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# Behavioural responses in uncertain conditions are influenced by the orbital prefrontal cortex

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**BEHAVIOURAL RESPONSES IN UNCERTAIN CONDITIONS ARE INFLUENCED BY THE ORBITAL PREFRONTAL CORTEX**

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**Bachelor of Science, University of Lethbridge, 2013**

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BEHAVIOURAL RESPONSES IN UNCERTAIN CONDITIONS ARE
INFLUENCED BY THE ORBITAL PREFRONTAL CORTEX


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Date of defence: Sept 12 2016


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# ABSTRACT

The orbital prefrontal cortex (OPFC) is implicated in generating outcome expectancies and in preventing the over-generalization of fear. Here, I investigate if the OPFC supports associative processes by determining the relevance of cues during behavioural tasks with relatively high levels of uncertainty. Two projects were conducted: one using appetitive and aversive context conditioning and another using a cue/place variant of the Morris water task. I observed that OPFC inactivation resulted in generalized responses on the appetitive and aversive context conditioning tasks. Further, I observed that after OPFC inactivation, rats favour spatial over cue responses in a competition test of the water task. These results support a role for the OPFC in influencing response strategies and suggest this region is critical for constraining responses during uncertain conditions. Through interactions with learning and memory systems, these results suggest the OPFC supports associative processes during uncertainty by mediating between discrimination and generalization.

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CPP – conditioned place preference
DFCTC – discriminative fear conditioning to context
GAD – Generalized Anxiety Disorder
HPC – hippocampus
IGT - Iowa Gambling Task
LER – Long-Evans rat
mPFC – medial prefrontal cortex
MWT – Morris water task
OFC – orbitofrontal cortex
OPFC – orbital prefrontal cortex
PFA – paraformaldehyde
PFC – prefrontal cortex
vmPFC – ventral medial prefrontal cortex

**Chapter 1**

**Introduction**

*Purpose*

  A function of the mammalian brain that is important for survival is the ability to learn which environmental cues predict biologically significant events. This function is thought to be supported by associative processes, however, the dynamic complexity of our environments creates uncertainty when learning about, and responding to predictors for biologically significant events. The orbital prefrontal cortex (OPFC) has been linked to encoding the emotional significance of stimuli and is thought to generate outcome expectancies (Gottfried, Schoenbaum, Roesch, Stalnaker, & Takahashi, 2011; Rempel-Clower, 2007; Schoenbaum, Saddoris, & Stalnaker, 2007). Here, I have investigated OPFC and its influence on associative processes during uncertainty by exploring cue relevance. Specifically, I hypothesize that the OPFC facilitates the ability to constrain responses when multiple cues provide associative information by determining the relevance of cues. To investigate this, I assessed the role of the OPFC during learning of a task with relatively high levels of uncertainty. Previous work from our laboratory supports this theory showing that damage to the OPFC impairs the ability to constrain fear responses during testing of a context discrimination task (Zelinski, Hong, Tyndall, Halsall, & McDonald, 2010). In this task, animals experience two distinct contexts throughout training; one context is always paired with a foot-shock, whereas the other context is paired with no event. The contexts differ in shape, colour, and smell, but they also share some similarities such as both have grid floors and are constructed from Plexiglas. In this previous study, rats were impaired at making constrained

responses, when OPFC lesions occurred before learning the task. The first experiment presented here will extend these findings by assessing the effects of temporary inactivation of OPFC after training acquisition. This design will determine whether OPFC must be active during expression of discriminative fear as well as during the acquisition process. I hypothesize that inactivation of the OPFC during testing will lead to generalized freezing responses without impairing the ability to express active avoidance; a pattern of effects similar to those reported following permanent lesions induced before training (Zelinski et al., 2010).

The OPFC is implicated in learning about both aversive and appetitive associations (Gallagher, McMahan, & Schoenbaum, 1999). However, rarely has the role of the OPFC in aversive and appetitive conditioning been compared on a similar task except in the domain of reversal learning. Therefore, it is important to determine if OPFC is involved in a general mechanism of response constraint during both appetitive and aversive conditioning. Experiment 2 will assess the role of the OPFC in a more general function of constraining responses to cues that have perfect predictive value for any biologically significant events, independent of their rewarding or aversive properties. I hypothesize that OPFC inactivation will result in generalized activity level responses, but will not impact preference during an appetitive version of the discriminative contextual conditioning task.

Although it is presumed that the OPFC functions to solve uncertainty by determining the relevance of presented environmental information, it is unlikely that this region encodes all this information and/or completes this function alone (Farovik et al., 2015; Keiflin, Reese, Woods, & Janak, 2013). I propose that the OPFC contributes to this function by interacting with brain systems implicated in

learning and memory functions such as the striatum, hippocampus, and amygdala. It is thought that instrumental responses can either be goal-directed or habitual, and that these two responses types are controlled by separate brain systems. The hippocampus is thought to be central to the goal-directed system which facilitates flexible behavioural responses such as spatial navigation whereas the dorsolateral striatum is facilitating habitual responses which are less flexible (McDonald & Hong, 2013). Certain behavioural tasks can be solved using different response strategies. For example, in the cue/place water task animals learn to locate a platform that is visible on some training days and invisible on others thus animals acquire both habitual and goal-directed response information (McDonald & White, 1993). On a subsequent competition test, I can determine if animals are more likely to respond according to cue or spatial information. I designed project two with the intention of determining if inactivating the OPFC before the competition test would induce a bias towards the use of one response strategy over the other; allowing me to infer how this region might function within the larger neural circuitry implicated in mammalian learning and memory processes. I hypothesize that after OPFC inactivation rats will favor cue responses over spatial.

### *Significance*

Understanding how uncertain environmental conditions influence behaviour is fundamental to understanding normal patterns of behaviour and their neural substrates. Further, determining how uncertainty affects behaviour is important for gaining a better understanding of the maladaptive behaviours exhibited in anxiety and mood disorders such as generalized anxiety disorder (GAD; Grupe & Nitschke, 2013). Patients with GAD are often highly intolerant of uncertainty, and exhibit

persistent uncontrollable worry in conjunction with continual avoidance of potentially adverse situations (Blair & Blair, 2013; Nutt, Ballenger, Sheehan, & Wittchen, 2002; Rowa & Antony, 2008). Patient's are impaired when required to form a judgement about likely outcomes and tend to overestimate the magnitude and likelihood of a potential threat (Behar, DiMarco, Hekler, Mohlman, & Staples, 2009). As compared to control subjects, GAD sufferers display only slightly amplified responses to direct threats. However, they show abnormally exaggerated fear responses to neutral, future-oriented and ambiguous events (Blair & Blair, 2013; Newman, Llera, Erickson, Przeworski, & Castonguay, 2013). Often, neutral situation or events can also be quite ambiguous, so there is likely a lot of overlap between these subjects augmented responses to these types of stimuli. For example, although a neutral facial expression does not clearly indicate threat in the same way a fearful expression might, the ambiguity of a neutral face subjects may create uncertainty for the subjects leading to similar fearful responses. This is consistent with the propensity that patients worry about highly unlikely, or not overly aversive events, whereas these same events would cause little or no worrisome thoughts for control subjects (Blair & Blair, 2013; Newman et al., 2013).

In many disorders, abnormal fear or anxiety of a perceived threat has been linked to amygdala hyperactivity, which subsequently results in enhanced fear conditioning (Etkin & Wager, 2007; Phan, Fitzgerald, Nathan, & Tancer, 2006). However, there is only minimal evidence that GAD patients condition more easily to threat stimuli (Blair & Blair, 2013; Lissek et al., 2005), and adult GAD patients often do not show heightened amygdala activation in response to a threat. Instead, GAD patients do show amygdala hyperactivity in response to ambiguous stimuli or in anticipation of viewing either ambiguous or negative stimuli (Lissek et al., 2005;

Nitschke et al., 2009). GAD sufferers also exhibit over-generalized fear responses to uncertain stimuli (Lissek, Kaczkurkin, et al., 2013). Therefore, excessive anxiety observed in GAD is not the result of overactive responses to threat, but to uncertainty (Dugas et al., 2005). Inspired by the abnormal anxiety observed in GAD, here I focus on understanding how uncertainty influences behaviours under various training conditions and explorations into the neural systems essential for these phenotypes.

### *Uncertainty*

An important function of the mammalian brain is the ability to predict biologically significant events according to predictive cues contained within the environment. Biologically significant events can be categorized as an occurrence that can impact an organism's survival and ultimate reproductive success, including rewards and punishments. Learning about cues that signal these events increases an organism's survival, fitness, and adaptive advantage by enabling access to nourishment and mates and avoiding threats and hazards. It is now widely accepted that animals learn about the world using associative learning processes (Rescorla & Wagner, 1972), of which there are two main subtypes: Pavlovian learning and instrumental learning. Pavlovian learning involves the association of cues with biologically significant events (Pavlov, 1927), and instrumental, or operant learning, involves forming associations between actions (responses) and outcomes (Skinner, 1938; Thorndike, 1898, 1913).

Classical conditioning, a form of Pavlovian learning, pairs the presentation of previously neutral, but now conditioned, stimuli with responses similar to what would occur following the presentation of a biologically significant event (Pavlov,

1927). Animals can be conditioned to respond to discrete cues as well as to the constellation of environmental stimuli in a process called contextual conditioning (Myers & Gluck, 1994; Phillips & LeDoux, 1992; Winocur, Rawlins, & Gray, 1987). The acquisition of contextual conditioning can be assessed in two ways: simple conditioning compares responses to the conditioned stimuli before and after conditioning, and differential conditioning involves exposure to two different contexts throughout training. In differential conditioning, the paired context is always associated with the biologically significant event (CS+), whereas the second context (CS-) is explicitly unpaired and remains neutral. After conditioning, the CS+ elicits a conditioned response and the CS- unpaired context elicits no response, given its neutrality (Büchel & Dolan, 2000; Clark & Squire, 1998). During differential conditioning, the subject forms an association between the paired context and an outcome and no association with the unpaired context. Differential conditioning is advantageous because this paradigm is sensitive to any stimulus generalization. For example, if animals are exhibiting generalized fear (responding fearfully to both the neutral and aversive contexts) this would not be apparent in a simple conditioning paradigm which is only able to demonstrate if animals are more fearful after conditioning than they were prior to conditioning. In a differential paradigm we are able to see if animals have learned to fear a shock context, and if they are able to differentiate between the contexts, then constrain their fear responses to just the threatening context.  Further, because animals have the same number of experiences in the two contexts, and we are comparing responses between the contexts, rather than a before and after measure, differential conditioning is advantageous for controlling for non-associative learning processes like sensitization (Antoniadis & McDonald, 1999).

Although animals easily learn associative tasks, because environments are composed of a vast array of stimuli, this can create uncertainty during learning and when responding to predictors for biologically significant events (Rescorla, 1968). It may initially seem like a simple process for animals to distinguish between stimuli that have no predictive value versus those indicating reward, threat or danger, but the complexity of diverse environments challenges the perceived simplicity of this function. Moreover, as we move through the world, the constellation of stimuli composing our surroundings is continually changing, and the significance of an individual cue can also vary; therefore, animals must distinguish relevant from irrelevant environmental components.

Too much or too little information leads to uncertainty when learning and responding to environments and novelty represents the most obvious example of the latter uncertain situation. When faced with a novel environment or cue, it is beneficial to be able to draw on information gained in previous similar situations to reduce uncertainty. Stimulus generalization occurs when a stimulus (or context) that resembles the original conditioned stimulus elicits a conditioned response (Ghosh & Chattarji, 2015; Pearce, 1987). Generalization affords a flexibility that can be adaptive in novel environments because similarities between past and present experiences can be used to generate predictive knowledge in an unknown situation. However, responses can become maladaptive if over-generalization occurs (Dunsmoor & Paz, 2015; Onat & Büchel, 2015).

In contrast to a novel situation, where uncertainty arises from insufficient information, a complex environment represents another form of uncertainty occurring because of an abundance of information. Complex environments include many different cues, therefore, animals must determine from the vast number of

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stimuli they are faced with at any given moment, which cues signal biologically

significant events and which do not. Further complicating matters, the biological

significance of stimuli can vary; for example, a cue might signal reward in one

context and punishment in another. Alternatively, the predictive value of stimuli

changes, such that a stimulus can be salient within certain contexts and irrelevant

or neutral in others. In the face of uncertainty and dynamic contexts, animals must

be able to balance generalization with discrimination; too much, or too little of either

process results in maladaptive learning and subsequent behavioural responses.

### The Orbitofrontal Cortex

*Anatomy and connectivity*

Homology of the human, monkey and rodent prefrontal cortex (PFC)

continues to be debated. However, based on the increasing acceptance of common

functions and thalamic connections across species, for the purpose of this thesis, I

assumed the rodent PFC is homologous to the monkey and human PFC

(Groenewegen, 1988; Kolb, Pellis, & Robinson, 2004; Rose & Woolsey, 1947; Uylings,

Groenewegen, & Kolb, 2003; Uylings & van Eden, 1990). The rodent PFC is divided

into two separate subdivisions: the medial prefrontal cortex (mPFC) and the

orbitofrontal cortex (OFC), which can be further segregated into several sub-regions.

The mPFC includes the infralimbic cortex, prelimbic cortex, anterior cingulate

cortex, and the medial agranular areas. Together these regions are implicated in

decision-making, attentional control, goal-directed behaviour, and working memory

(Birrell & Brown, 2000; Heidbreder & Groenewegen, 2003). The OFC is located

ventral to the mPFC and subdivided into the medial orbital, ventral orbital, lateral

orbital, dorsolateral orbital areas and along the lateral wall, and slightly dorsal is the agranular insular area (Paxinos & Watson, 1997).

