT; van den Bergh et al., 2006). The D2 agonist sumanirole has no observable effect on DD in a DRT with DS and no CDP (Koffarnus et al., 2011). Pramipexole, a mixed D2/D3 agonist, increased immediate reward selection on a DCT with DS and a CDP during a short delay condition but not during a long delay condition, but its use in a DRT by the same researchers causes a “flattening” of choice behavior where it increases and decreases immediate reward selection at the no delay and longest delay condition respectively such that choice behavior no longer varies as a function of delay (Madden et al., 2010). However the D4 partial agonist ABT-724 causes a modest increase DD in a DRT (Koffarnus et al., 2011).

The effects of D2 antagonists on DD tend to have no effects or globally disrupt behavior unless a CDP is used. When a CDP is used D2 antagonists tend to increase DD. Eticlopride does not affect choice behavior on a DRT with DS and no CDP (van Gaalen et al., 2006). Both PG01037, a D3 preferring antagonist, and L-741,626, a D2 preferring antagonist, on a DRT with DS but no CDP decrease delayed reward selection overall including when it is not delayed (Koffarnus et al., 2011). The typical antipsychotic haloperidol increases DD on a T-maze which can be argued to have a CDP (Denk et al., 2005), has no effect on a DRT without DS or CDP (Evenden & Ryan, 1996), increases immediate reward selection but also disrupts magnitude discrimination on a DRT with DS but not a CDP (Koffarnus et al., 2011), and in a DRT with DS and a CDP it increases DD selectively (Boomhower & Rasmussen, 2014). Finally raclopride administered in an AA task with DS and a CDP increased DD (Wade et al., 2000).

While no receptor selective drug can truly mimic clozapine’s polypharmacology, examination of clozapine’s two main mechanisms of action, antagonizing D2 and 5-HT<sub>2A</sub> receptors, suggests clozapine may not affect DD or at least change it in the opposite direction observed in the current study (a decrease in DD). 5-HT<sub>2A</sub> receptor antagonists seem not to effect DD, and D2 receptor antagonists tend to have no effects or globally disrupt behavior unless a CDP is used wherein an increase in DD is usually observed. Indeed in a study that has very similar methods to the current body of work (DS, CDP, AA task), raclopride causes an increase in DD (Wade et al., 2000). Based on these two

mechanisms, CNO back metabolizing into clozapine would be predicted to either have no effect on DD or increase DD, and not be a likely alternative explanation for the current findings. While much of this work has been conducted in the DRT which is susceptible to false positives for a decrease in DD, a susceptibility to false negatives for decreases has not been demonstrated. Consequently it may be relatively safe to infer that clozapine's two main pharmacodynamic mechanisms do not result in increased DD.

To recapitulate, CNO has been shown to back metabolize into clozapine, and injections of CNO influence behavior and stimulated VTA DA release when amphetamine is administered but not without amphetamine (MacLaren et al., 2016). This raises the possibility that activation of the hM4Di-mCherry receptor in the mPFC may not be responsible for the observed increase in IDPs (i.e., decreased DD). There are three pieces of evidence which argue against this possibility. First, clozapine's two main mechanisms of action, antagonizing the D2 and 5-HT<sub>2A</sub> receptors, appear to either increase DD or have no effect. Second, in AA task upon which the current task was directly based and matches almost exactly, clozapine has no effect on DD when tested in mice (1.32 & 5mg/kg; Halcomb & Grahame, personal communication). Finally, animals classified as misses did not exhibit any change in IDPs. If CNO administration were causing a decrease in IDPs, it would be expected to do so in all animals, not just animals that have hM4Di-mCherry expressed bilaterally in the PL cortex and surrounding areas. As the decrease in IDP was only seen in animals classified as hits, it strongly suggests that activation of the hM4Di-mCherry in the PL cortex caused the increase in IDPs/decrease in DD.

There is some indication that activating the hM4Di-mCherry caused a small decrease in motivation, vigilance, and/or attention. The 3mg/kg dose trended towards increased session completion times on the first day, and the number of trials completed showed a trend towards an effect of dose with CNO tending to decrease trial completion. However both effects were relatively small, but the effect on trials may have been limited by a ceiling effect at the 0mg/kg dose. A decrease in motivation or vigilance is also corroborated by the TIL. Activation of the hM4Di-mCherry construct caused increased TIL with the median TIL increasing by several seconds. However a large decrease in motivation would have affected CL as well, and no differences in CL were observed.

Unlike TIL, CL are not subject to a large attentional demand because the animal is already attenuating to the task and the overall timeframe they have to pay attention for is relatively short. By contrast, in order to have shorter TIL, an animal must maintain sustained attention without action during a long ITI (>20 seconds) in which nothing occurs. As a dependent variable influenced by both attention and motivation versus motivation but not attention was affected, it suggests that an attentional deficit was induced. Although, a small motivation deficit cannot be excluded. Indeed a decrease in attention cannot completely account for the decrease in trial completion. Nevertheless, decreased attentional capacity following mPFC disruption is supported by lesions and glutamateric antagonist microinjections of/into the mPFC causing attentional deficits on the 5-CSRTT (Maddux & Holland, 2011; Pozzi et al., 2011; Murphy et al., 2005).

Interestingly TIL showed a dose by day interaction in which the 3 and 9mg/kg, but not the 0mg/kg dose showed a decrease across days. This could represent some form of tolerance to the effects of hM4Di activation or a habituation to any possible interoceptive sensations arising from mPFC inactivation. Session completion time showed a similar, but far less robust pattern where the 3mg/kg dose trended towards elevated time only on the first day, but it is important to note that if animals are taking longer to initiate trials, then it should take them longer to complete the session with all other factors being equal. Licks also showed a dose by day interaction with the 3mg/kg dose causing decreased licks on the 1<sup>st</sup> and 5<sup>th</sup> days.

There is some minimal support in the literature for decreasing effects of DREADD receptor activation with multiple CNO administrations. Inhibiting serotonergic neurons repeatedly to cause a decrease in body temperature has been shown to provide diminishing effects with repeated administration (Ray et al., 2011). However body temperature regulation is a critical homeostatic function, and a conditioned compensatory response is a plausible explanation for this effect. Other studies have not seen anything resembling tolerance or habituation. Change in gamma power does not reduce with a second administration (Alexander et al., 2009), and chronic inhibition of PBelo CGRP neurons causes large, repeatable decreases in food intake to the point of starvation (Carter et al., 2013).

Regardless, how this potential tolerance or habituation effect may alter decision making and DD is an important consideration. The strongest “tolerance” effects, represented by a dose by day interaction were driven by the 3mg/kg dose which did not affect IDPs. Two of the three variables, TIL and session time completion, are fairly distant and unrelated to the actual “choice,” and critically no effect of day or dose by day was seen or even trending for IDPs. Consequently, if tolerance to the effects hM4Di were present, they failed to cause systematic variation in IDPs.

Counter to expectations based on an increase in IDP, activation of the DREADD receptor decreased licks and intake of 10S. The increased IDP should result/be a consequence of greater sipper access per trial. This increase in access to the sipper tube should in turn result in increased licks and intake. However a decrease in trial completion was observed as well. Completing fewer trials would reduce the total sipper access time and potentially offset any gains resulting from increased sipper access per trial. Moreover if animals were in fact less motivated, they may simply drink less per unit of access time. Another point to consider is that the decrease in licks was only significant at the 3mg/kg dose in which there were not significant effects on IDP.

In sum, activation of the hM4Di-mCherry DREADD receptor caused a dose dependent change in IDPs as well as several secondary variables. However, the changes in the secondary variables did not covary with the changes in IDPs, but they do suggest a small decrease in motivation and attention that does not approach problematic levels. Electrophysiological confirmation of the DREADD receptors mechanism of action led to the unexpected finding that some neurons increase their firing rate in response to activation of hM4Di via CNO. This unexpected result is consistent with a disinhibition effect and may explain the other unexpected finding. IDP increased versus decreased as was hypothesized. It is possible that hM4Di-mCherry activation actually strengthened a representation of the delayed reward via “tuning” the mPFC to favor strong signals.