For clarity, throughout this thesis the orbitofrontal cortex (OFC) designates the general region regardless of species. A secondary term, orbital prefrontal cortex (OPFC) refers to a specific region used here and in previous work from our group (Zelinski et al., 2010), targeting primarily the lateral orbital, and ventral orbital areas. Specifically, the OPFC does not include medial orbital areas.

The OFC receives extensive sensory input from all the sensory modalities as well as direct projections from the olfactory bulb (Bedwell, Billett, Crofts, & Tinsley, 2014; Datiche & Cattarelli, 1996). The OFC shares reciprocal connections with many brain regions including the hippocampus, entorhinal cortex, and the inferior temporal cortex (Kondo & Witter, 2014), as well as the hypothalamus, the periaqueductal grey, other prefrontal regions, and the anterior cingulate cortex (Floyd, Price, Ferry, Keay, & Bandler, 2000; Hoover & Vertes, 2011). The OFC is extensively connected with the amygdala, particularly with the basolateral amygdala (Kita & Kitai, 1990; Reep, Corwin, & King, 1996). Further, the OFC is connected with the striatum (Berendse, Graaf, & Groenewegen, 1992; Brog, Salyapongse, Deutch, & Zahm, 1993; Hoover & Vertes, 2011).

*OFC function*

Initial research examining orbitofrontal cortex function emerged from case studies of patients with prefrontal damage encompassing the entire ventral medial prefrontal (vmPFC) region (including the OFC), and implicated this region in decision making, planning, flexible responding, and emotional control. Following prefrontal damage, patients initially would appear normal, with relatively average

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cognitive capacities, motor abilities, and speech. However, it was quickly recognised that damage resulted in personality and behavioural changes, such as, increased impulsively and inappropriate social behaviours (Bechara & Damasio, 2005; Bechara, Damasio, & Damasio, 2000; Eslinger & Damasio, 1985; Hornak et al., 2003; Saver & Damasio, 1991; Wallis, 2007). The OFC was implicated in value guided learning and decision making, when patients with OFC damage displayed impairments in minimizing long-term losses in the Iowa Gambling Task (IGT; Bechara, Damasio, Damasio, & Anderson, 1994; Bechara, Damasio, Damasio, & Lee, 1999; Bechara, Damasio, Tranel, & Damasio, 2005). During this task, patients are asked to select a card from one of four different decks to receive either a reward or loss depending on the card selected. On these initial trials, the cards chosen from all the decks lead to rewards, but two of the decks consistently lead to larger rewards and the other two decks cards lead to smaller rewards. After the initial reward only trials, now some cards begin to lead to losses. The losses incurred in the high reward decks are larger than losses received by choosing from the lower reward decks. Control subjects would initially select from high reward decks, but quickly learn once the losses were introduced, that choosing from the high reward decks lead to larger losses than rewards and switch their choices to lower reward (but less risky) decks. Patients with damage to vmPFC perseverated on large gain choices even though this action would ultimately result in larger losses overall.

Initially, Damasio et al. (1997) hypothesized that IGT impairments were a result of the abnormal anticipatory somatic responses vmPFC damaged patients exhibited. When considering risky choices normal participants generated skin conductance responses, however, vmPFC subjects would not (Bechara et al., 1999; Bechara, Damasio, Tranel, & Damasio, 1997). Known as the Somatic Marker

Hypothesis, it was suggested that the vmPFC forms associations between internal

emotional signals (for example skin conductance responses) with predictive

environmental stimuli (events or outcomes). In other words, healthy subjects were

biased by the representation of the association between the previous emotional

outcome (loss or reward), and that choice was stored in the vmPFC. Decisions made

by patients with damage to the vmPFC were not biased by anticipated emotional

outcomes (via activation of a somatic state), resulting in riskier choices (Bechara &

Damasio, 2005). Although vmPFC lesions do impair emotional processing, the

Somatic Marker hypothesis does not fully encompass the deficits associated with

vmPFC damage. Self –reports indicate an awareness of risky choices, and vmPFC

lesioned patients experience normal immediate somatic responses to rewards and

punishments. They are also able to choose between different reward values. Thus,

these patients respond normally to immediate rewards and punishments and

understand different reward values but are unable to respond appropriately to

future, anticipated or hypothetical outcomes (Fellows, 2007; Fellows & Farah, 2007;

Maia & McClelland, 2004).

Neurons in the OFC encode a wide array of information regarding positive

and negative outcomes (Padoa-Schioppa & Assad, 2006; Tremblay & Schultz, 2000).

OFC neurons fire initially when a reward is received then begin to fire in response to

cues that signal reward (Hosokawa, Kato, Inoue, & Mikami, 2007). Neuronal firing

in this region increases in anticipation of larger rewards over smaller and to rewards

with shorter over longer delays (Roesch & Olson, 2005; Roesch, Taylor, &

Schoenbaum, 2006; Wallis & Miller, 2003), suggesting that the OFC represents

reward value and/or associations with stimuli predicting rewards. However,

behavioural deficits following OFC damage do not impair sensitivity to direct

rewards and punishments (Hosokawa et al., 2007). Humans, primates, and rodents with OFC lesions can select a preferred reward over less preferred reward (Keiflin et al., 2013; McDannald et al., 2014), are able to learn stimulus-outcome associations, and can discriminate between two different stimuli associated with different valued outcomes (Tait & Brown, 2007).

Following OFC lesions, in non-human animals, the most prominent deficits are in behavioural inflexibility, specifically with impairments in reversal learning (Bissonette et al., 2008; Boulougouris, Dalley, & Robbins, 2007; Butter, 1969; Chudasama & Robbins, 2003; Izquierdo, Suda, & Murray, 2004; Schoenbaum, Nugent, Saddoris, & Setlow, 2002; Tait & Brown, 2007; Teitelbaum, 1964). Reversal deficits are accounted for by several explanations, some suggesting that OFC damage diminishes sensitivity to the value of different outcomes. An early view of OFC function, supported by evidence found in the electrophysiological literature, states that the OFC integrates reward information to generate a common currency and compare different rewards (Padoa-Schioppa, 2009; Padoa-Schioppa & Assad, 2006). However, this concept has been called into question by studies demonstrating that OFC neurons fire in response to variables that do not impact the value of an outcome. For example, OFC neurons fire differentially to right or left responses even if they lead to the same reward (Feierstein, Quirk, Uchida, Sosulski, & Mainen, 2006; Roesch et al., 2006), and after OFC lesions subjects remain sensitive to the different values of outcomes (Kennerley & Wallis, 2009; O'Neill & Schultz, 2010). Alternative theories posit that the OFC encodes outcome information, including the value of the outcome along with aspects of reward identity and parameters relevant to receiving rewards including but not limited to the probability of reward or if there is a delay to reward (Steiner & Redish, 2012; Wilson, Takahashi, Schoenbaum, &

Niv, 2014). A recent study by McDannald et al. (2014) demonstrated that the OFC represents the features of a juice reward, independent of information regarding the value of the juice. Another explanation for reversal deficits is that subjects are unable to realize that they are no longer receiving an expected reward after contingency changes because the OFC is responsible for generating prediction error signals. However, although the OFC contributes outcomes expectancy information to the generation of theses signals, this function more likely involves several brain areas with signals ultimately occurring in other brain areas (such as the ventral tegmental area and ventral striatum; Schultz, Dayan, & Montague, 1997; Takahashi et al., 2011). Taken together, although the OFC appears to represent value information, this region also represents the specific properties that differentiate outcomes independent of value, indicating that the OFC is more likely to be involved in outcome expectations rather than general economic value encoding (Feierstein et al., 2006; Roesch et al., 2006).

A competing theory of OFC function suggests that reversal deficits are due to impaired inhibitory control over responses (Stalnaker, Cooch, & Schoenbaum, 2015). According to this view, subjects are aware they are no longer receiving rewards but are unable to disengage from, or inhibit established responses to previously rewarded choices. This theory is supported by the observation that after OFC lesions, rats are impaired at reversals due to perseverative responses to the previously rewarded stimulus (Boulougouris et al., 2007; Tait & Brown, 2007). However, this has been called into question by experiments using more complex learning tasks. It was thought that if the complexity of a reversal task is increased, this allows for better identification of the exact nature of the errors being made by subjects with OFC damage. A study that used a four choice discrimination reversal

rather than two choice found that rats were impaired on the task because of multiple types of errors including perseverative. The animals make a similar number of irrelevant (selecting an option that was not previously rewarded), regressive (not maintaining the selection of a rewarded option) and perseverative errors (Kim & Ragozzino, 2005). Several other studies using a complex reversal model have found a similar variety of errors occurring as frequently as perseverative errors (Chudasama & Robbins, 2003; Riceberg & Shapiro, 2012; Walton, Behrens, Buckley, Rudebeck, & Rushworth, 2010). Therefore, the role of the OFC is more complex than just response inhibition, particularly when task complexity increases.

*Outcome Expectancies*

The outcome expectancy theory of OFC function suggests that this region integrates information stored in multiple brain regions to determine the most likely outcome of the current situation (Gottfried et al., 2011; Schoenbaum & Roesch, 2005; Takahashi et al., 2013). This theory posits that the OFC integrates information regarding previous outcomes, cues relevant to outcomes, and associative information stored in other brain regions allowing expectancies to be based on all available past and present information. Further, because the OFC receives sensory input from all modalities, expectancies are specific to the current situation. Thus, expectancies do not uniquely signal that a cue signaled an outcome previously. Instead, expectancies are a judgement or an evaluation that an outcome will occur in the future (Zald & Kim, 2001). This concept is best summed by Zald & Kim (2001):

"Expectancies differ from simple associative encoding in two important ways. First they provide internalized model of future reality that can be used to guide

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behaviour, which does not require external cues for its maintenance, and second, they provide an expectation of likely outcomes that can be compared to actual outcomes to facilitate learning in other brain regions" (Zald, 2001 p. 206).

Expectancies generated in the OFC assist with the generation of reward prediction error signalling in other regions (Takahashi et al., 2009). Another important aspect of the outcome expectancy theory is that the OFC signals outcomes according to internalized information rather than just according to the cues included in the surrounding context (Farovik et al., 2015; Levens et al., 2014). This is important for guiding behavioural responses because animals can infer the most likely outcomes based on similar past experiences. For example, rats with OFC lesions are impaired in tasks that require subjects to integrate multiple previously learned associations to infer likely outcomes. In a Pavlovian over-expectation task, rats are presented with two cues that have been previously trained to predict reward but have never been presented together. Although the two cues have never been experienced together, non-lesioned animals are able to infer the anticipated abstract value of the new cue combination and exhibit summation or increased responding to the combination of cues based on the expectation that both the rewards will be offered (Rescorla, 1970). Following OFC lesions, animals do not exhibit summation, nor do they extinguish their responses when the cue is presented independently of reward (Lucantonio et al., 2015; Takahashi et al., 2013). This highlights two important roles of the OFC. The ability to integrate multiple associations, and the ability to integrate abstract information with current information in order to infer outcomes when faced with a novel cue combination.

In addition to these two functions, here, I hypothesized that generating expectancies also requires the ability to determine the relevance of information. The OFC is not required for discriminative responding, Pavlovian conditioning, or instrumental responding so long as simple, unambiguous association provide task solution, but instead the OFC is essential when appropriate responses cannot be made using simple associative information (Zald & Kim, 2001). In these situations, the OFC reduces uncertainty by determining the relevance of information, meaning that rather than OFC-dependent impairments being the result of an insensitivity to reward, instead, they occur because animals are unable to focus attention towards the information that is the most relevant (Diekhof, Falkai, & Gruber, 2011; Young & Shapiro, 2011b). Above, I presented a situation where all the information for task solution was not immediately available; therefore, the OFC functioned to integrate relevant previous experiences to infer likely outcomes. In the next section, evidence will be presented supporting the idea that the OFC functions to determine the relevance of cues thereby facilitating discrimination.

*Relevance*

Uncertainty due to excess information challenges neurobiological processes designed for determining cue relevance. OFC activity increases with increasing uncertainty (Hsu, Bhatt, Adolphs, Tranel, & Camerer, 2005), and following OFC lesions subjects are insensitive to ambiguity and exhibit deficits in identifying relevant cues particularly after unexpected outcomes (Chase, Tait, & Brown, 2012). Importantly, the OFC receives current sensory input along with information about the animals current motivational state (Morris & Dolan, 2001), allowing this area to determine cue relevance within the current task context (Wilson et al., 2014).

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Neuronal recording evidence supports the conclusion that the OFC encodes and/or signals information according to its relevance. When a task requires rats to discriminate between two odours, 77% of OFC neurons are odor selective. Conversely, if rats are rewarded for identifying if an odor presented on the current trial is the same as the odor presented on the previous trial then only 15% of OFC neurons are odor selective, and instead the majority of OFC neurons encode match information. Therefore, OFC neurons primarily encode the specific cue information relevant to receiving a reward, odor in the former task and match information in the later. Similarly, if background information was relevant to the task, then it was encoded by 50% of OFC neurons compared to the 25% of neurons that encoded the same information when it was irrelevant (Ramus & Eichenbaum, 2000; Schoenbaum & Eichenbaum, 1995; Schoenbaum, Setlow, Saddoris, & Gallagher, 2003).

The OFC is linked to discrimination processes, and lesions of this area result in generalized responses (Farovik et al., 2015; Pellis et al., 2006; Ward, Winiger, Kandel, Balsam, & Simpson, 2015; Wilson et al., 2014; Zelinski et al., 2010). For example, human subject's navigating through familiar routes with overlap demonstrate hippocampal activation throughout the task, but when navigating through overlapping areas, OFC activation is observed suggesting the OFC aids in disambiguating overlapping spatial information (Brown, Ross, Keller, Hasselmo, & Stern, 2010). During play, rats with OFC lesions do not alter their play behaviours according to whether they are playing with a dominant and subordinate partner (Pellis et al., 2006). Similarly, after OFC damage, human patients exhibit inappropriate social behaviours and impairments in discriminating between social stimuli such as facial expression (Haxby, Hoffman, & Gobbini, 2000; LoPresti et al., 2008). Previously, we observed that rats with OPFC lesions exhibited generalized

freezing in a discriminative contextual conditioning task thought to be a result of an impaired ability to constrain fear responses in the face of conflicting cues. Similar generalized fear responses have been recorded in primates with OFC lesions (Agustín-Pavón et al., 2012). Generalized fear may be a result of an inability of rats or primates with OFC dysfunction to determine cue relevance (Agustín-Pavón et al., 2012; Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013; Zelinski et al., 2010).

Behavioural studies have not clarified whether the OPFC is essential for learning the relevance of cues, or if this region is crucial only when constraining responses to uncertain cues. Some evidence suggests that the OFC is essential for determining cue significance while learning (Walton, Behrens, Noonan, & Rushworth, 2011), while others support a role for the OFC during responding (Diekhof et al., 2011; O. Gruber, Diekhof, Kirchenbauer, & Goschke, 2010). For example, conclusions from one study suggest after OFC lesions macaques are unable to specify which choice to credit for receiving a reward when learning a reversal (Walton et al., 2011). Alternatively, studies done by Hosokawa and colleagues show that the OFC calculates the importance of information when making responses (Hosokawa, Kennerley, Sloan, & Wallis, 2013).

Taken together one consistent effect is maintained across the literature: the OFC is crucial for uncertain or ambiguous tasks. Uncertainty may be due to task complexity, with conflicting cue outcomes (Walton et al., 2010; Zelinski et al., 2010), or uncertainty might be present because all the information required for task solution is not immediately available (Jones et al., 2012). Presented here, in agreement with others, I proposed that the OPFC is focal to solving uncertainty because of this region's role in determining the relevance of cues and directing behavioural attention towards relevant cues (Diekhof et al., 2011; Walton et al.,

2011; Walton, Chau, & Kennerley, 2015; Young & Shapiro, 2011b). According to the current situational demands and the determined relevance of cues, I proposed the OPFC moderates between the need for generalization or discrimination in order to resolve uncertainty (Farovik et al., 2015; Wilson et al., 2014). I hypothesize that during complex situations, this region functions to constrain responses to the most relevant stimuli (Diekhof et al., 2011; Kim & Ragozzino, 2005; Riceberg & Shapiro, 2012; Young & Shapiro, 2011b; Zelinski et al., 2010), and when the situation lacks ample information, the OPFC determines the relevance of previously gained knowledge in order to infer likely outcomes (Jones et al., 2012; Schoenbaum & Esber, 2010; Takahashi et al., 2013).

However, the OFC likely cannot store all the information relevant to all decisions, and likely does not function independently. The complex functions of the OFC are supported by the sensory, reward, context, and motivational input the OFC receives from a wide array of neural circuits including all the main learning and memory systems. In the next section, I will review evidence for the idea of multiple memory systems and how these complex parallel loops might interact with neocortical areas like the OFC.

### Multiple Memory System Interactions

The multiple memory systems theory proposes that there are multiple learning and memory systems found in different parts of the brain that interact in a competitive and cooperative manner to influence behaviour (McDonald, Devan, & Hong, 2004). The systems simultaneously acquire information about the world, but, individually, each system is specialized to obtain and store specific information details relevant for producing specific behaviours (Leong & Packard, 2014;

McDonald et al., 2004). Each learning and memory system centers on a focal brain structure including but not limited to the hippocampus, striatum, and amygdala.

The hippocampus (HPC) is thought to form a complex, and detailed memory representations and is essential for forming episodic memories. This area is most often linked to the ability to perform spatial navigation (Shapiro, Tanila, & Eichenbaum, 1997; Sutherland & Rudy, 1989), but is also important for discrimination tasks (Antoniadis & McDonald, 2000; Rudy & O'Reilly, 2001; Sutherland & Rudy, 1989), and temporal ordering (Fortin, Agster, & Eichenbaum, 2002). With the dorsomedial striatum and prefrontal cortex, the hippocampus forms representations that are part of a goal-directed learning and memory system that facilitates flexible behavioural responses (A. Gruber & McDonald, 2012; Schwabe & Wolf, 2011). The amygdala specializes in the formation of emotionally relevant memories by associating stimuli with biologically significant events like the memories formed during simple classical conditioning (McDonald & White, 1993; Phillips & LeDoux, 1992). The striatum plays a fundamental role in instrumental learning, linking actions to cues and outcomes. Like that which occurs during operant conditioning (Featherstone & McDonald, 2004, 2005; McDonald & White, 1993). Striatal sub-regions form parts of separate learning systems including the goal-directed system (mentioned above) and a habit system that are both proposed to be modulated by a third ventral emotional network that includes ventral HPC, ventral striatum, and vmPFC (A. Gruber & McDonald, 2012). The dorsolateral striatum (DLS) is part of the habitual learning system, forming associations between stimuli and specific motor responses. This system is habitual because the associations formed are inflexible due to their insensitivity to changes in outcome

values (Devan, Hong, & McDonald, 2011; McDonald, King, & Hong, 2001; Schwabe & Wolf, 2013).

Many behavioural tasks can be solved using multiple strategies that are each supported by different learning and memory system. For example, rats learning to navigate to a reward can use either a spatial or a cue-response strategy to solve the task (Packard & Wingard, 2004). Spatial strategies are dependent on the hippocampus and considered part of the goal-directed system because they are flexible to task changes. Alternatively, animals can use cue-response strategies which are dependent on the DLS, and part of the habitual response system. Using a cue-response strategy means that animals learn relationships between specific motor responses and cues. For example, in the T-maze, a rat using a cue-response strategy might learn to walk straight then turn right when the wall ends, and in the water task animals would learn to swim towards the visible platform. If animals were only able to use cue-response strategies, then task changes such as rotating the T-maze (so animals now have to turn the opposite direction) or making the visible platform invisible in the water task would result in maladaptive perseverative responses.

There is substantial evidence that animals can learn multiple strategies. Both the goal-directed and habitual systems acquire and store information throughout learning that can be used depending on the current situational demands. For the most part, animals usually first learn tasks using goal-directed spatial strategies, then, as tasks become familiar, switch to making habitual responses using simple stimulus-response representations (Packard, 2009; Ritchie, Aeschliman, & Pierce, 1950). However, should the task contingencies change behavioural control can switch back to the goal-directed system if hippocampal spatial representations are required.

Two brain regions have been proposed to influence switching between these two learning and memory systems: the prefrontal cortex and amygdala. Stress or infusion of stress hormones into the basolateral amygdala induces a bias towards the use of cue-response strategies (Packard & Wingard, 2004), and damage to the mPFC in the rat impairs the ability to switch between different response strategies (Gemmell & O'Mara, 1999; Killcross & Coutureau, 2003; Ragozzino, Detrick, & Kesner, 1999). Lesions to the infralimbic cortex of mPFC induce a bias for the use of goal-directed strategies (Coutureau & Killcross, 2003; Whishaw, Zeeb, Erickson, & McDonald, 2007), whereas prelimbic inactivation results in habitual strategies being favoured in initial task learning (Killcross & Coutureau, 2003). Therefore, the medial prefrontal cortex has been proposed to act as a modulatory switch between different learning and memory systems (A. Gruber & McDonald, 2012; McDonald & Hong, 2013). The OFC is thought to be a part of the goal-directed system, and activity in the OFC facilitates the initiation of goal-directed responses (Gremel & Costa, 2013; Young & Shapiro, 2011a). However, no studies similar to the studies mentioned above have been completed to investigate if damaging this area will influence response strategies used.

The tasks described above focus on determining which memory system gains control over behaviour, however, other in behavioural task appropriate responses might require cooperation between several systems. Next, I will introduce an uncertain learning task where the OFC might function to integrate information from different learning and memory systems in order to reduce uncertainty.

### *Discriminative Fear Conditioning to Context*

The discriminative fear conditioning to context (DFCTC) task assess adaptive responding during uncertain situations. Uncertainty is inherent in this task as the paradigm consists of exposing rats to two distinct contexts that differ in various dimensions. In one context, an aversive event (foot-shock) consistently occurs and in the other context, no stimulus pairing occurs. Despite the differences between the contexts, there are common features, including construction materials of the floors, walls and covers, location in the same room and association with the same experimenter. Similarities and differences between the contexts make certain cues perfect predictors (only found in the paired context) of the aversive event, other cues partial predictors (common to both contexts), and other cues perfect predictors of no event (only found in the unpaired context).

Learning is inferred on the DFCTC task by assessing two response measures, time spent freezing, and preference. Under normal circumstances, rats that associate the paired context with the foot-shock will differentiate the paired from unpaired context and spend more time freezing within the paired context and prefer to dwell within unpaired context (Antoniadis & McDonald, 1999). Lesions of certain brain areas, such as the hippocampus, amygdala, and OPFC, can disrupt these normal behavioural responses (Antoniadis & McDonald, 2000; Zelinski et al., 2010).

As mentioned above the hippocampus specializes in forming complex and detailed representations of the stimuli that compose our environments (context; Shapiro et al., 1997; Sutherland & Rudy, 1989; White & McDonald, 2002). After hippocampal lesions, rats exhibit generalized freezing in the DFCTC task and do not exhibit a preference for either context. Because the freezing levels of hippocampal lesioned rats within both the paired and unpaired contexts are similar to levels

exhibited by controls in the paired context, we interpret this to demonstrate that these animals have learned the contexts are associated with foot-shock but are unable to discriminate between the contexts (Antoniadis & McDonald, 2000).

The amygdala forms and stores emotional memories, such as learned associations between cues and biologically significant events (McDonald & White, 1993; Phillips & LeDoux, 1992), meaning this region is essential for classical conditioning tasks (LeDoux, 2007; Phillips & LeDoux, 1992). In the DFCTC task, amygdala lesions result in depressed freezing in both contexts and no preference for either context (Alvarez, Biggs, Chen, Pine, & Grillon, 2008; Antoniadis & McDonald, 2000; Phillips & LeDoux, 1992), suggesting that these animals did not learn to associate fear with the paired context. Anatomical, electrophysiological and behavioural evidence suggests that the hippocampus and amygdala synergistically interact to produce appropriate responding in the fear conditioning to context paradigm (Antoniadis & McDonald, 2000; Maren, 2001; Phillips & LeDoux, 1992; Winocur et al., 1987). The hippocampus provides a complex representation of the context, and this information is processed by the amygdala to access fear response systems (LeDoux, 2003). Thus, during DFCTC, the amygdala and hippocampus must interact cooperatively, and normal behavioural responses require input from both structures for rapid acquisition (Antoniadis & McDonald, 2000).

OPFC lesions alter normal behavioural expression of discriminative fear. Rats with OPFC lesions exhibit high levels of freezing in both context chambers that persists over several testing blocks. Although these rats exhibit generalized fear and impaired extinction, they maintain the ability to avoid the paired context (Zelinski et al., 2010). This suggests these animals are able to learn the shock-context association and are able to discriminate between the contexts, but without OPFC

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input are unable to constrain their responses according to the most relevant context cues during testing (Zelinski et al., 2010). Therefore, in this task, the OPFC functions to reduce uncertainty by determining the relevance of the cues presented during testing in order to constrain fear responses only to those cues that are perfect predictors of the shock.

### *Purpose of this thesis and research questions*

The OPFC has been implicated in determining the relevance of cues and in preventing the over-generalization of fearful responses. However, these functions are reliant on multiple brain regions including the OPFC and other neural systems important for learning and memory. Here, I hypothesize that the OPFC supports associative processes during uncertainty by mediating the balance between discrimination and generalization according to the relevance of information. To address this theory, I assessed the role of the OPFC during learning of tasks with relatively high levels of uncertainty.

*Project 1: Role of OPFC in generalization to moderate predictors*

Project 1 consisted of two parts. The first experiment is based on previous work from our laboratory showing that OPFC lesions prior to learning impaired constrained fear responses. The behavioural effect was pronounced, rats with OPFC damaged showed elevated levels of fear that generalized to both contexts and did not extinguish, although they actively avoided the aversive context (Zelinski et al., 2010). Experiment 1 extended these findings by assessing the effects of temporary inactivation, using local infusions of muscimol into OPFC, after acquisition training to determine whether OPFC must be active during expression of discriminative fear

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and during acquisition. I hypothesized that inactivation of the OPFC during behavioural assessment will result in generalized freezing responses but will not impact the ability to exhibit active avoidance. Experiment 2 followed the same experimental design as experiment 1, but rats were trained in an appetitive version of the discriminative context conditioning task. Experiment 2 was conducted to determine if the OPFC has a more general function of constraining responses to cues that have perfect predictive value for aversive and appetitive biologically significant events. Accordingly, animals completed the training phase of the task, and the OPFC was inactivated during the behavioural assessment phase. I hypothesized that OPFC inactivation will result in generalized activity level responses, but will not impact preference.

*Project 2: OPFC interactions with multiple memory systems*

Although I proposed that the OPFC functions to solve uncertainty by determining the relevance of presented environmental information, it is unlikely that this region encodes all this information and/or completes this function solitarily. I proposed that the OPFC interacts with other neural systems implicated in learning and memory functions such as the striatum, hippocampus, and amygdala. Some behavioural tasks can be solved using different response strategies creating uncertainty regarding which specific cues will be relevant. Animals may exhibit an innate preference for the use of one response strategy over the other, but many different influences can sway these preferences. Therefore, Project 2 was designed to determine if OPFC inactivation induces a bias in response strategies allowing us to infer on this region's influence and interactions with learning and memory structures.