## EXPERIMENT 2

### Experiment 2: Introduction

Experiment 2's goal was to build upon and differentiate between experiment 1's possible interpretations. Complete inactivation of the rat mPFC via microinjection of tetrotoxin (TTX), a very potent and efficacious voltage gated sodium channel blocker, was used as an experimental manipulation. It is possible that the decrease in indifference points seen in experiment 1 was due to a disinhibition of pyramidal cell output caused by incomplete inactivation of the mPFC. This new manipulation that completely silences the mPFC should prevent this from occurring. It also has the advantage of not using systemic CNO injections combined with the DREADD virus, working around the largest caveat from experiment 1<sup>28</sup>. If it is truly the pyramidal cell output to other structures that is the critical element for the functional effects of a representation of the delayed reward, then complete inactivation of their activity should increase DD. Conversely if the results indicate a decrease in DD, this would speak to the other prior interpretations of experiment 1 (i.e., disruption in time perception; simpler decision strategy).

TTX is a voltage gated sodium channel blocker most commonly known as the poison in Japanese puffer fish and is classified as guanidinium toxin (Narahashi, 2008; Fozzard & Lipkin, 2010; Moczydlowski, 2013; Bane et al., 2014). It is a small polar molecule with a dioxo-adamantane carbon skeleton with a cyclic guanidinium moiety and numerous hydroxyl groups (Moczydlowski, 2013; Bane et al., 2014). TTX's positively charged guanidinium moiety interacts ionically with the negatively charged carboxylate groups on the extracellular pore loops of voltage gated sodium channels (Stevens et al., 2011; Bane et al., 2014). This interaction results in occlusion of voltage gated sodium channel's pore with a 1:1 stoichiometry and blocks Na<sup>+</sup> passage through the channel (Fozzard & Lipkind, 2010; Moczydlowski, 2013, Bane et al., 2014). TTX application

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<sup>28</sup> This serendipitous advantage was not initially planned. At the time this experiment was initially designed and the committee last met in early September of 2016, the MacLaren et al. (2016) paper was not yet in press. It became available for early access in mid-October of 2016 after animals had been trained and surgeries were underway.

results in a complete loss of voltage-dependent sodium current across neuronal membranes, and full loss of action potentials (Kaneda et al., 1989). Hence, TTX is a highly potent and efficacious sodium channel blocker that completely silences neural activity.

There is timecourse data on TTX's duration of action indicating a timescale of hours, but there are no biotransformation studies, possibly because it is lethal at very small doses. In animals undergoing multi-unit recording in the hypothalamus under urethane anesthesia, TTX microinjection caused a full suppression of spike activity within 6 minutes which lasted "many hours" and up to 10 hours post injection in one subject when recordings were stopped (Harlan et al., 1983). When injected into the dorsal midbrain of conscious, both 10ng and 3.3ng of TTX per hemisphere inhibited lordosis reflex in female rats after 10 minutes with no decrease in effect until 4 hours post injection and no significant effects after 12 hours post injection (Rothfeld et al., 1986). Synchronized lever pressing-drinking behavior was impaired immediately by 10ng TTX microinjections into the motor cortex. Animals only began lever pressing again after 3 hours, had lingering effects on some variables for 24 hours, and trends towards effects were present up to 2-3 days post injection (Zhuravin et al., 1994). In sum, TTX appears to have an onset of effects within 10 minutes which does not degrade for at least several hours, and trace effects may be present for several days.

When it is microinjected into the CNS, TTX diffusion remains localized (1-2mm diameter from point of injection). Zhuravin and Bures (1991) microinjected 10ng of TTX directly into, 1mm away from, and 1.5mm away from the Edinger-Westphal nucleus and then measured the resulting pupil dilation. When microinjected directly into the nucleus, 75% of maximal effects was seen by 6 minutes. At the next timepoint (40 minutes), maximum pupil dilation was observed that lasted until 90 minutes before effects began a slow decline. When the injection was 1mm away from the nucleus, the onset of effects was right-shifted by 8 minutes, but the decline of effects occurred at the same time-points as the 0mm condition. At 1.5mm away, only 50% of maximum dilation was seen after 120 minutes (Zhuravin & Bures, 1991). In a more complex behavioral paradigm, microinjection of TTX (5ng/side) blocked conditioned-cue reinstatement of cocaine seeking when microinjected into the PL cortex but not the adjacent infralimbic cortex

(McLaughlin & See, 2002). Consequently, microinjection of TTX in the 10-5ng range can produce dissociable changes in behavior in adjacent brain areas and the high end of that range produces submaximal effects 1.5mm from the site of injection.

## Experiment 2: Methods

### Animals

Thirty-two male Long Evans rats were obtained from Envigo<sup>29</sup> (Indianapolis, IN) were used as research subjects. Animals began experimental procedures at approximately 50 days of age, and weighed between 273g and 327g ( $M=297.9$ ,  $SD=14.2$ ). Animals were housed in polypropylene shoebox cages under a 12 hour reverse light/dark cycle. Animals had ad libitum access to water throughout, and were food restricted to 85% of their free feeding weight. All behavioral sessions were run during the dark phase.

### Apparatus and Delay Discounting Task

Experiment 2 used the same operant chambers as experiment 1 with no modifications. Similarly, the same DD paradigm, along with the subsequent training was employed. After animals demonstrated magnitude discrimination, cannulation surgeries occurred. Then magnitude discrimination was reassessed. Following successful redetermination of magnitude discrimination in all subjects, the 4sD was in place for 6 sessions before the first sham microinjections. All microinjections were tested at a delay of 4 seconds.

### Surgeries

Rats underwent isoflurane anesthesia, placed in a stereotaxic apparatus (Benchmark Digital Stereotaxic; myNeuroLab, St. Louis, MO), and were administered 5mg/kg/ml Carprofen in sterile saline (s.c.) and .1ml of 5mg/ml Marcaine at the site of the incision. Stainless steel guide cannulae (26 gauge) were lowered to 1mm above the mPFC (A.P. +3.2mm, M.L.  $\pm$ .7mm, and D.V. -2.mm; from brain). Three cranial screws

<sup>29</sup> Between the completion of experiment 1 and start experiment 2 Huntingdon Life Sciences, and Harlan Laboratories merged to form Envigo.

combined with cranioplastic cement were used to hold the cannulae in place. Finally, removable wire obturators (33 gauge) extending the full length of the cannulae were inserted. Obturators were checked daily and replaced at need. Post-surgery, animals were given 10ml/kg of sterile saline (s.c.) and wet food to aid in recovery.

### **Microinjection Procedures**

Microinjections occurred over the course of five weeks. Each week had three no injection days (normal sessions) followed by microinjections occurring on days 4 and 5 of each week. The same type of microinjection was given both days in a particular week. Sham microinjections occurred on weeks 1, 3, and 5 (i.e., before, after, and between all experimental conditions). Microinjections (.5 $\mu$ L/side) of TTX (10ng/ $\mu$ L; 5ng/side; Sigma-Aldrich, St. Louis, MO) and aCSF (Fisher Scientific) were microinjected on weeks 2 and 4 in a counterbalanced order (order 1= TTX week 2 & aCSF week 4; order 2 aCSF week 2 & TTX week 4).

For all microinjections, animals were gently restrained and placed in a 27cm x 17cm x 12cm clear acrylic holding tub. Obturators were removed, and 33 gauge stainless steel microinjectors extending 1mm beyond the end of the cannula were inserted. Over the course of 1 minute, a volume of .5 $\mu$ l was injected. Afterwards, microinjections were left in place for an additional minute to allow for diffusion. Then obturators were replaced, and animals underwent a 10 minute waiting period in their homecages before beginning operant sessions. For sham micorinjections, everything occurred exactly as previously described, except sham wire microinjectors that did not extend beyond the cannulae were used, and no solution was injected.