I addressed this question using the cue/place water task. During training for this task, animals learn to navigate to a platform that remains in the same location throughout training, but is visible on some training days, and invisible on others. Therefore, rats acquire information for both goal-oriented spatial responses and habitual cue responses. After training, the visible platform is moved to the opposite quadrant in the pool to assess if rats are responding using spatial or cue strategies. Rats that swim first to the previously trained platform location are using spatial strategies, whereas those that swim directly to the visible platform are using cue-response strategies. Prior to the competition test rats will receive infusions of muscimol or saline into the OPFC. I hypothesize that rats with an inactivated OPFC will exhibit a cue response bias.

## Chapter 2

## Evidence of a role for orbital prefrontal cortex in preventing over-generalization to moderate predictors of biologically significant events

## Introduction

Contextual conditioning is a form of classical conditioning where the collective sensory properties of a context become conditioned to elicit a response rather than a single discrete stimulus (Antoniadis & McDonald, 1999; Winocur et al., 1987). Discriminative context paradigms are of interest because it represents a learning situation that is challenging for the organism. Exposure to uncertain environmental contingencies is a challenge encountered during daily activities when learning about a new situation or environments (Rescorla, 1968). Uncertainty is inherent in the discriminative fear conditioning to context task (DFCTC) because the paradigm consists of exposing rats to two distinct contexts differing on various dimensions including shape, colour, and smell. In one context, an aversive event (foot-shock) consistently occurs, and in the other context, nothing happens. Despite the differences between the discriminative contexts, there are also many common features such as the materials of the floor, walls and roof among other (listed in the general introduction). The differences and similarities between the context make certain cues perfect predictors (only found in the paired context) of the aversive event, other cues partial predictors (common to both contexts), and other cues perfect predictors of no-event (only found in the unpaired context).

The formation of contextual associations requires synergistic input from complex neural circuits including the amygdala and hippocampus (Antoniadis & McDonald, 2000; Phillips & LeDoux, 1992), alongside involvement from prefrontal

regions (Alvarez et al., 2008; Maren, Phan, & Liberzon, 2013; Zelinski et al., 2010). The OPFC has been implicated in overgeneralization and amplification of fear in rats (Zelinski et al., 2010), marmosets (Agustín-Pavón et al., 2012), and human patients with anxiety disorders (Greenberg et al., 2013). Rats with OPFC lesions exhibit generalized and enhanced freezing in the DFCTC task suggesting that input from the OPFC facilitates the ability to constrain fear responses to the most relevant cues. The ability to constrain fearful responses is particularly relevant for this task because the overlapping cues within the paired, and unpaired contexts create uncertainty regarding the predictive value of the constellations of cues composing the two contexts (Zelinski et al., 2010).

For the present experiments, I was interested in answering two questions related to the role of OPFC in over-generalization. First, I expand on our previous work directly implicating OPFC dysfunction in over-generalized fear responses. This work had lesioned OPFC before training occurred, demonstrating that OPFC function was necessary for the task acquisition and/or the expression of discriminative freezing. However, these experiments do not speak to whether OPFC function is necessary for the expression of discriminative freezing if the task is acquired with the OPFC online and functional. To address this question rats were trained on the DFCTC task, and before testing, the OPFC was inactivated using muscimol. Second, because the OPFC has also been implicated in encoding information about rewards (Gottfried, O'Doherty, & Dolan, 2003; McDannald et al., 2014; Padoa-Schioppa & Assad, 2006; Tremblay & Schultz, 2000), it is important to determine if this region might have a more general function of constraining responses to cues that have perfect predictive value for biologically significant events. To answer this question, rats were trained on an appetitive version of the

discriminative context task using a highly palatable food as the reward and during testing the OPFC was inactivated using muscimol.

## Experiment 1

For Experiment 1, I will dissociate the role of the OPFC in acquisition and expression of discriminative contextual fear conditioning by temporarily inactivating it with muscimol. This will provide clear information about whether the OPFC must be intact and functioning to exhibit normal discriminative fear responses even if this neural system was functioning during acquisition.

## Methods

### *Subjects and Handling*

Male Long-Evans rats obtained from Charles River Colony (Raleigh NC, USA and Laval QC) were housed in pairs and allowed to acclimated in their home cages for approximately one week. Animals were housed on a 12:12 dark light cycle with food and water available *ad libitum*. Handling occurred for 5 minutes daily for the 3 days before experimental start.

Typically, acquisition of the DFCTC task is assessed using two measures over three testing days. For the first two days of testing, time spent freezing is assessed, one day in the paired context and the second in the unpaired context followed by a preference test day. However, I wanted to assess freezing in rats after they received an infusion and then again with no infusions given. Since no shocks are delivered during testing each test day also serves as an extinction day. Therefore, I used two separate groups of rats to complete the two different assessments (Fig 1). Animals in both groups followed the same surgical, infusion (aside from whether they received

muscimol or saline during the infusion), and training procedures. Each group was separated into treatment groups according to whether they received an infusion of either muscimol or saline before assessment. Therefore, animals in Group A (muscimol N = 10; and saline N = 10) were assessed using two testing blocks, and prior to the first testing block, they received infusions. Group B animals (muscimol N = 17; saline N = 17) were assessed by one testing block and two preference tests during which they received infusions prior to the first preference test only. All procedures were in accordance with the regulations set out by the Canadian Council of Animal Care and approved by the University of Lethbridge Animal Care Committee.

*Surgery*

All surgical procedures were carried out under aseptic conditions. Subjects were given a 0.03 mg/kg dose of buprenorphine subcutaneously 30 min before being anesthetized using isoflurane anesthesia (4% with 2l/min of oxygen for induction and reduced accordingly to maintain a surgical plane throughout the procedure). Once anesthetized, rat heads were shaved and placed in a standard stereotaxic apparatus. Using a scalpel blade, an incision was made anterior to posterior along the midline of the scalp. Four hemostats were used to retract the skin, and the periosteum was cut laterally to expose the skull bone. Using a high-speed drill (0.7mm drill bit) two holes were drilled in the skull at the following coordinates relative to Bregma: AP +3.7mm, ML +/-3mm. An additional three holes were drilled (1mm drill bit) into three different skull plates in order to attach three self-tapping screws to the skull allowing for better attachment of an acrylic resin skull cap. Two cannulae (each 11 mm, 26 ga) were lowered (DV -3.5mm) into the first two holes drilled. Grip cement

(Chaulk Division and Dentsply) was applied to the surface of the skull in order to hold the cannulae in position. Removable dummy wires (bent at a 70º angle) were inserted into the implanted cannulae in order to prevent any clogging or contamination from debris passing through the cannulae. The dummy wires were flush with the implanted cannulae. Directly after surgery, each animal was given a subcutaneous injection of Metacam© (1mg/kg) for postoperative pain and inflammation, and saline (3 mL). Animals were monitored in recovery cages that were placed on a low-heat heating pad until awake and active. Once recovered, animals were placed back into paired housing and monitored twice daily for a total of 72 hours. The rats were allowed at least one week of postoperative recovery time before behavioural testing began.

### Infusions

Infusion cannulae (12mm) made of 32 gauge cannulae cut to extend 1mm beyond the guide cannulae tip were used to infuse muscimol or saline on the infusion day. Clear tubing was attached to the top end of the infusion cannulae connecting the infusion cannulae to a 10 ul Hamilton syringe on a Hamilton Infusion Pump. Rats were restrained in a towel and their dummy wires removed prior to the infusion cannulae being inserted into the rats implanted cannulae. Infusions of saline (0.9%) or muscimol (1ug/ul) occurred at a rate of 0.5ul/min over 70 seconds. The infusion cannulae were left in place for an additional one minute following the infusion period to allow for drug diffusion. Once the infusion procedure was complete, the removable dummy cannulae were replaced, and the rat was returned to his home cage for 20 minutes prior to testing.

### Discriminative Fear Conditioning to Context

*Chambers*

Two context chambers were used that differed in three dimensions: colour, shape, and odour (see Fig 2 for pictorial representation). Both context chambers were opaque with floors made of metal bars spaced 1.5 cm apart. A small plastic cylinder containing a distinct odorant was mounted on one wall of each chamber. Daily, each odorant, serving as an olfactory cue, was placed on a cotton ball that was inserted into the cylinder container. The white square-shaped chamber (41 cm × 41 cm × 20 cm) was paired with a eucalyptus scent. The other context was a black triangle-shaped chamber (61 cm × 61 cm × 30 cm) with amylacetate as the scent cue. During pre-exposure and preference, the two chambers were connected by a grey alley (16.5 cm long × 11 cm wide × 11 cm high). The entire structure was placed on a clear Plexiglas table with a height of 100 cm. A mirror (91 cm long × 61 cm wide), inclined by 45°, was placed on the floor under the clear table, allowing the experimenter to see the interior of the chambers. A video camera was placed in front of the mirror to record the testing and preference phases of the experiment. The entire apparatus was cleaned with a soap solution after each rat.

*Pre-exposure*

To allow animals to acclimate to the testing apparatus, each rat was placed in the middle alley and allowed to explore the entire apparatus freely for 10 minutes. An observer recorded dwell time which was amassed when both forepaws were past the threshold of the doorway into the chamber and stopped when both forepaws were back in the alleyway. Pre-exposure occurred in room B.

*Training*

Training began approximately 24 hours following pre-exposure. The rats were counterbalanced such that half the animals from each group were assigned the white square as their paired context and the other half was paired with the black triangle. The animals were further counterbalanced so that half the animals would begin training in their paired context and the other half would start in their unpaired training context. All paired days occurred in room A, and all unpaired days occurred in room B. During training, Plexiglas panels were inserted into the doors of the chambers to block access to the middle alley. In the unpaired condition, each animal was placed in its assigned context individually and remained there for 5 minutes. For the paired (foot-shock) condition, 0.6 mA of current (scrambled shock) was delivered for 2 seconds through the grid flooring at minute 2, 3, and 4. Animals experienced their contexts on alternating days, such that animals that were assigned to begin training in their paired context on training day one would then experience their unpaired context on training day two, whereas, those assigned to begin in the unpaired context, would be placed in the paired context on the second day. This alternating training sequence was repeated over 8 training days so that all animals received four training sessions in their paired (shock) context and four training days in their unpaired (neutral) context.

*Testing*

Testing was conducted to record the amount of time rats spent freezing within each chamber as a measure of whether the animals learned to associate the context with the aversive foot-shock and whether they were able to discriminate the shock associated context from the neutral context. Normal animals exhibit discriminative freezing evidenced as spending more time freezing within their paired than unpaired context. Testing began approximately 24 hours following the final

training session. No shocks were administered throughout testing and all testing sessions occurred in room B. According to their counterbalanced group's rats were placed within either the paired or unpaired context on the first testing day, then were placed in the opposite context on the second testing day. A testing block consisted of one test day within the paired and another in the unpaired context. During testing, rats were placed into one of the enclosed contexts for 5 minutes and an observer recorded time spent freezing. Freezing constituted total immobility of the rat's body and whiskers, other than the movement required for breathing. All testing sessions were filmed so that freezing scores could be later verified from the recording.

**Group A:** Animals in group A received two testing blocks. Infusions were given 20 minutes before the first testing block (test days 1 & 2). No infusions occurred before testing block two (test days 3 & 4).

**Group B:** To determine if animals in group B acquired the context-shock association (before their intended preference assessment) these animals received one standard testing block with no infusion.

*Preference test*

Preference testing was conducted to establish if the rats would show an aversion to the context previously paired with shock. Normal rats easily learn to avoid the paired (foot-shock) context as exhibited by spending more time within the unpaired context. Preference began approximately 24 hours after test day 2. The doors restricting exit from the chambers to the alley were removed and each rat was placed within the middle alleyway and allowed to explore both chambers freely for 10 minutes. Dwell time in each context was recorded by an observer and later verified from video recordings. Time was accumulated when both forepaws and half

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the body were past the threshold of the doorway into one of the chambers and ended when both forepaws and half the body exited the chamber into the alleyway. The preference test occurred in room B.

**Group A:** Animals in group A did not complete a preference test.

**Group B:** Animals in group B were assessed by two preference tests occurring approximately 24 hours apart. Infusions were given 20 minutes before the first preference test. No infusions occurred before the second preference test.

### *Histology*

After completion of behavioural testing, animals were euthanized with a single intraperitoneal injection of sodium pentobarbital (300mg/kg) and transcardially perfused with approximately 150mL of 1x phosphate-buffered saline (PBS) followed by approximately 150mL of 4% paraformaldehyde in 1xPBS. After decapitation, brains were removed from the skull and immersed in 4% paraformaldehyde in 1xPBS for approximately 24 hours. Brains were then transferred to a 30% sucrose and 0.2% sodium azide in 1xPBS until sectioning. Brains were sectioned in a series of 3 at 40μm using a cryostat (CM1900, Leica, Germany) and stained using Cresyl Violet. Cannulae placement was then verified under a microscope. All subjects' cannulae were confirmed to be in the appropriate position.

### *Statistical Analysis*

Effects are reported as significant at $p < .05$, and all statistical analyses were two-tailed. For each of the test or preference test days, a mixed design ANOVA was conducted with group (muscimol or saline) as the between subject factor, and context

(paired or unpaired) as the within subjects factor. Planned Fisher's LSD comparisons were also conducted to analyze time spent freezing or dwell time between and within the groups in the paired and unpaired contexts. All statistical analyses were conducted using SPSS ver 21 (IBM, USA) and GraphPad Prism software (GraphPad, La Jolla, CA), and all graphs were created using GraphPad Prism software.