### **Histology**

Animals were given 100-150mg/kg sodium pentobarbital (i.p.) and placed into a deep plane of anesthesia before being transcardially perfused with 1xPBS followed by 10% formalin (Fisher Scientific). Brains were extracted and post-fixed in 10% formalin for 24 hours and soaked in 30% sucrose for 72-96 hours before being SNAP frozen in isopentane chilled via dry ice.

Brains were sectioned on a cryostat at  $-20^{\circ}\text{C}$ , collected in PBS, and then mounted on subbed microscope slides. Sections were stained with cresyl violet by immersion in a 33mg/ml cresyl violet acetate-Milli-Q water solution followed by 70% ethanol and Milli-Q water rinses. Images were acquired via light microscopy on a Leica LMD 6500 system (Leica Microsystems, Buffalo Grove, IL). Cannula placement was determined by the most ventral extent of tissue damage. Hits, defined by placement within the PL area (Paxinos & Watson, 1998), were dichotomously coded when experimenters were blind to behavioral results.

### **Data Analysis**

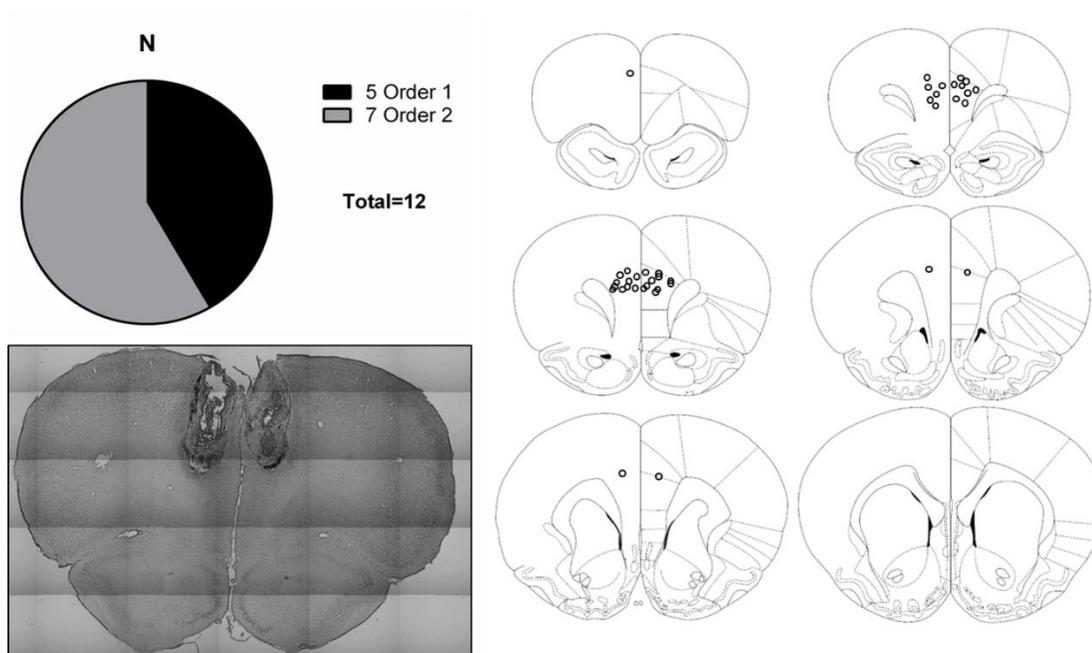
For behavioral data, normality assumptions were tested via visual inspection of histograms and q-q plots as well as Shapiro-Wilks tests. If normality and homogeneity of variance assumptions were violated, log<sub>10</sub> transformations were used for right skewed data, and for left skewed the data was reflected, anchored at 1, and log<sub>10</sub> transformed. If data transformations were unable to normalize the data, non-parametric tests were used, Wilcoxon signed rank's tests & Friedman's ANOVA. For the non-parametric tests the normal approximations were used.

Primary data analysis was conducted using repeated measures analysis of variance (ANVOA) with factors of TTX and session. Interactions were followed up with Bonferroni corrected student t-tests. To examine week to week variation a RMANOVA was first conducted for data from sham microinjection, no injection, all microinjection sessions, and all sessions with factors of week and session using Bonferroni corrected paired samples t-tests as post hoc tests.

### **Experiment 2: Results**

The final n for the main analysis, TTX microinjection sessions versus aCSF microinjection sessions, was 12 animals. Two animals failed to learn to lever press, and one animal failed to meet magnitude discrimination resulting in 29 microinjection surgeries. Among these animals, 16 animals were classified as hits, 12 were misses, and 1 animal died as a result of surgery. Figure 14 displays a hit map for all 16 hits. Inside the 16 hits, 4 animals did not meet performance criteria during all TTX and aCSF sessions.

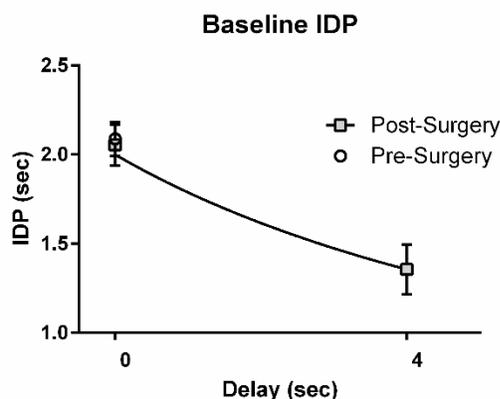
These animals were approximately evenly distributed between the two orders (5 order 1 TTX first, 7 order 2 aCSF first),  $\chi^2(1)=.333$ ,  $p=.564$ . Inside sham sessions only 2 of the animals failed to reach performance criteria across all sessions, and on no injection days only one animal failed to reach performance criteria during all sessions. Any animal that did not meet performance criteria across all sessions for a particular analysis was excluded from only that analysis. This left final n's of 12, 14, and 15 respectively for analyzing TTX effects in comparison to aCSF, comparing sham injections week to week, and comparing no injection sessions week to week.



**Figure 14:** Experiment 2 Cannula placement. **Top Left)** Pie chart showing the number of animals in each order used in the main analysis. **Bottom Left)** Representative image of cannula placement created by stitching adjacent 5x images together. **Right)** Hit map showing cannula placements.

Inside all animals classified as hits, the number of sessions required to reach magnitude discrimination both before ( $Mdn=4$ ,  $Range=9$ ) and after ( $Mdn=4$ ,  $Range=4$ ) was heavily right skewed by several extreme outliers. A Wilcoxon signed ranks test found no pre- vs. post-surgery difference with the vast majority of animals demonstrating magnitude discrimination within the minimum number of sessions required,  $Z=-.071$ ,  $p=.943$ . Similarly, IDPs when animals met magnitude discrimination criteria did not differ before ( $M=2.09$ ,  $SD=.38$ ) or after surgery ( $M=2.05$ ,  $SD=.47$ ),  $t(15)=.222$ ,  $p=.827$ .

However increasing the delay to the standard reward to 4 seconds decreased IDPs ( $M=1.34$ ,  $SD=.56$ ) compared to the post-surgery 0sD,  $t(15)=3.434$ ,  $p=.004$ . Fitting to the mean group IDP, the best fitting  $k$  value was 0.12 with a standard error of the estimate of 0.01, and a strong model fit,  $R^2=.99$ . Figure 15 displays the baseline IDPs both pre- and post-surgery.