## Results

### Group A

*Pre-exposure*

Neither group showed an initial preference for one of the chambers and an analysis of dwell time accumulated in each context during pre-exposure confirmed this observation (Fig 4A). No significant effects of Group, Context or a Group by Context interaction were found ($p$'s > 0.05).

*Testing with infusion*

Figure 4B illustrates that the saline infused group spent more time freezing within the paired chamber indicating they learned that the paired context was associated with the receipt of a foot-shock. In contrast, the muscimol infused group generalized the contexts and even spent slightly longer freezing within the unpaired than paired context. There was no significant main effect of Context [$F_{(1, 18)}$ = 1.229, $p$ < 0.282], nor Group [$F_{(1, 18)}$ = 2.080, $p$ = 0.166], however there was a significant Group by Context interaction [$F_{(1, 18)}$ = 14.36, $p$ = 0.001]. The saline infused rats spent more time freezing within their paired context ($p$ = 0.003), whereas the muscimol infused group did not spend significantly more time freezing within either context ($p$ = 0.074). To further address the interaction effect, a posthoc analysis revealed that the

muscimol infused animals froze significantly more than the saline infused animals within the unpaired context ($p = 0.005$). There were no significant differences in time spent freezing within the paired context ($p = 0.683$). Overall, inactivation of the OPFC resulted in elevated freezing within the unpaired context compared to the saline infused group leading to what could be described as generalization of the fear response after muscimol infusions.

*Test with no infusion*

Both groups froze longer within the paired compared to the unpaired context (Fig 4C). There was a significant difference in time spent freezing within the Contexts [$F_{(1, 18)} = 14.30$, $p = 0.001$], but not across the Groups [$F_{(1, 18)} = 0.016$, $p = 0.902$], and no significant Group by Context interaction [$F_{(1, 18)} = 0.011$, $p = 0.918$]. Animals in both groups spent more time freezing within the paired than unpaired context (muscimol ($p = 0.013$), and saline ($p = 0.018$)). Therefore, although on the previous testing block the muscimol group exhibited generalized freezing, during this test block when no infusions were given, both groups showed normal discriminative freezing. Thus, the muscimol group acquired the association between the paired context and the aversive foot-shock received throughout training within that context but was only able to express that learning with a functioning OPFC.

**Group B**

*Pre-exposure*

As can be seen in Fig 5A, the groups of rats did not exhibit a bias for either context during the pre-exposure phase, which was confirmed by no significant Group, Context or Group by Context effects ($p$'s $> 0.05$).

*Testing*

Both groups froze longer within the paired than unpaired context (Fig 5B) indicating they learned to associate the paired context with the foot-shocks and were able to differentiate that context from the neutral unpaired context. There was a significant difference in freezing within the Contexts [$F_{(1, 32)}$ = 19.05, $p < 0.001$], however, no significant differences across Groups [$F_{(1, 32)}$ = 0.614, $p$ = 0.439], and no Group by Context interaction [$F_{(1, 32)}$ = 0.499, $p$ = 0.485] was observed. Both groups froze significantly longer within the paired contexts (muscimol ($p$ = 0.007), saline ($p$ = 0.010)).

*Preference Test Following Infusion*

When given free access to both contexts, infusions of muscimol or saline did not impact the animals' ability to exhibit a preference for the unpaired context. Both groups spent longer in the unpaired than paired context (Fig 5C). There was a significant difference in dwell time within the Contexts [$F_{(1, 32)}$ = 16.74, $p < 0.001$], but no significant differences in dwell time between the Groups [$F_{(1, 32)}$ = 1.089, $p$ = 0.304], nor a Group by Context interaction [$F_{(1, 32)}$ = 0.423, $p$ = 0.423]. Both the muscimol ($p$ = 0.002) and saline ($p$ = 0.027) groups demonstrated a significant preference for the unpaired context. Therefore, inactivation of the OPFC did not interfere with the animals' ability to actively avoid the paired context.

*Preference Test with No Infusion*

Both groups spent more time within the unpaired than paired context (Fig 5D). There was a significant difference in dwell time within the Contexts [$F_{(1, 32)}$ =

8.963, $p < 0.0053$], however no significant main effect across the Groups [$F_{(1, 32)} = 3.476, p = 0.072$], or a significant Group by Context interaction [$F_{(1, 32)} = 0.151, p = 0.700$]. The muscimol group spent significantly longer in the unpaired than paired context ($p = 0.023$), however the saline group did not spend significantly longer within the unpaired compared to paired context ($p = 0.075$).

**Cannulae Placement**

Correct cannulae placement was confirmed histologically. The approximate locations of the infusion cannulae tips for all animals included in Experiment 1 are shown in Fig 6, modified from a standard brain atlas (Paxinos & Watson, 1997).

**Summary**

When the OPFC was inactivated following infusions, rats froze equally between the two contexts whereas saline infused rats spent significantly longer freezing within the paired than unpaired context. When no infusions were given before the second testing block, the generalization effect that occurred after muscimol infusions disappeared. Therefore, on second test block, both groups froze for longer in the paired when no infusions occurred. Before training, neither group exhibited a preference for either of the context chambers. Following conditioning, animals in both groups preferred to dwell within the unpaired context, with and without infusion. Therefore, when allowed to move freely between the safe and shock context, inactivation of the OPFC does not alter a rat's ability to express an aversion for a context previously paired with shock.

## Experiment 2

The OPFC is implicated in learning about both aversive and appetitive associations. Therefore, it is important to determine if OPFC is involved in a general mechanism of response constraint during both appetitive and aversive conditioning. Experiment 2 is intended to assess if OPFC inactivation will result in an over-generalization during an appetitive version of the discriminative contextual conditioning task.

## Methods

### Subjects and Handling

Animals were housed and handled as described in Experiment 1. Similar to Experiment 1, two separate groups of animals completed each of the different assessment measures both with and without infusion. Both groups completed the same surgical, infusion, and behavioural procedures. Animals in group B completed a 14-day water task that ended approximately one month before the CPP pre-exposure day. Rats in group A were naïve. The animals from each group were segregated into two different treatment groups according to whether they received infusions of muscimol or saline before assessment. Animals in group A (muscimol n = 8, and saline n = 8) were assessed using two testing blocks. Infusions were given only before the first testing block. Two preference tests were used to assess subjects in group B animals (muscimol n = 4; saline n = 4) with infusions occurring before the first but not second preference test.

### Surgery & Infusions

Surgical and infusion procedures were identical to those described in Experiment 1.

### *Conditioned place preference (CPP)*

*Apparatus*

The same apparatus was used for CPP (Fig 3) as that described for the DFCTC task in Experiment 1, except that a clear plastic Plexiglas insert was placed directly below the metal bar floor to contain the cookie pieces within the apparatus. Further, the inserts were marked with black tape lines spaced 1" apart that were used to measure activity level during the test blocks.

*Pre-exposure*

Pre- exposure procedures are identical to those described in Experiment 1, except that rats were allowed to explore the two chambers freely for 20 minutes. Dwell time within each chamber was recorded.

*Training*

The training schedule followed that described for the DFCTC task in Experiment 1 such that animals were counterbalanced to begin in either their paired or unpaired context then alternated between contexts on the subsequent training days. All animals received a total of 4 training sessions in the unpaired context and 4 training days within the paired context. In the unpaired condition, each animal was placed into the assigned context individually and remained there for 30 minutes. For the paired condition 10 g of Chips Ahoy Original Chocolate Chip Cookie was placed on the floor opposite the entry way. Rats were placed in the paired context for 30 minutes and allowed to consume the cookie reward.

*Activity Level Test (ALT) Days:* **Group A**

Rats in group A were assessed by comparing levels of activity within the paired to unpaired context. Normal animals are expected to exhibit higher levels of activity within the paired than unpaired contexts (Bolles & Stokes, 1965; Ito, Everitt, & Robbins, 2005). ALT occurred approximately 24 hours after the final (8th) training day. During ALT, animals were placed within the contexts for 10 minutes, and the number of times the rats moved across the black tape floor markers was recorded. No cookies were present during any of the activity level testing days. According to their counterbalanced group's rats experience either the paired or unpaired context on the first day, followed by the opposite context on the second ALT day. Group A received two ALT blocks (4 ALT days). Infusions of muscimol or saline occurred 20 minutes before the first ALT block (test sessions 1 & 2). No infusions occurred before the second ALT block (test sessions 3 & 4).

*Preference test:* **Group B**

Rats in group B were given a preference test approximately 24 hours after the 8th training day. Infusions of either saline or muscimol were given 20 minutes before the preference test. The rats were placed individually in the middle alley and given free access to both chambers for 20 minutes. No food was located in either context. Dwell time was determined as described in Experiment 1 preference test procedures. Approximately 24 hours later a second preference test was administered, but animals did not receive infusions before the second preference test.

### Histology & Statistical Analysis

Procedures were identical as those described in Experiment 1.

# Results

## Group A

### *Pre-exposure*

Neither group showed an initial preference for either chamber before training (Fig 7A). Dwell time in each context during pre-exposure demonstrated no significant effects of Group, Context, or Group by Context interaction ($p$'s > 0.05).

### *Activity Level Testing with Infusion*

All animals were more active within the paired than unpaired context, however the difference in activity levels was larger for animals in the saline than muscimol group (Fig 7B). There was a significant difference in activity levels within the Contexts [$F_{(1,14)}$ = 13.49, $p$ = 0.003], however no significant difference across the Groups [$F_{(1,14)}$ = 0.497 , $p$ = 0.493], nor a Group by Context interaction [$F_{(1,14)}$ = 0.853, $p$ = 0.371]. Only animals in the saline group were significantly more active within the paired than the unpaired context ($p$ = 0.006), and muscimol ($p$ = 0.072).

### *Activity Level Testing with No Infusion*

Both groups were more active within the paired context (Fig 7C), and there was a significant main effect of Context [$F_{(1,14)}$ = 4.753, $p$ = 0.047]. No differences in activity levels across the Groups [$F_{(1,14)}$ = 0.031, $p$ = 0.862] or a significant Group by Context interaction [$F_{(1,14)}$ = 0.307 , $p$ = 0.588] were observed. When comparing activity levels in the paired to unpaired context, neither group exhibited a significant difference in activity levels across the contexts (muscimol ($p$ = 0.074), and saline ($p$ = 0.270)).

**Group B**

*Pre-Exposure*

Neither group exhibited an initial preference for either context before training (Fig 8A). No significant effects of Group, Context, or Group by Context interaction were observed ($p$'s > 0.05).

*Preference after Infusion*

Inactivation of the OPFC or infusion of saline into the same region did not impact either group's ability to show a significant preference for the paired context (Fig 8B). Dwell time in each context were compared revealing a significant difference within the Contexts [$F_{(1,6)} = 29.64$, $p = 0.002$], but no significant difference between Group [$F_{(1,6)} = 0.555$, $p = 0.485$] or a significant Group by Context interaction [$F_{(1,6)} = 1.531$, $p = 0.262$] were observed. Both groups spent significantly more time within the paired than unpaired context (muscimol ($p = 0.003$), and saline ($p = 0.025$)).

*Preference No Infusion*

Although it appears that both groups spent more time within the paired context on the second preference test (Fig 8C), an analysis revealed that there was no significant effect of Group, Context, or a Group by Context interaction ($p$'s > 0.05).

**Cannulae Placement**

Correct cannulae placement was confirmed histologically. Fig 9 depicts the approximate locations of the infusion cannulae tips for all animals included in Experiment 2, modified from a standard brain atlas (Paxinos and Watson, 1997).

*Summary*

Saline infused rats learned the task and were able to discriminate between contexts during the first testing block. However, this effect does not persist into the second test block; the saline group was no longer significantly more active within the paired than unpaired context likely an extinction effect. Muscimol infused animals did not exhibit a significant difference in activity level on either testing block. After receiving infusions of either saline or muscimol, both groups exhibited a preference for the paired context during the first preference test. On the second off-drug preference test, both groups spent longer within the paired than unpaired context, although that difference was not statistically significant. OPFC inactivation did not prevent rats from expressing a preference for a previously rewarding context when given free access to choose between the paired and unpaired context, however, did result in generalized activity levels across the two contexts.

## Discussion

The over-generalization of fearful responding has been linked to a dysfunctional OPFC across species (Agustín-Pavón et al., 2012; Greenberg et al., 2013; Zelinski et al., 2010). Previous work in rats by our group has shown that OPFC lesions elicit amplified and generalized freezing in a discriminative contextual conditioning task. As OPFC lesioned rats were able to differentiate the contexts when allowed to move freely between them, it was assumed that impairments were not due to an inability to learn the association between the context and aversive event. Rather, rats were impaired at constraining responses to those cues that perfectly predicted the aversive event (Zelinski et al., 2010). The current study was intended to answer two open questions about the role of OPFC in the

overgeneralization of responses to biologically significant events. First, experiment 1 demonstrated that the OPFC must be functional during expression of the context discrimination even if it was operational during acquisition. Experiment 2 showed that the OPFC might aid in constraining appetitive responses to cues. In both experiments, OPFC inactivation had no impact on the subject's ability to actively choose their preferred chamber when given free access to both chambers during preference testing.

### *Generalized responses after OPFC inactivation*

Experiment 1 demonstrated that rats with an inactivated OPFC induced via an intracranial muscimol infusion exhibit indiscriminate freezing between the paired and unpaired contexts. This generalization effect reverses in the same subjects the following day when the OPFC is active. Saline infused animals froze for longer within the paired context regardless of whether or not they received an infusion. Because animals learned the task while the OPFC was active, I can conclude that the generalization is not a result of learning impairments, but instead, a functional OPFC is required during the expression of freezing. Our results are consistent with the generalization observed by Zelinski et.al. (2010), except here, there was only a strong amplification of freezing within the unpaired context rather than across both contexts. This suggests that chronic OPFC dysfunction may enhance overall fearful behaviours, whereas acute OPFC inactivation enhances fearful responding to neutral, or ambiguous contexts. Nevertheless, further investigation is required to address this finding.