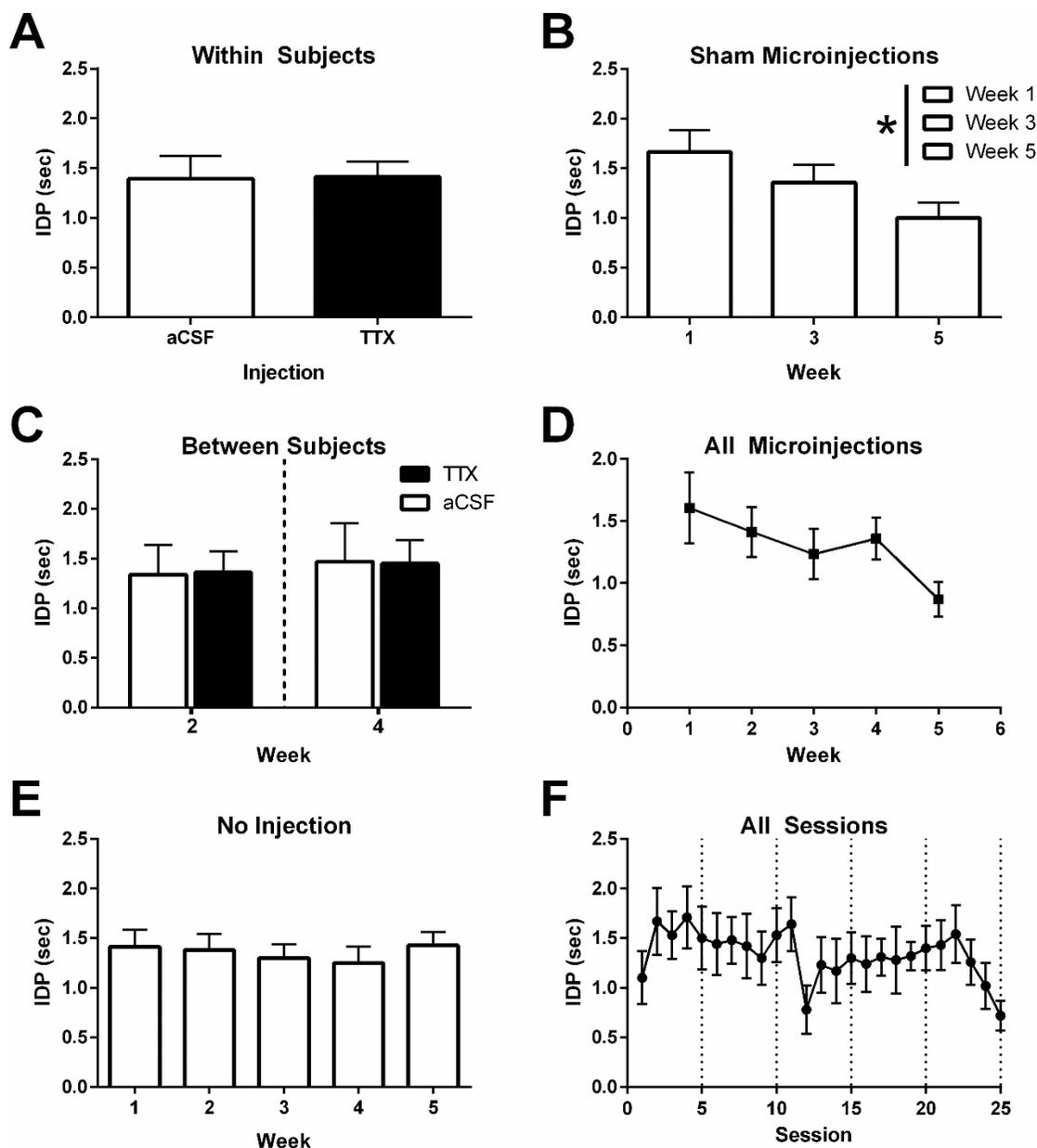
**Figure 15:** Experiment 2 Baseline Indifference Points (IDP). Mean IDP ( $\pm$ SEM) both pre- and post-surgery. A hyperbolic equation, whose slope is defined by  $k$  (Mazur, 1987), was fitted to the post-surgery data.

In the main analysis IDP were normally distributed across all aCSF and TTX microinjection sessions. The RMANOVA found no effects of TTX,  $F(1,11)=.007$ ,  $p=.936$ , session,  $F(1,11)=.337$ ,  $p=.573$ , nor an interaction of TTX and session,  $F(1,11)=1.523$ ,  $p=.243$ . Whether IDP were stable across sham session was examined with a RMANOVA with factors of week (3 levels) and session (2 levels). This analysis revealed a main effect of week,  $F(2,26)=4.498$ ,  $p=.021$ , but not session,  $F(1,13)=.008$ ,  $p=.932$ , nor an interaction of week by day,  $F(2,26)=.247$ ,  $p=.783$ . Overall IDP tended to decrease across weeks, but Bonferroni corrected paired samples t-tests failed to pull out any significant pairwise differences. As a potential effect of time could possibly obscure the effect of TTX, between subject analyses were conducted comparing the effect of TTX and aCSF with a mixed ANOVA with factors of session (2) and TTX (2). During the first week of TTX versus aCSF microinjections there was no effect of session,  $F(1,10)=.015$ ,  $p=.904$ , or TTX,  $F(1,10)=.004$ ,  $p=.953$ , but there was a trend towards a TTX by session

interaction,  $F(1,10)=4.809$ ,  $p=.053$ . During the second week of microinjections there were no effects of session,  $F(1,10)=.619$ ,  $p=.450$ , TTX,  $F(1,10)=.002$ ,  $p=.964$ , nor an interaction of the two,  $F(1,10)=1.788$ ,  $p=.211$ .

To investigate the possibility that IDP simply tended to increase across time, several analysis were conducted testing the effect of time. Subjects were excluded based on performance in a pairwise fashion for each analysis resulting in a final n of 10 for each analysis. First the week to week change on all microinjection sessions collapsed across type (sham, TTX, aCSF) was examined with a RMANOVA with factors of week (5) and session (2). A trend towards an effect of week,  $F(4,36)=2.617$ ,  $p=.051$ , but not session,  $F(1,9)=2.617$ ,  $p=.937$ , or week by session,  $F(4,36)=.628$ ,  $p=.645$ , was present. Next, a RMANOVA on no injection sessions was conducted with factors of week (5) and session (3). This analysis revealed no effects of either week,  $F(4,52)=.730$ ,  $p=.576$ , session,  $F(2,26)=.858$ ,  $p=.436$ , nor their interaction,  $F(8,104)=1.497$ ,  $p=.167$ . Finally looking at all sessions (no injection, sham, TTX, and aCSF) simply as a function of week (5) and session (5) found no significant effects of week,  $F(4,36)=1.081$ ,  $p=.380$ , session,  $F(4,36)=.085$ ,  $p=.987$ , or week by session,  $F(16,144)=1.568$ ,  $p=.085$ .

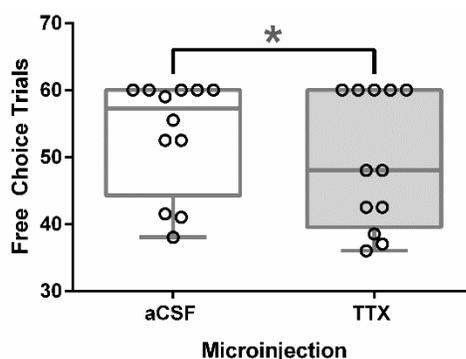
The IPD CRs were normally distributed. A RMANOVA found no effect of TTX,  $F(1,11)=.178$ ,  $p=.681$ , session,  $F(1,11)=.590$ ,  $p=.458$ , nor their interaction,  $F(1,11)=.006$ ,  $p=.941$ . Analysis of sham microinjections found no effect of week,  $F(2,26)=.653$ ,  $p=.529$ , session,  $F(1,13)=.001$ ,  $p=.973$ , nor their interaction,  $F(2,26)=.594$ ,  $p=.559$ . One sample t-tests compared the mean IDP CR on each session to a test value of .5. On the first,  $M=.52$ ,  $SD=.28$ ,  $t(11)=-.256$ ,  $p=.803$ , and second,  $M=.56$ ,  $SD=.26$ ,  $t(11)=-.823$ ,  $p=.428$ , sessions of aCSF and the first,  $M=.49$ ,  $SD=.16$ ,  $t(11)=.271$ ,  $p=.791$ , and second,  $M=.52$ ,  $SD=.19$ ,  $t(11)=-.387$ ,  $p=.706$ , sessions of TTX IDP CRs did not differ from .5. The same pattern was seen after sham microinjections,  $p>.05$ , on all sessions.