### *Similar role for OPFC in constraining aversive and appetitive responses*

The orbitofrontal cortex encodes information regarding reward (Gottfried et al., 2003; McDannald et al., 2014; Padoa-Schioppa & Assad, 2006; Tremblay & Schultz, 2000), however this area is not essential for preference of one reward over another (Keiflin et al., 2013; McDannald et al., 2014), or to discriminate between two cues that are perfect predictors of reward and non-reward (Tait & Brown, 2007). Instead, the predominant view is that the OPFC is critical for flexible responding when reward contingencies must be changed during tasks such as reversal learning (Murray, O'Doherty, & Schoenbaum, 2007; Stalnaker, Franz, Singh, & Schoenbaum, 2007), or outcome devaluation (Pickens, Saddoris, Gallagher, & Holland, 2005). The OPFC facilitates reversal learning by generating current outcome expectancies based on the rewards received when similar stimuli and responses were encountered. If an unexpected outcome occurs, such as receiving no reward when one is expected, the OPFC produces an error signal that guides the animal to change their behaviour. Therefore, rats with OPFC lesions are insensitive to reward changes because they are unable to update internal contingency information and thus continue to make inflexible perseverative responses (McDannald, Jones, Takahashi, & Schoenbaum, 2013; Schoenbaum, Roesch, Stalnaker, & Takahashi, 2009; Tsuchida, Doll, & Fellows, 2010). However, rats with OPFC lesions are not impaired solely due to continued perseveration. Instead, they are impaired at disregarding irrelevant stimuli, initiating a novel selection, and repeating advantageous choices (Kim & Ragozzino, 2005; Riceberg & Shapiro, 2012; Walton et al., 2010). Moreover, when rats are directed to the relevant cues for reversal by intermixing cued trials with choice trials, rats with OFC lesions can reverse contingencies (Keiflin et al., 2013). This points to a role for the OPFC beyond flexible reward processing towards controlled responding in complex environments where correct choices require

animals to identify which stimuli are currently relevant from the numerous predictors available (Diekhof et al., 2011; Walton et al., 2010).

Here I show impairments following OPFC inactivation in a contextual conditioning task that requires rats to differentiate stimuli that are perfect predictors for a reward from those that are only moderate predictors. During the first testing block of the appetitive conditioning task activity levels were higher within the paired than unpaired context, only for the saline infused group. The muscimol infused group was not significantly more active within either context. On the second test block, neither group exhibited a significant difference in activity levels between the contexts. This differs from results of the second test block in Experiment 1 where both groups exhibited differentiated freezing. It is likely that during the appetitive version of the task rats more quickly realize that rewards will not be presented thus extinguishing the conditioned response. Accelerated extinction during the appetitive task is likely because the cookie that was used as a reward throughout training acted as an additional visual cue signalling reward within the paired context, and now this cue is absent during testing. During appetitive training days each time animals enter the paired context the cookie reward is immediately known and available to them, decreasing uncertainty, and the need to anticipate when the outcome will occur. In contrast, during the training for the fear task shocks are administered only at certain time points throughout training meaning they anticipate the onset of the shocks. Therefore, animals may experience less uncertainty on the appetitive version of the task because the chocolate chip cookie acted as an immediate and unambiguous reward signal. Alternatively, in a natural environment missing a threat could have more impact on the organism than missing

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a potential food source. Therefore, animal's responses may be more conservative in threatening situations to reduce the potential of missing a threat.

***Why is OPFC necessary to constrain responses?***

The results of the current set of experiments are somewhat surprising because other neural systems are thought to mediate these kinds of context discrimination tasks (Antoniadis & McDonald, 2000). During both aversive and appetitive discriminative conditioning paradigms, subjects were exposed to uncertainty about the predictive value of various cues in the paired and unpaired contexts. Although there are key sensory features that differ between the contexts, more importantly for the present results, there are also commonalities. The similarities and differences between the contexts make certain cues perfect predictors of the presence or absence of the biologically significant event while another subset of cues were partial predictors (common to both contexts). The brain copes with the uncertainty inherent to this task in a variety of ways and identified learning and memory systems and the OPFC might contribute in some complex way to resolve these tasks.

One way to deal with this uncertainty is to create orthogonal representations of the two contexts with unique associations with the reinforcer. Specific cortical nodes for the different modalities probably represent the distinct patterns of sensory activity, and the hippocampus forms a representation of this unique pattern of cortical sensory activity that provides an index for memory retrieval (McDonald et al., 1997; McDonald & White, 1995; Sutherland & McDonald, 1990; Sutherland & Rudy, 1989; White & McDonald, 2002). One confusing point about the animal's indiscriminate responses to the contexts when the OPFC is offline is that the

hippocampus is thought to participate in context discrimination learning and memory (Antoniadis & McDonald, 2000) by providing a complex representation of the relationships among the cues in each context (Shapiro et al., 1997; Sutherland & Rudy, 1989). This representation is created by a hippocampal-mediated pattern separation function that reduces or eliminates generalization precisely because of the orthogonal nature of the two context representations thought to be represented in the hippocampal structure (Gilbert, Kesner, & DeCoteau, 1998; Rudy, 2009; Sutherland & McDonald, 1990; Sutherland, McDonald, Hill, & Rudy, 1989; Sutherland & Rudy, 1989). These representations are retrieved when the cues defining that context are re-experienced or even just elements of the context are presented. This retrieval process reactivates the cortical nodes representing the original sensory representations defining each context via a mechanism called pattern completion (Rudy & O'Reilly, 2001; Sutherland & Rudy, 1989). Retrieval of these context representations then accesses various response/effector systems like attentional/general approach systems in the case of appetitive conditioning (A. Gruber & McDonald, 2012; White & McDonald, 2002), and autonomic/freezing systems in the case of aversive conditioning (White & McDonald, 2002). The OPFC does encode some spatial and contextual information (Feierstein et al., 2006; Vafaei & Rashidy-Pour, 2004; Young & Shapiro, 2011a), but it is much less detailed than that stored in the hippocampus. Further, lesions of the hippocampus and amygdala result in indiscriminative responses during both testing and preference (Antoniadis & McDonald, 2000), unlike the testing specific generalization seen with OPFC damage. Therefore, it could be assumed that without a functioning OPFC, rats maintain the ability to differentiate the contexts supported by the hippocampus and

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amygdala, but that the parameters of the testing assessment highlight a specific OPFC dependent function.

The amygdala can also acquire elemental associations during context conditioning that can support context learning via sensory projections directly to this nucleus, possibly independent of hippocampal input (see Antoniadis and McDonald, 2000 for a full explanation). This form of learning requires more training trials compared to the rapid acquisition thought to be acquired by the synergistic interactions between the hippocampus and amygdala (Lehmann et al., 2009). However, the representation constructed by the networks in the amygdala may not be sufficient to resolve the discrimination when the contexts are defined by some overlapping cues, a situation in which those cues would be moderate predictors of the food or shock. Consequently, this system might be supported by the OPFC by reducing the responses elicited by the presence of those moderate predictors during testing.

The OPFC may bias the animal to use the hippocampal/amygdala representation over the simpler amygdala representation during learning and expression of conditioned responding. With extensive training, it is possible that either of these representations could control responding. However, when the OPFC is removed before training or during expression of discriminative context conditioning, the amygdala representation gains control over responding but is less efficient because of the presence of overlapping cues in the two contexts, resulting in generalization. Consistent with this idea, during reversal learning lesions of both the amygdala and OPFC eliminate the standard reversal deficit associated with OPFC damage, suggesting that another system can take control of this behaviour when both regions are inactive (Stalnaker et al., 2007). Rats can learn a reversal with the

OPFC inactivated but will not recall that reversal if the OPFC is re-activated, demonstrating that OPFC representation can interfere with other learning and memory systems (Keiflin et al., 2013). This might explain the generalization when the animal is forced to stay in one of the contexts during the test, but not why rats with OPFC dysfunction actively avoid or approach the appropriate context during the preference test. It might be the case that the hippocampal/amygdala representation is required to reduce responding in the unpaired context when the animal is given a forced exposure to those cues.

### *Importance of the OPFC during testing but not preference*

During the testing phase, animals are confined within one of the contexts without access to any of the stimuli associated with the other context and must recall the features of the secondary context. This retrieval process likely requires the hippocampus, whereas, in the preference, they are presented with two known options that they can actively compare and react to. Further, during testing they must determine their expectation of threat or reward, whereas in preference, they are directly faced with a known risk. In other words, during testing, the rats are in an anticipatory state that requires internalized information, whereas, during preference, animals are actively responding to currently available cues.

The OPFC is important in abstract anticipatory circumstances like the testing procedure here. For example, neuronal firing in the basolateral amygdala and OPFC suggests that the OPFC supports responses when internal representations of cues are required, whereas the basolateral amygdala can maintain behaviour when external cues are available (Saddoris, Gallagher, & Schoenbaum, 2005; Schoenbaum, Chiba, & Gallagher, 1999; Schoenbaum et al.,

2003; Zald & Kim, 2001). This might explain why the amygdalar and hippocampal circuit can only support discriminative behaviour during preference but not testing.

Therefore, under normal circumstances with an intact OPFC, rats can integrate previously acquired information with the current sensory input of the various cues. Integration of multiple levels of information allows animals to generate an expected outcome based on the stimuli perceived to be most relevant in the current context. Without an intact OPFC, animals are unable to determine the predictive value of the individual context features, and so they generalize their responses because moderate and perfect predictors are no different. Evidently, more work needs to be completed to understand the fundamental role of OPFC in context discriminations demonstrated in the present work.

### *Conclusions*

Overall, the OPFC was not essential when rats are presented with two known options and must immediately react to those options. However, when the rats are confined within one of the contexts without access to the other and are required to determine their behavioural responses during uncertainty, they do not express discriminative behaviours and instead, generalize their responding. This occurs even if the OPFC is intact throughout learning, in both appetitive and aversive context conditioning tasks. I propose that the OPFC functions to guide behaviour according to the relevance of the varying stimuli that make up a context. This is facilitated through interactions with other learning and memory systems, such as the hippocampus and amygdala that support learning the context-outcome association and discrimination. The OPFC was essential during testing, which requires retrieval of abstract information not available in the presented context. Our results suggest

that the OPFC is required when behaviour is shaped according to the rat's determination of the predictive value of multiple stimuli signalling a biologically significant event.

## Chapter 3

## Preliminary evidence that the rat orbital prefrontal cortex can influence interactions amongst multiple memory systems

### Introduction

A key way to investigate interactions between learning and memory systems is to use behavioural tasks like the cue/place water task, plus-maze task, or stimulus-response version of the radial arm task that can be solved using multiple learning strategies (McDonald & White, 1994; Packard, Hirsh, & White, 1989; Packard & McGaugh, 1996; (Sutherland & Rudy, 1988). During training for these tasks, animals acquire information for both goal-oriented spatial responses and habitual cue responses. Then a subsequent competition test that can be solved using either strategy reveals whether behavioural responses are biased towards using either spatial or cue-response strategies. Under normal conditions, rats will exhibit an innate preference for the use of one strategy over the other on these competition tests. Approximately 50% of rats prefer to solve tasks using spatial strategies, and the other 50% prefer using cue response strategies. These biases, however, can be altered by changing the parameters of the task, inducing damage to specific brain regions, or from environmental influences (such as stress). For example, hippocampal lesions cause rats to use cue-response strategies, whereas the reverse occurs after striatal lesions (Packard & McGaugh, 1996)McDonald & White, 1993; McDonald & White, 1994; Packard et al., 1989).

Here, I focused on the cue/place water task. It represents a task that can induce a competitive interaction between the hippocampus and dorsolateral striatum. The parameters of this task induce a level of uncertainty because on

visible platform days' animals can use either cue or spatial information to solve the task, but when the platform is invisible, only spatial responses lead to efficient task solution. Therefore, each day, animals do not know which information will be relevant, creating uncertainty. Further, during the competition test, uncertainty arises when the visible platform is moved to the opposite position in the pool. Moving the visible platform creates uncertainty regarding whether the visible cue in a new spatial location or the previously trained spatial location will be more likely to lead to escape from the pool.

The prefrontal cortex may influence multiple memory system interactions (Gemmell & O'Mara, 1999; A. Gruber & McDonald, 2012; Ragozzino et al., 1999; (Euston, Gruber, & McNaughton, 2012), after mPFC lesions or specific lesions to the infralimbic cortex behaviours are biased towards the use of hippocampal goal-directed strategies (Coutureau & Killcross, 2003; McDonald, Foong, Ray, Rizos, & Hong, 2007). However, when just the prelimbic cortex was inactivated, subjects immediately use habitual strategies to learn tasks without first learning a goal-directed solution (Killcross & Coutureau, 2003). Therefore, the medial prefrontal cortex may support switching between the use of different response strategies (Killcross & Coutureau, 2003), however, although evidence does supports that the OFC can exert hierarchical control over behavioural responses, the exact influence of this region remains unclear (Keiflin et al., 2013). Some suggest that the OPFC functions within the goal-directed system because this region is activated when making goal-directed responses (Gremel & Costa, 2013; Pickens et al., 2005; Pickens et al., 2003). Further, the OFC is active when navigating overlapping spatial routes, as measured in human fMRI; therefore, the OFC may aid in disambiguating spatial information (Brown et al., 2010). Alternatively, the OPFC and amygdala are known

to share many similar functions such as guiding behaviour according to the value of stimuli (Stalnaker et al., 2007; Zeeb & Winstanley, 2011), therefore, these two areas might also have complimentary influences on multiple memory system interactions. Alternatively, the OPFC is thought to reduce anxiety through inhibitory control of amygdalar activity. Therefore, dysregulation between the OPFC and amygdala might impact a subject's ability to downregulate the influence of stress leading to a habitual response bias.

This study was designed to gain an understanding of how the OPFC might influence learning and memory system interactions. To determine if the OPFC influences multiple memory system interactions by facilitating goal-directed behaviours, male Long-Evans rats received bilateral OPFC cannulation. They were trained on the cue/place water task and then were tested on a competition test. Before testing, separate animals received infusions of either saline of muscimol into the OPFC allowing assessment of whether spatial or cue information guided responses after OPFC inactivation. I hypothesized that the OPFC will influence interactions between the habitual and goal-directed response systems during the completion task and that rats will be more likely to make cue responses after OPFC inactivation on the cue/place water task.

## Methods

### Subjects and Handling

Male Long-Evans rats obtained from Charles River Colony (Raleigh NC, USA) were housed in pairs and allowed to acclimate in their home cages for approximately one week. Animals were housed on a 12:12 dark light cycle with food and water available *ad libitum*. Before the experiment start, rats were handled for

five minutes daily over 4 days. All animals underwent the identical procedures throughout the study aside from the type of infusion received on the competition test day. According to the type of infusions the rats received on this day, animals were split into two treatment groups, muscimol (N = 8), or saline (N = 7). All procedures were carried out in accordance with the standards set by the Canadian Council on Animal Care as well as the University of Lethbridge animal welfare committee.

### Surgery and Infusions

All surgical and infusion procedures were carried as described in Chapter 2.

### Mock Infusions

To habituate animals to the infusion procedure, mock infusion`s occurred before the three final water maze training days. Approximately 20 minutes before commencing training, rats were transported from their home cages to the infusion room. Animals were restrained in a towel for three minutes. For the first 70 seconds of the restraint period, the infusion pump was allowed to run to acclimate the animals to the noise. After the mock infusion, animals were returned to their home cages.

### Cue/Place Water Task

*Apparatus*

A white plastic pool 150cm in diameter, 50 cm in height was filled with room temperature water (20-22ºC) that was made opaque by adding non-toxic white paint. The training room was 310cm × 610cm, and the pool was raised 48cm above ground in the center of the room. All extra-maze cues remained unchanged throughout all

trials and included posters of varying size, a computer desk, chair, the experimenter, a sink cabinet, and door. A computer tracking system (Ethovision 3.1, Noldus, Leesburg, USA) was used to collect data obtained from an overhead video camera. Two Plexiglas platforms were used as escape platforms throughout training (Fig 10). The invisible platform was made of clear plastic measuring 28cm tall, with a 12 cm × 12 cm top and was always submerged 2-3 cm under the water. The visible platform had a white plastic top and black plastic siding measuring 36 cm tall with a 13 cm × 13 cm top. This platform always protruded the water surface by approximately 3-4 cm.

*Training*

Training occurred daily over 12 consecutive days following a 3:1 training schedule. Although the platform remained in the same position throughout all the training days, on the first three days of training, the visible platform was used. On the fourth day, the invisible platform replaced the visible platform. The 3:1 visible to invisible training sequence was repeated three times such that the animals received a total of 12 training days, 9 of which used the visible platform, and 3 days using the invisible platform (always remaining in the same location). Each subject received 4 trials per day separated by a 2-4 minute inter-trail interval. A trial began when the experimenter gently placed the rat into the pool facing the pool wall at one of the 4 predetermined start points. The trial ended when the subject climbed onto the platform, or after 30 s had elapsed. In the case of the latter event, the experimenter would use their hand to guide the rat towards the platform. Once the rats had climbed onto the escape platform, they remained there for 10 seconds before the experimenter returned them to their holding cage. Each of the four daily trials began

from a different start point, however the four start locations were the same on each training day.

*Competition Test*

The competition test occurred approximately 24 hours following the last (12th) training day. Rats received infusions of muscimol or saline 20 minutes before the competition test. For the competition test, the visible platform was placed in the quadrant opposite from where the platform was located throughout training. There was only one platform in the pool during the competition test, and there was no platform in the trained location. The rats started the competition trial from the start point that always occurred 2nd throughout training. This start point was equal distance from the trained platform location and the location that the visible platform was placed during the competition test. Similar to training, rats were gently placed into the water facing the wall of the pool and allowed to swim until they found and climbed onto the visible platform.

### Statistical Analysis

Effects are reported as significant at $p < .05$, and all statistical analyses were two-tailed. All statistical analyses were conducted using SPSS ver 21 (IBM, USA) and GraphPad Prism software (GraphPad, La Jolla, CA), all graphs were created using GraphPad Prism software.

## Results

*Day 1-12 – Training*

Animals quickly learned to swim to the either the visible or invisible platform over the training days. As seen in Figures 11 and 12 there were no differences in the

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overall learning rate between the groups. Individual rats escape latencies were averaged over the 4 daily trials such that analysis was performed on the average daily escape latency for each animal. The cue and place days were analyzed separately but represented on the same graph to allow for a simple summary (Fig 10). A mixed model ANOVA was performed on the daily mean escape latency to find the visible platform (days 1-3, 5-7, 9-11). Mauchly's Test of Sphericity was violated and so a Greenhouse-Geisser correction was used revealing a significant main effect of Days [$F_{(8, 104)}$ = 137.449, $p$ = 0.000], but no significant main effect of Group [$F_{(1, 13)}$ = 1.203, $p$ = 0.293], nor a Days by Group interaction [$F_{(8, 104)}$ = 2.611, $p$ = 0.075]. Escape latencies increased on the invisible platform days but escape latencies for both groups decreased from the first invisible day (day 4) to the final day of training (day 12). A mixed model ANOVA was performed on the mean escape latency to find the invisible platform (Days 4, 8, 12) indicating a significant main effect of Days [$F_{(2, 26)}$ = 5.914, $p$ = 0.018], however there was no significant main effect of Group [$F_{(1, 13)}$ = 0.007, $p$ = 0.937], or a significant Days by Group interaction [$F_{(2, 26)}$ = 0.904, $p$ = 0.387]. Mixed model ANOVA's were also performed on the distance travelled following the same procedures as described for latency (Fig 12). Corrected analysis of the distance travelled on the visible platform days revealed a significant main effect of Days [$F_{(8, 104)}$ = 80.572, $p$ = 0.000], but no significant main effect of Group [$F_{(1, 13)}$ = 0.003, $p$ = 0.966], or a significant Days by Group interaction [$F_{(8, 104)}$ = 2.854, $p$ = 0.082]. On the invisible platform days analysis of the distance travelled revealed a significant main effect of Days [$F_{(2, 26)}$ = 4.057, $p$ = 0.047], but no significant main effect of Group [$F_{(1,13)}$ = 0.123, $p$ = 0.732], and no significant Days by Group interaction [$F_{(2, 26)}$ = 1.454, $p$ = 0.253].

*Day 13 - Competition Test with infusion*

To determine if rats exhibited a bias towards the use of a place or cue-response strategy, a competition test was completed on the thirteenth day of the task. Before commencing the competition test, the visible platform was moved to the quadrant opposite of where the platform was located throughout training. As the platform acted as a visible cue rats that swam directly to the visible platform quadrant were scored as cue responders. Conversely, rats that entered the previously trained quadrant first were qualified as place responders. After receiving a muscimol infusion inactivating the OPFC, rats made more annulus crossing of the previously trained platform location than did the saline infused rats (Fig 13A). Fig 13B shows that OPFC inactivated rats were more likely to make a place response whereas saline infused rats were equally as likely to make a place as a cue response. Specifically, 75% of the OPFC inactivated rats swam first to the previously trained quadrant (6 of the 8 subjects). In contrast, with 43% of saline infused animals exhibited a place response (3 of the 7 controls). However, a $X^2$ test performed on the number of cue compared to place responders in each group did not reveal a significant difference between the groups [$X^2 = 1.607$, $p = 0.205$]. Therefore, after inactivating the OPFC rats made more annulus crossings and were more likely to swim first to the previously trained quadrant suggesting these animals were biased towards using spatial response strategies.

*Histology*

Animals underwent the same histological procedures as described in chapter two, however, were not euthanized immediately after completing this project. Instead, animals rested for approximately 1 month before completing the appetitive

discriminative context conditioning behavioural (chapter 2 of this thesis).

Unfortunately, the headcaps of several animals fell off unexpectedly before

completion of the second behavioural task and had to be immediately euthanized (N

= 9), therefore I was only able to verify the cannulae placement for 8 of the 17

subjects included in this task (Fig 9). Cannulae placement was confirmed to be in the

correct position for these remaining subjects'.

## Discussion

The orbital prefrontal cortex can exert hierarchical control over learning and

memory systems (Keiflin et al., 2013), however to my knowledge no evidence has

determined if inactivating this region might influence competitive interactions

between the hippocampal and striatal system. Using the cue/place water task, I

determined if rats' responses are biased towards using either spatial or cue

strategies which indicate which of the hippocampus or striatum are controlling

behavioural outputs (McDonald & White, 1993). It has been suggested that the

OPFC functions within the goal-directed system and is active when making goal-

directed responses (Gremel & Costa, 2013), and so I hypothesized that after OPFC

inactivation, subjects would be more likely to make cue responses.

During training, all rats learned to locate the visible and invisible platforms

efficiently and at a similar rate. On the competition test, as expected, 57% of saline

infused rats swam first to the visible platform and 43% to the invisible. Surprisingly,

after infusion of muscimol inactivating the OPFC, 75% of rats navigated first to the

invisible platform and made significantly more annulus crossings of the previously

trained platform location. These results do not support our initial hypothesis but

suggest that OPFC inactivation induces a spatial response bias. These results

provide preliminary evidence that inactivation of the OPFC facilitates hippocampal spatial response strategies, although replication of this study and further investigation into this conclusion should be pursued.

Considering that the prefrontal cortex including the OPFC is primarily thought to be important for goal-directed responses, the present results suggesting the opposite are surprising (Dahmani & Bohbot, 2015). Inflexible and perseverative responses are consistently linked to OFC damage, particularly during reversal learning tasks, although a subset of studies have shown that certain habitual type responses that presumably should require the OPFC are unimpaired after lesions to this area. For example, rats are able to learn to inhibit prepotent responses (Murray, Kralik, & Wise, 2005), that is that these animals learn to inhibit a naturally favourable response, in favour of making a selection that is naturally less favourable, but will result in a larger reward in this task structure. The ability for animals with OPFC lesions to learn to inhibit these responses suggests that OPFC damage does not always result in perseverative responses. One interesting feature of this task could provide some explanation for our results. The last training that the subjects experienced was with the invisible platform, therefore, OFC damage may not reflect a facilitation of spatial strategy use, but instead simply that the animals are responding according to their most recent experience in the maze. However, this description is weakened because animals receive more training days with the visible than the invisible platform (nine to three respectively). Alternatively, recordings from OFC suggest that this region encodes rough spatial information (Farovik et al., 2015; Feierstein et al., 2006; Young & Shapiro, 2011a), and aids in disambiguating overlapping spatial routes (Brown et al., 2010). Therefore, perhaps when the OPFC is functioning normally, it exerts inhibitory control over the hippocampus. Another

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possible explanation for these effects arises from the understanding that there are at least two cortical systems influencing responses, one system emphasises OFC and striatal circuits, whereas the other emphasises medial PFC and hippocampal circuitry (Goto & Grace, 2008). Therefore, when the OPFC is inhibited the mPFC and hippocampal system may be enhanced leading to the spatial responses biases seen here.

*Uncertainty pushing rats towards using default HPC system*

When first learning a task, rats initially use goal-directed strategies, then as the task becomes well-known responses will begin to favor habitual strategies (Packard, 2009). This has lead to the suggestion that the hippocampal goal-directed system functions as the default learning and memory system (Driscoll, Howard, Prusky, Rudy, & Sutherland, 2005; McDonald & Hong, 2013; Ritchie et al., 1950). When initially learning a task subjects also experience high levels of uncertainty. Therefore, in addition to training modulating when goal-directed strategies are favoured, uncertainty could similarly influence the type of responses animals are likely to make. A predisposition to make goal-directed responses when conditions are uncertain would be advantageous because of the flexibility afforded by the goal-directed system. Here, rats may have favoured goal-directed responses after OPFC inactivation because without the OPFC generating outcomes expectancies, they may have become more uncertain about which responses will lead to escape from the pool. Extending this concept, during well trained conditions when cues provide a faster more efficient task solution, the OPFC might inhibit the hippocampal system or facilitate the striatal system. Of these two possibilities, it may be more likely that the OPFC exerts influence through interactions with the hippocampus rather than

facilitating cue responses as based on the other outcomes associated with OPFC damage such as perseveration, or impaired reversal learning the habitual responses seem unimpaired. Evidently more research will be required to gain a full understanding of this unexpected effect.

*Conclusion*

These results suggest that the OPFC does not function to modulate multiple memory systems in the hypothesized fashion; instead, these results provide evidence that without OPFC input rat's favor spatial responses. These results are in agreement with previous evidence suggesting that the OPFC functions to exert hierarchical control over other brain regions (Keiflin et al., 2013).