**Figure 16:** Experiment 2 Indifference Points (IDP). **A)** Mean IDP ( $\pm$ SEM) collapsed across session from the within subject analysis comparing TTX to aCSF microinjections. **B)** Mean IDP ( $\pm$ SEM) after sham microinjections as a function of week and collapsed across session. \*Indicates a main effect of week. **C)** Mean IDP ( $\pm$ SEM) from the between subject analysis after TTX or aCSF microinjections. The left side displays data after the first round of microinjections. The right side displays data after the second round of microinjections. **D)** Mean IDP ( $\pm$ SEM) after all microinjections regardless of type as a function of week. **E)** Mean IDP ( $\pm$ SEM) from no injection days as a function of week. **F)** Mean IDP ( $\pm$ SEM) as a function of session regardless of condition.

The total time it took for subjects to complete a session in minutes was heavily left skewed and bounded by 60 minutes on the right side of the distribution. Data transformations failed to correct for the deviations from normality and non-parametric statistics were used. A Wilcoxon signed rank test found no effect of TTX compared to aCSF microinjections,  $Z=-1.177$ ,  $p=.239$ , and a Friedman's test found no week to week variation after sham microinjections,  $\chi^2(2)=1.564$ ,  $p=.458$ . Mean values for session time are reported in table 9.

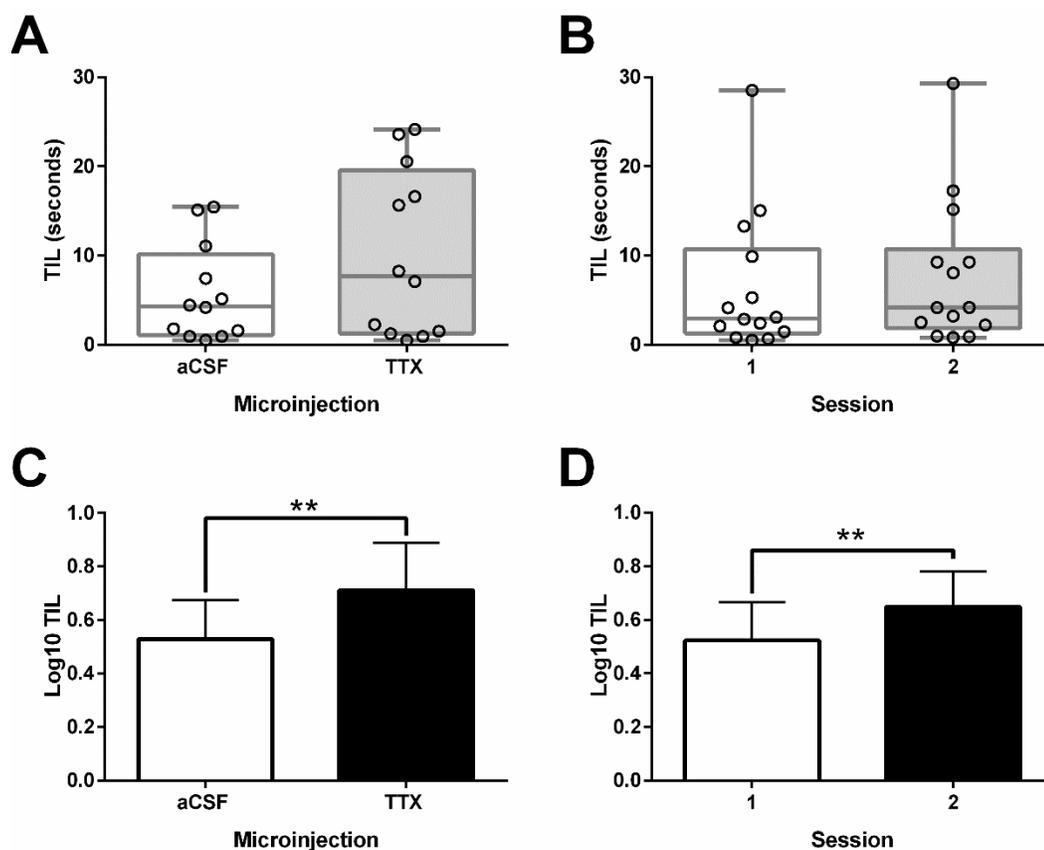
The number of free choice trials inside each session were left skewed and transformations were unable to correct for the deviation from normality. A Wilcoxon signed ranks test revealed a trend for TTX microinjection ( $Mdn=48$ ,  $Range=24$ ) to decrease the number of trials completed compared to aCSF microinjections ( $Mdn=57.25$ ,  $Range=22$ ),  $Z=-1.859$ ,  $p=.063$ . A Friedman's test failed to find any week to week variation after sham microinjections,  $\chi^2(2)=1.366$ ,  $p=.505$ .



**Figure 17:** Experiment 2 Free Choice Trial Completion. Boxplots of the number of free choice trials completed as a function of microinjection solution. \*(grey) trend for aCSF vs. TTX.

Trial initiation latencies (TIL) were right skewed. Log 10 transformation corrected for the deviation from normality and parametric statistics were carried out on the transformed values. The log 10 transformed TILs showed an effect of TTX,  $F(1,11)=10.212$ ,  $p=.009$ , but not session,  $F(1,11)=2.737$ ,  $p=.126$ , or an interaction of TTX and session,  $F(1,11)=.662$ ,  $p=.433$ . Analysis of sham microinjections found that

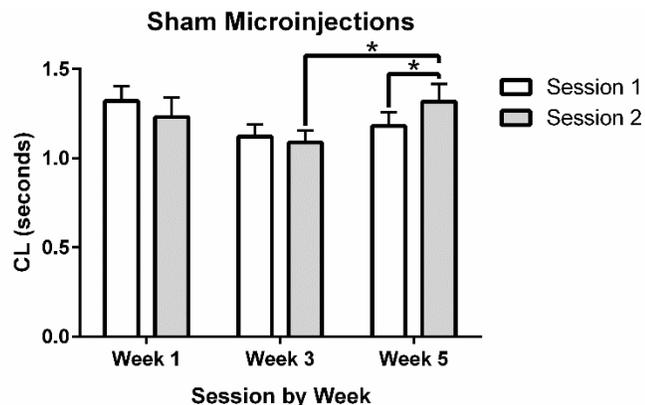
TIL did not vary from week to week,  $F(2,26)=2.251$ ,  $p=.125$ , but did change across sessions,  $F(1,13)=9.459$ ,  $p=.009$ . There was no interaction between session and week,  $F(2,26)=.283$ ,  $p=.756$ .



**Figure 18:** Experiment 2 Trial Initiation Latencies (TIL). **A)** Boxplots of untransformed TIL as a function of microinjection. **B)** Boxplots of untransformed TIL after sham microinjections as a function of session. **C)** Mean ( $\pm$ SEM) Log<sub>10</sub> transformed TIL as function of microinjection  $**p<.01$  main effect of TTX. **D)** Mean ( $\pm$ SEM) Log<sub>10</sub> transformed TIL after sham microinjections as function of session.  $**p<.01$  main effect of session.

Free trial CL were normally distributed. A RMANOVA found no effects of TTX,  $F(1,11)=.015$ ,  $p=.904$ , session,  $F(1,11)=.075$ ,  $p=.790$ , nor of TTX by session,  $F(1,11)=.408$ ,  $p=.536$ . Analysis of sham microinjection data found an effect of week,  $F(2,26)=3.535$ ,  $p=.044$ , but not session,  $F(1,13)=.031$ ,  $p=.863$ . An interaction of week and session was present,  $F(2,26)=4.085$ ,  $p=.029$ . Although week 3 tended to have lower CLs, comparing the week to week mean CLs with Bonferroni corrected paired sample t-

tests failed to find any significant pairwise differences. However, the second session of week 3 exhibited shorter choice latencies than the second session of week 5,  $t(13)=-3.800$ ,  $p=.002$ , and the first session of week 5 had significantly shorter CL than the second session of week 5,  $t(13)=-3.689$ ,  $p=.003$ . Analysis of sessions in which no injection occurred found no significant effects [Week:  $F(4,56)=1.001$ ,  $p=.415$ ; Session:  $F(2,28)=1.009$ ,  $p=.378$ ; Week x Session:  $F(8,112)=1.000$ ,  $p=.440$ ], and examining all sessions irrespective of injections found no effects [Week:  $F(4,56)=.996$ ,  $p=.418$ ; Session:  $F(4,56)=.978$ ,  $p=.427$ ; Week x Session:  $F(16,224)=1.000$ ,  $p=.457$ ]. Mean CL values as a function of injection are displayed in table 9, and the week by session interaction for sham microinjections is graphed in figure 19.

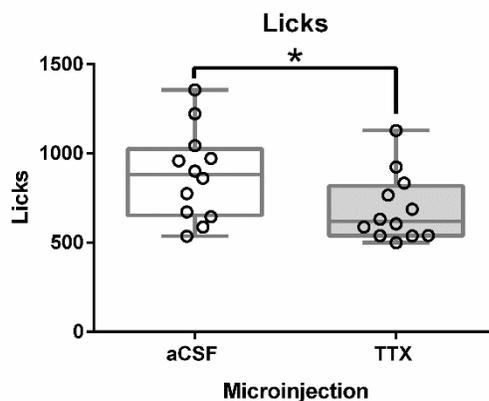


**Figure 19:** Experiment 2 Choice Latencies (CL). Mean ( $\pm$ SEM) CL after sham microinjections as a function of week and session. \*significantly different from Week 5 session 2 on paired sample t-test with Bonferroni corrected alpha level.

The intake of 10S in milliliters (ml) was normally distributed in spite of two strong outliers on TTX administration days. In the main analysis no effects of TTX,  $F(1,11)=.246$ ,  $p=.629$ , session,  $F(1,11)=.562$ ,  $p=.469$ , or TTX by session,  $F(1,11)=.003$ ,  $p=.957$ , were observed. Examining intake after sham microinjections<sup>30</sup> also found no significant effects of week,  $F(2, 24)=2.468$ ,  $p=.106$ , session,  $F(1,12)=1.003$ ,  $p=.336$ , nor week by day,  $F(2,24)=2.528$ ,  $p=.101$ . Mean intake is reported in table 9.

<sup>30</sup> Note: An additional animal was excluded due to a missing data point on the second session of week 1.

The number of licks animals earned was non-normally distributed after TTX microinjections, and log transformations failed to correct for this deviation from normality. A Wilcoxon signed rank test revealed that after TTX microinjection ( $Mdn=617.5$ ;  $Range=629$ ) the number of licks decreased compared to after aCSF microinjection ( $Mdn=880.8$ ;  $Range=820$ ),  $Z(-2.197)$ ,  $p=.028$ . However, a Friedman's test failed to find a week to week variation after sham microinjections,  $\chi^2(2)=.167$ ,  $p=.92$ .



**Figure 20:** Experiment 2 Licks. Boxplots of the total number of earned licks as a function of microinjection. \* $p < .05$  Wilcoxon signed rank test

**Table 9:** Experiment 2 Secondary Variables. Mean ( $\pm$ SEM)

Variable	Sham	aCSF	TTX
Session Time (minutes)	54.4(1.5)	54.2(1.9)	54.7(1.8)
Choice Latency (seconds; CL)	1.22(.07)	1.13(.20)	1.13(.27)
Intake 10% Sucrose (milliliters; 10S)	9.0(0.8)	9.0(0.9)	9.6(1.1)

## Experiment 2: Discussion

The findings of experiment 1 in the context of prior literature suggest that hM4Di DREADD receptor activation in the mPFC may actually strengthen a representation of the delayed reward via a disinhibition mechanism. If this representation is critical for DD and proper valuation of the delayed reward, then its complete abolishment with a very efficacious manipulation should increase the rate of DD. To this end, TTX was microinjected into the mPFC of rats performing an AA DD task. However, contrary to predictions, no significant change in DD behavior was observed. As such the results of experiment 2 suggest that a possible representation of the delayed reward in the mPFC and the mPFC in general, is not *critical or necessary* for DD.

Animals quickly learned the AA task and displayed typical DD behavior as evidenced by their rapidly reaching magnitude discrimination criteria and exhibiting decreased IDP with increases in delay to the standard reward. Moreover, IDP CR were not altered or different from .5 under any condition. Microinjections of TTX compared to aCSF, in both within and between groups analyses, caused no systematic difference in IDP. However, there was a week-to-week change on sham microinjection days. As intracerebral microinjections damage the target structure, the effect in shams could be due to progressive damage being done to the mPFC. However such progressive damage would be expected to cause increased DD across all conditions and not just after sham injections, and this pattern was not observed. On no injection sessions there was not an effect of week, and when all sessions irrespective of condition were examined there were no differences between sessions. Consequently progressive damage being done to the mPFC causing an increase in DD appears to be an unlikely scenario.

Similar to experiment 1, disrupting the mPFC appears to have caused a decrease in motivation and/or sustained attention. Once again, there was a trend towards decreased trial completion with TTX microinjections suggesting decreased motivation. Also, TIL were increased with TTX, but TTX did not affect CL suggesting a decrease in rats ability to sustain attention. Further paralleling experiment 1, neither of these decreases were to problematic levels. Free choice trial completion decreased from just under 60 trials ( $Mdn=57.25$ ) to just under 50 trials ( $Mdn=48$ ). The effects on TIL and free choice trial completion do appear to be larger in experiment 2 versus 1. This difference may reflect the greater efficacy of TTX at decreasing neural activity compared to hM4Di activation. Critically this finding shows that the TTX microinjections had behavioral effects and services as a quasi-manipulation check.

There was some unique variation in CL as a function of session and week inside sham microinjections, but its meaning and interpretation is not readily apparent. Week 1 had a relative elevation of CL while week 3 a general reduction. Finally in week 5 the second session had a sharp increase. The increase in CL on week 1 may be due to animals first time experiencing the microinjection procedure. The decrease on week 3 thus may represent that animals have habituated to the procedure. The subsequent rise on week 5's second session is much more anomalous, and has no readily apparent explanation.

In sum, the results of experiment 2 replicate the decrease in motivation and attention with mPFC disruption. They also discredit the idea that the mPFC is *necessarily* involved in the valuation of the delayed reward and *critical* for DD. TTX, which completely abolishes neural activity, when microinjected into the mPFC produces no detectable change in IDP. Importantly, the current results do not suggest that the mPFC is not *sufficient* to sustain or manipulate delayed reward valuation and DD.

## GENERAL DISCUSSION AND CONCLUSIONS

Prior investigations of the rat mPFC's involvement in DD suggested that the mPFC may generate an outcome representation of the delayed reward. The PL cortex exhibits outcome specific delay period activity (Burton et al., 2009; Miyazaki et al., 2003) and is necessary for outcome directed responding (Balleine & Dickinson, 1998; Balleine & O'Doherty, 2010; Killcross & Coutureau, 2003). Finally, the lesion and inactivation studies in the literature have been limited and suffer from potential response inhibition and behavioral perseveration confounds (Cardinal et al., 2001; Churchwell et al., 2009; Feja & Koch, 2014), leaving a hole to fill in the literature.

If the mPFC and this potential delayed reward outcome representation is necessary for appropriately attributing value to the delayed reward, then inactivating the mPFC should increase DD. To test this hypothesis, two experiments were conducted. Activation of an hM4Di inhibitory DREADD receptor and microinjections of the voltage gated sodium channel blocker TTX were used to site-specifically disrupt the mPFC while animals completed an AA DD task. This task does not suffer from the same potential response inhibition and behavioral perseveration confounds that prior lesion and inactivation studies possessed. In experiment 1, activation of the hM4Di receptor caused a decrease in DD, the opposite of what was predicted. This finding was coupled with a potential disinhibition effect on a subpopulation of mPFC neurons. The potential disinhibition effect raises the possibility that an outcome representation of the delayed reward was actually strengthened. Experiment 2 microinjected TTX to ensure no disinhibition effect would be possible, and the mPFC would be completely silenced. In this case, no effect on DD was observed. Combined, these results indicate that altering mPFC activity is sufficient to alter DD, but the mPFC and any outcome representation it may generate are not necessary for adaptively valuing the delayed reward.

One likely interpretation is that the rat mPFC plays a secondary or modulatory role in DD. If the rat mPFC was a primary contributor to DD and necessary for delayed reward valuation, inactivating it with TTX microinjections should have increased DD, but this manipulation did not alter indifference points. The implication is that the mPFC

is not critical for attributing value to and choosing delayed rewards. However, DA agonist and antagonist mPFC microinjections increasing DD (Loos et al., 2010; Pardey et al., 2013; Sonntag et al., 2014; Yates et al., 2014), and activating the hM4Di DREADD receptor decreasing DD show that when you alter mPFC function, versus simply abolishing it, you can change DD. Hence the mPFC is capable of altering DD, and is likely to play a secondary, or perhaps situation-specific role.

The rat HPC is thought to play a very similar role to the one originally hypothesized for the mPFC. Namely, the HPC has been proposed to contribute by representing future outcomes which are converted to expected value signals by other downstream areas such as the BLA and the OFC (Johnson et al., 2007; Schacter et al., 2007). HPC lesion and inactivation studies do show that the HPC is necessary for DD in rats more consistently than any other structure (Abela & Chudasama, 2012; 2013; Cheung & Cardinal, 2005; Mariano et al., 2009; Rawlins et al., 1985). Consequently, the HPC may be the primary contributor to delayed outcome representations that the mPFC supports and backs up. Ironically, evidence of this secondary role for the mPFC is provided by studies showing the mPFC exhibits outcome specific delay period activity (Burton et al., 2009; Miyazaki et al., 2003). Lesioning the HPC abolishes the PL cortex's delay period activity (Burton et al., 2009).

The mPFC may be recruited when the animal is challenged or when the outcome representation needs to be strengthened. The HPC and mPFC have overlapping efferent projections to the OFC (Cenquizca & Swanson, 2007; Sesack et al., 1989), BLA (Gabbot et al., 2005; Ishikawa & Nakamura, 2006), and NAcc core (Groenewegen et al., 1987; Berendse et al., 1992). The HPC may send the outcome representation to these areas where it is converted to an expected value signal (Johnson et al., 2007; Schacter et al., 2007). Then the expected value signals are then compared in the NAcc core, action is selected/the choice is made, and the action necessary to achieve the chosen outcome is initiated. The mPFC's role may be to act as a secondary amplification step wherein it receives the outcome representation, amplifies it, and then sends it to the OFC, BLA, and NAcc. In this fashion, these value attribution areas receive a greater outcome representation signal overall via one input from the HPC and then a second, possibly

amplified, representation from the mPFC. This bolstering of the outcome representation may be needed when there are particular barriers to overcome such as when the delay is particularly long.

This possible secondary outcome representation role for the mPFC can explain the results of both experiments 1 and 2. In experiment 1, the decrease in DD was accompanied by a potential disinhibition of the mPFC pyramidal cells. If the mPFC's pyramidal cells did in fact have their inputs tuned to stronger signals, an outcome representation from the HPC that normally drives activity might be favored (Burton et al., 2009). This effect could functionally mimic the mPFC amplifying the outcome representation and then sending an artificially strengthened outcome representation to downstream valuation areas. Accordingly, the greater outcome representation received by areas such as the OFC and BLA could result in a greater expected value signal. Such a greater value signal would be expected to facilitate choice of the delayed alternative. By contrast, removing this polysynaptic pathway for the outcome representation by silencing the mPFC with TTX may have limited effect. Such an amplification mechanism may not be necessary under nominal conditions, or the HPC is normally capable of compensating for the loss of the mPFC. Consequently, no change in DD would be observed.

A stronger outcome representation causing a greater expected value signal and subsequently decreased DD is critical to this new hypothesis. In support of this processes being possible, episodic future tags decrease DD (Peters & Büchel, 2010). Specifically when individuals are presented with text describing what they plan to do on the day the delayed reward would be delivered, their choices shift towards the delayed alternative. In theory, such an episodic tag would aid in the generation of outcome representations, and the effect of the episodic tags was moderated by functional coupling strength between the hippocampus/amygdala and the ACC (Peters & Büchel, 2010).

An alternative secondary role for the mPFC in DD may be contributing additional working memory resources. The mPFC is involved in working memory (Zahrt et al., 1997), and working memory may have a causal relationship with DD. The rate of DD is inversely related to working memory in healthy adults (Shamosh et al., 2008). MAD scores on an AD task in rats are related to working memory assessed via a delayed

matching to position task (Renda et al., 2014). A more direct causal role is supported by increasing working memory load, by having participants remember a series of digits or by offering more options, increasing DD (Hinson et al., 2003), and working memory training decreases DD in treatment seeking stimulant addicts (Bickel et al., 2011). Also, both DD (Boettiger et al., 2007; Gianotti et al., 2012; Smith & Boettiger, 2012; Kayser et al., 2012) and working memory (Goldman-Rakic et al., 2000; Zahrt et al., 1997) have been shown to exhibit an inverted “U” shaped dose response function for DA in the rodent mPFC/primate dlPFC. Both dopamine agonists and antagonists microinjected into the rat mPFC, as well as altering receptor expression with a lenti virus, increase DD mirroring a similar inverted “U” shape function (Loos et al., 2010; Pardey et al., 2013; Sonntag et al., 2014; Yates et al., 2014). In sum there is a case to be made for the mPFC affecting DD via working memory.

However, TTX microinjections would cause a similar if not greater working memory impairment as DA agonist and antagonist microinjections. This manipulation should have caused increased DD as well. One possible explanation for why TTX microinjections did not center on the length of the delay used in the current study: four seconds. Compared to other discounting studies, 4 seconds is a very short delay (Loos et al., 2010; Pardey et al., 2013). With such a short delay, the HPC may be able to completely fulfill all of the outcome representation demands on working memory. However, the mPFC and HPC may both be necessary at longer delays. In support of this interpretation, independent mPFC and HPC inactivations do not increase errors on a spatial delayed non-matching to position task with short delays (10 seconds), but inactivation of both structures simultaneously increases errors (Churchwell & Kesner, 2011). Conversely at long delays (10 minutes) inactivation of either structure or functionally disconnecting them increased errors (Churchwell & Kesner, 2011). Consequently the four second delay to the standard reward may not have been long enough for TTX to increase DD.

Delay length does account for results in the mPFC and DD literature to a moderate degree. Studies which have shown that inactivating the mPFC (Churchwell et al., 2009) and injecting dopaminergic drugs increases DD (Loos et al., 2010; Pardey et al., 2013) have used longer delays of at least 15 seconds. Yates et al., (2014) found D2,

but not D1 antagonists, increased DD in an AD task. The baseline MAD scores were approximately 10 seconds which correspond to a longer delay on most AA tasks in rats (Beckwith & Czackowski, 2014; Richards et al., 1997) and a shorter delay on DRT tasks (Loos et al., 2010 Pardey et al., 2013). Of the null results, the two lesion studies did use longer delays, but they also used DRTs with only ascending delays so there is a legitimate concern these studies may be a false negatives for increased DD (Cardinal et al., 2001; Feja & Koch, 2014). One study did find a result with a shorter delay; overexpressing D1 receptors with a lenti-viral vector caused an increase in DD at a delay of 5 seconds in a T-maze (Sonntag et al., 2014). In sum, delay length does explain some, but not all, mPFC DD results.

However there are several holes in the relationship between DD and working memory as well as in the working memory explanation. Spatial working memory assessed in an eight arm radial arm maze does not correlate with DD on a DRT (Dellahagedorn, 2006). Also the working memory training that decreased DD, did not change working memory raising questions as to what exactly about the training actually affected DD (Bickel et al., 2011). With regards to the current experiments, whether or not disinhibiting mPFC pyramidal cells could cause increased working memory to decrease DD does not appear to have a clear mechanism. Increasing the working capacity of a network is likely a much more complex process than simply amplifying a signal that is already being sent.

Assuming that the rat PL/IL cortices primarily correspond to the human dIPFC, two system accounts of DD would predict that their inactivation would cause an increase in DD. In experiment 1, the opposite effect was found. However, it is possible the output of this structure was increased. As the dIPFC and other executive system areas likely would need an effector structure of some type, increasing the output of a structure via disinhibiting its projection neurons, may functionally equate to increasing its activity level. In this case, two system accounts would predict the observed decrease in DD (Bickel et al., 2007; Bechara et al., 2005). However, the results of experiment 2 are completely incongruent with two system accounts. Silencing an executive system area

should decrease activity in the executive system and result in an increase in DD (Bickel et al., 2007; Bechara et al., 2005). In this sense, the current results detract from two system accounts.

The interpretation that the rat mPFC may serve a secondary role in outcome representation to the HPC is also discordant with the human literature. Disrupting the dlPFC with either TMS or DCS increases the rate of DD (Figner et al., 2010; Hect et al., 2013). These results suggest, independent of any larger theory of DD, that the dlPFC is critical for delayed reward valuation.

However when fitting the current results in with human neuroimaging studies, it is important to consider what the rat mPFC may correspond to from a translational standpoint. Given that the primate brain is unique in its amount and differentiation of neocortical development; both structures may be derived from a common ancestor but underwent a divergent evolutionary path where human cortical areas underwent extensive expanding, differentiation, and parceling into more specific subregions. Hence the rat mPFC may be considerably closer to an ancestral structure that areas such as the dlPFC evolved from. As such, one could consider the essential functions mediated by the human dlPFC to be subserved by the rat mPFC at a lower level in addition to the functions of other cortical structures such as the cingulate cortex. There is some evidence of this as that rat mPFC performs the same common class functions as the human dlPFC (Uylings et al., 2003).

As the rat mPFC may correspond to the human dlPFC but not be as advanced, it may not be as influential on rat behavior as it is on human behavior. Consequently, the current findings not matching up with two system accounts of the neurobiology of DD is far from a death blow to these conceptualizations. It may simply be a species difference. Also as the human neocortex underwent extensive development and expanding, it may have wrested the primary role in delayed outcome representation from the HPC. This could explain why, despite being consistently shown to be necessary for DD in rodent studies (Abela & Chudasama, 2012; 2013; Cheung & Cardinal, 2005; Mariano et al., 2009; Rawlins et al., 1985), the HPC does not take center stage in human neuroimaging studies with some studies not even identifying it as important (McClure et al., 2004; 2007; Kable & Glimcher 2010).

In summary, these findings extend those of prior studies by showing that the mPFC is able to affect the rate of DD, but it is not a necessary contributor to DD under certain conditions. Critically showing that the mPFC is not necessary for DD was accomplished in a task, unlike prior studies, that has no response inhibition or behavioral perseveration confounds. These results detract from the hypothesis that the mPFC generates a *necessary* outcome representation of the delayed reward. However, it does not preclude the mPFC from producing such a representation *sufficient* to increase delayed reward value. Rather it suggests that it may be possible to amplify this hypothesized representation to decrease DD. The current body of work is unable to address if under different conditions, such a longer delay to the larger-later reward, the mPFC is necessary to properly value the delayed reward; nor, is it able to confirm if the mPFC plays a secondary role to a structure such as the HPC. Future research should attempt to determine if the mPFC is critical at longer delays as well as if HPC-mPFC interactions are needed at longer delays to confer value to the delayed reward.

### **Limitations & Future Directions**

Like any other study, there are limitations to the current body of work. First, the effects of hm4Di activation and TTX microinjections into the mPFC were only tested at a single delay. It is entirely possible that different results could be obtained at a longer or shorter delay. Indeed the differential effects of mPFC inactivation on working memory based on the delay (Chuchwell & Kesner, 2011) suggest that a longer delay may have yielded a significant result in experiment 2. Along the same lines, the current study is not able to detect if the sensitivity to reinforcer magnitude or magnitude discrimination was disrupted by the acute manipulations. Another methodological element to the AA DD task was the use of a CDP. Using a CDP is not a bad practice but doing so likely alters which neural mechanisms are engaged. Different results could possibility be obtained if a CDP was not used. That being said, delayed rewards often have predictive cues and conditioned reinforcers in real life, but not always. Consequently the current investigation is limited in that it did not also include a group without a CDP. Furthermore the

electrophysiological techniques and data analysis used are unable to determine if the different responses to CNO occurred in different populations of neurons, such as the disinhibition occurring in pyramidal cells and not interneurons.

Both of the manipulations used in experiments 1 and 2 have limitations. For experiment 1, CNO may back metabolize into clozapine (MacLaren et al., 2016). It is possible that this occurred in the current study. Three pieces of evidence suggest that if it did occur, it did not alter DD. First and foremost, there was no change in DD in misses who received the same dose of CNO, but did not have hM4Di-mCherry expressed in the PL cortex bilaterally. Clozapine's main mechanisms of action are antagonism of 5-HT<sub>2A</sub> and D2 receptors. Selective antagonists for both of these receptors do not alter DD, and finally when tested in mice, clozapine does not affect DD on an AA task (Halcomb & Grahame, personal communication). Also the spread of the hM4Di-mCherry encompassed several areas of the mPFC. The effects of transfecting a single area, such as the only the PL cortex, may be different than the whole area. For experiment 2, TTX microinjections also have drawbacks. TTX will not spare the fibers of passage. Consequently, TTX would impair any projections that are simply traveling through the mPFC. Microinjections also do permanent damage to the target structure altering its functionality. Consequently there was a limit to how many times TTX microinjections could be delivered.

The extent to which a rat's neurobiology relates to a humans is another limitation. The rat brain's areas are not a one-to-one match with the human brain. This is particularly true of cortical structures. The rat lacks many neocortical areas that the human brain possesses, including the dlPFC. While the mPFC appears to correspond to the primate dlPFC based on anatomical connections and the "common class functions" it subserves (Uylings et al., 2003) and a combination of the dlPFC and ACC based on electrophysiological evidence (Seamans et al., 2008), it is ultimately not the human dlPFC. Consequently, the current findings may not necessarily generalize to the human dlPFC.

One possible interpretation of the current findings is that the mPFC plays a secondary role to the HPC and that it may only be necessary when a longer delay is used. However the current study is unable to substantiate this hypothesis. Future work should

look to determine if the mPFC is critical at longer delays, and if mPFC-HPC communication is needed at longer delays as well. Also, if the mPFC does truly play a secondary role to the HPC, the importance of different HPC efferents to DD is worth investigating. Conceptualizations of HPC contribution to decision making involve sending an outcome representation to downstream areas which then generate an expected value signal (Johnson et al., 2007; Schacter et al., 2007). This outcome representation could be sent to the OFC, BLA, or directly to the NAcc. However, if the NAcc is truly the final common output of the entire circuitry, conversion to an expected value signal may need to occur in advance of the NAcc. To test this hypothesis, functional disconnection studies breaking HPC to BLA and HPC to OFC connections would be needed.

The current conceptualization of the neurobiology of DD centers around the NAcc core being a final common output pathway that compares expected value signals and helps initiate motor behavior to select an outcome. Consequently, the inputs to the NAcc core should be examined with the use of functional disconnection studies. Particular areas which may be important inputs to the NAcc for DD are those suggested to generate expected value signals, the OFC and the BLA.

Another area that deserves further investigation is the dorsal striatum as a number of human neuroimaging results have implicated it, and it likely plays a role in action selection similar to the NAcc (Nicola, 2007). Frontostriatal WMI between the dorsal striatum and the dlPFC is related to increased DD (Hampton et al., in press). Increased *fMRI* BOLD in the head of the caudate nucleus and putamen is seen when DD choices involve a long versus short delay (Wittman et al., 2007). Also, individuals with  $\alpha$ -synuclein gene duplication before the development of Parkinson's disease have normal caudate volume and no difference between controls on DD, but once symptoms begin to onset, caudate volume is decreased, DD is increased, and caudate volume is negatively correlated with the rate of DD. Accordingly, the dorsal striatum needs some attention from preclinical investigations.

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