## Chapter 4

## General Discussion

The dynamic complexity of our environments creates uncertainty regarding the likelihood of future outcomes. The significance of cues can vary, and we face diverse cues signalling multiple outcomes. This question remains: how do animals determine which cues signal biologically significant events? This function is thought to be mediated through associative learning processes. The amygdala encodes cue valence, and the hippocampus encodes in concert with the amygdala when complex representations of cues are required, (such as a context; Antoniadis & McDonald, 2000; Phillips & LeDoux, 1992). The OFC also encodes information regarding cues that signal outcomes and value (Gallagher et al., 1999; Schoenbaum & Roesch, 2005), but unlike other brain regions, the behavioural evidence does not consistently support a direct role for the OFC in associative processes. OFC lesions do not impair associative learning during simple and certain conditions, instead, impairments arise when tasks are somehow uncertain, whether due to changing contingencies (Keiflin et al., 2013; Tait & Brown, 2007) complexity (Farovik et al., 2015; Kim & Ragozzino, 2005; Ward et al., 2015) or novelty (Lucantonio et al., 2015; Takahashi et al., 2009). Here, I investigated if the OPFC supports associative processes during uncertainty because of this region's role in determining the relevance of cues.

First, I investigated the role of the OPFC in constraining responses according to the relevance of cues in an aversive and an appetitive discriminative context conditioning task. After OPFC inactivation, rats froze equally in both contexts during the aversive task, and in the appetitive task, OPFC inactivated animals were not significantly more active within either context. Inactivation of the OPFC did not

impact preference suggesting that impairments were not due to a lack of awareness that one context signalled a biologically significant event, or that the contexts differed. Instead, I proposed that rats are unable to constrain their responses during the more uncertain testing conditions because they cannot determine the relevance of cues.

The inability of rats to constrain responses to perfect predictors in the context conditioning tasks could be due to a role of the OPFC in adjusting how information from other brain regions influences behaviour. The OPFC exerts hierarchical control over learning and memory systems (Gremel & Costa, 2013; Keiflin et al., 2013), but the exact nature of these influences is unknown. The second project aimed to determine if OPFC inactivation resulted in favored response strategies over another. I hypothesised that rats would exhibit a cue response bias because the PFC is thought to be part of the goal-directed system (Gremel & Costa, 2013; Young & Shapiro, 2011a). Counter to my hypothesis, I observed that after OPFC inactivation, rats preferentially swam first to the trained platform location and made more annulus crossings of this location.

### *Relevance of cues*

After OPFC inactivation, animals exhibited similar behaviours in both contexts suggesting they are impaired at constraining responses according to the most predictive context cues. I interpret this to support the role of the OPFC in cue relevance. Similarly, OFC lesioned rats exhibited unrestrained responses when faced with many different predictive cues in a rewarded digging task (Kim & Ragozzino, 2005). Therefore, without input from the OPFC, rats are unable to differentiate

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between the predictive values of perfect from moderate predictors leading to generalized responses.

Another example of a highly complex and uncertain situation that involves the OFC is social interactions. Humans with OFC damage are classically known to exhibit impulsive or inappropriate social responses and are impaired at discriminating facial expressions (LoPresti et al., 2008; Tsuchida & Fellows, 2012). Further, rats with OFC damage do not alter their play behaviour according to the social standing of their playmates (Beer, Heerey, Keltner, Scabini, & Knight, 2003; Pellis et al., 2006). Appropriate social responses are based on many different predictive cues that can vary according to the current situation, and who's significance can also vary according to different combinations of cue and contextual information. The social impairments described above could be the result of an inability to adjust behaviour according to cue relevance leading to impairments in constrained responses. Interestingly, humans with OFC damage often make faux-pas errors (when someone does not realize they said or did something they should not have) suggesting they are unaware of the significance of their responses within the current social setting (Shamay-Tsoory, Tomer, Berger, Goldsher, & Aharon-Peretz, 2005). Together, these effects suggest the OFC plays a critical role when appropriate responses cannot be made using simple associative information, but instead is important when complex information must be analyzed to determine optimal outcomes (Zald & Kim, 2001). Therefore, input from the OPFC allows animals to make constrained behavioural responses according to the relevance of information when they encounter complex environments.

Another, real-world scenario may help to clarify the OPFC's role in determining the relevance of cues. Say an animals was navigating through their

regular territory and encountered a predator odor, however, nearby to the predator odor there was also a valuable food source. In this situation the OPFC might function to guide the animal's responses according the relevance of either of these cues. For example, if the animal was starving and had not eaten in several days, then the food may become more relevant than the possibility of a predator. On the other hand, if the animal had previously encountered predator odor in this area of their environment and then encountered the predator themselves, then the predator odor may become more relevant. In this sense the OPFC is integrating past experiences, with the current environment in order to infer the most likely outcomes and make the most advantageous responses according to those anticipated outcomes.

### *Determine appetitive and aversive cue relevance*

Previous research investigating the function of OFC typically limits its experiments to either reward or aversive responding, with few investigations looking at both types of learning situations. Rarely are appetitive and aversive responding investigated in the same or similar tasks. Here, I examined the role of the OPFC in an aversive and appetitive version of the same context task. I observed that after OPFC inactivation, rats exhibit similar generalization, regardless of the appetitive or aversive stimuli, suggesting a common role of the OFC in determining the relevance of all cues regardless of valence.

Interestingly, previous research has suggested that the OFC might be important for dampening fear and anxiety behaviours (Motzkin, Philippi, Wolf, Baskaya, & Koenigs, 2015). However, animals studies have reported conflicting effects with certain studies reporting no changes in fearful, or anxiety-like behaviour after OPFC damage (Morgan & LeDoux, 1999; Orsini, Trotta, Bizon, & Setlow, 2015;

Rudebeck et al., 2007), but heightened negative responding after OFC lesions has also been observed in others (Shiba, Kim, Santangelo, & Roberts, 2015; Zelinski et al., 2010). Neuroimaging evidence from human subjects suggests that the vmPFC may regulate fear or anxiety behaviours through inhibitory control over the amygdala (Greenberg et al., 2013; Motzkin et al., 2015; Shiba et al., 2015). Here we find that although rats exhibited generalized fear after OPFC inactivation, the generalization was the result of enhanced freezing in the neutral context, whereas the freezing levels within the paired context were similar for rats in both groups. Thus, OPFC inactivation did not heighten negative responding to threat but instead heightened responding to a similar neutral or uncertain context. The same generalization was seen on the appetitive version of the task suggesting that rather than a specific impairment in rat's ability to inhibit negative responding or heightened reactivity to threat, OPFC inactivation resulted in an inability to constrain responses to the predictive cues in a familiar, neutral but perhaps uncertain context.

The effects seen here are only in part consistent with the effects seen previously following OPFC lesions in Zelinski et al. (2010). In that study after OPFC lesions rats exhibited exaggerates and generalized fear responses, whereas here, after OPFC inactivation rats exhibit generalization, but not an overall amplification of fear. One explanation for these differing effects could be that the OPFC must be inactivated over longer durations for exaggerated fear to arise. A malfunctioning OPFC may reduce the ability to anticipate the likelihood of outcomes leading to more unpredictable experiences and more generally increasing overall uncertainty. Uncertainty and unpredictability whether due to impairments in prediction, or just an unpredictable environment will more quickly lead to a state of chronic stress

(Herman, 2013). Therefore, similar to how unpredictable stress leads to long-term

elevations in fear and anxiety responses (Bondi, Rodriguez, Gould, Frazer, &

Morilak, 2007; McGuire, Herman, Horn, Sallee, & Sah, 2010; Simpkiss & Devine,

2003), OPFC lesions may similarly result in exaggerated fear responses. This

highlights an interesting question to address in future research, how, or if OPFC

inactivation and lesions might alter HPA axis reactivity.

### *Learning or responding*

It is unclear whether the OPFC determines the relevance of cues during

learning, or during responding. Some suggest that the OFC is crucial for identifying

which specific cue or choice lead to receiving a reward suggesting a larger role

during learning (Walton et al., 2010; Walton et al., 2011), whereas others imply a

larger role when making decisions or responding (Diekhof et al., 2011; Hosokawa et

al., 2013). Previous work from our laboratory observed that damage to the OPFC,

that occurred before learning, impaired the ability to constrain fear responses

(Zelinski et al., 2010). In the previous study, lesions occurred before DFCTC

training, leaving the precise role of the OPFC in learning and expression unclear.

Here we find that inactivation of the OPFC during testing lead to generalized

freezing responses suggesting that this region is important for determining the

relevance of cues when making responses, or that this region encodes information

regarding relevance that is not transmitted to other brain areas.

### What is the OPFC doing? Outcome expectancies, inhibition, or economic value?

There is continued debate surrounding the specific role of the OFC in guiding behaviour, however, most agree this region is important for behavioural flexibility (Stalnaker et al., 2015). Some suggest this region encodes economic value information (Gallagher et al., 1999; Schoenbaum & Roesch, 2005), others suggest this region facilitates behavioural inhibition (Chudasama & Robbins, 2003), and finally this area is proposed to generate outcomes expectancies (Rudebeck & Murray, 2014). Here, I provide additional support for the outcomes expectancies theory of OFC function. Specifically, our results suggest that the OPFC guides behaviour according to the relevance of cues predicting those outcomes. After OPFC inactivation, rats are aware of the value associated with the contexts and able to differentiate the two contexts. This corresponds with previous evidence showing that without OFC input, rats and primates can select preferred over non-preferred rewards (Keiflin et al., 2013; McDannald et al., 2014) and able learn simple discrimination tasks (Tait & Brown, 2007). Rats, however, do not exhibit differentiated responses during testing after OPFC inactivation on both the appetitive and aversive tasks, but on the aversive task, animals regain the ability to exhibit differentiated responses the following day when no infusions occur. Therefore, the generalized responses are not because the animals did not learn the values of the specific contexts, but instead because the OPFC must provide a specific function that is essential for testing but not preference.

Our results from the context discrimination task could be explained as an inability to inhibit responding in the face of partial predictors. However, the OFC could function to determine the relevance of cues, and therefore influences behaviour

by either inhibiting responses or facilitating them according to cue relevance. The inhibition theory is supported by the interpretation that without OFC, input behaviours are inflexible, and responses are perseverative (Tait & Brown, 2007). Although the design of the context tasks used in these experiments does not allow us to determine if impairments are the result of an inability to inhibit responding; the results from our second project do challenge this idea. Rats did not exhibit a habitual response bias after OPFC inactivation as expected suggesting that inactivation of this region does not impair the ability to inhibit responses. Further, results from several other studies conflict with the inhibition theory, particularly as complexity increases. In tasks of increasing complexity, rodents, and primates with OFC lesions are impaired, and responses become less constrained or specific (Kim & Ragozzino, 2005; Walton et al., 2010; Zelinski et al., 2010). Without OFC input, subjects respond equally to previously rewarded cues and to cues that have never been rewarded, and after making a rewarded selection, they do not maintain that selection. In reversal learning tasks, if contingencies are reversed quickly, rats with OFC lesions perform better than controls, as OFC lesioned rats employ a win-shift strategy (Riceberg & Shapiro, 2012). Together with previous studies, our results support the outcomes expectancies theory over the economic value or inhibition theories of OFC function.

### *Interactions with other brain areas*

Our findings show that this region is important for rats to respond appropriately to contexts composed of perfect predictors mixed with moderate predictors. During exposure to uncertain environments when all the required information is not currently available I believe that the OPFC guides memory retrieval from other regions towards information that better predicts biologically

significant events while inhibiting the influence of less predictive stimuli. The OFC shares connections with the hippocampus, striatum, and amygdala as well as receives input from sensory modalities (McDonald, 1991; Reep et al., 1996; Kringelbach and Rolls, 2004). Therefore, the OFC is anatomically well positioned to exert hierarchical control over behaviour in the face of uncertainty based on the determined relevance of the current information. In Chapter 2, I hypothesized that response generalization could be because when properly functioning the OPFC facilitates the integration of hippocampal context representations. Therefore, after inactivation animals are behaving according to the less detailed amygdala representations. This is not supported by the results presented in Chapter 3, as after OPFC inactivation's, rats favor hippocampal based spatial response strategies. Although these results do not confirm the exact role of the OPFC, they do demonstrate that the OPFC can influence interactions between learning and memory systems, which strengthens previous conclusions that the OPFC exerts hierarchical control over other brain areas (Keiflin et al., 2013).

The OFC and hippocampus both represent contextual or spatial information although that information is far more detailed within the hippocampus. Interestingly, electrophysiological recordings from rats in a context dependent task highlight differences in how the OFC and hippocampus represent context information. Hippocampal networks separated events according to the context in which they were experienced whereas OFC networks separated events according to value. If objects from different contexts did not signal value, they would be encoded similarly, whereas objects or contexts associated with a rewarded event were encoded in their own "value based schema" (Farovik et al., 2015), whereas hippocampal representations would encode objects contained in different contexts

separately regardless of their predictive value. Therefore, within our task, OPFC represented schemas could aid in reducing interference from moderate predictors signalling different biologically significant events allowing animals to differentiate the contexts and their associated outcomes (Farovik et al., 2015). Therefore, the OFC and hippocampus may function together when representing situations that include complex value and contextual information. The hippocampus may function to disambiguate sensory uncertainty whereas OFC handles outcome uncertainty.

These interactions between the hippocampus and OFC may also provide insight into the unexpected spatial bias demonstrated in chapter three. There we discussed that the OPFC may facilitate striatal, or inhibit hippocampal influence when cues provide more efficient task solution and that although speculative it is likely that inhibition of the hippocampus is more probable. The hippocampus plays a clear role when animals are disambiguating uncertain environments, and here we are suggesting that the OPFC plays a similar role. It could be that these two regions interact during uncertainty such that when faced with environmental uncertainty the OPFC might facilitate the hippocampus in order to facilitate disambiguation, but when faced with outcomes uncertainty the OPFC may inhibit the hippocampus should cue responses provide more certain outcomes.

### *Uncertainty*

Although the orbitofrontal cortex is thought to enable behavioural flexibility, this idea largely comes from research showing this cortical region's role in flexibly updating reward contingencies during reversal learning tasks (Murray et al., 2007; Stalnaker et al., 2007). The results presented from the present experiments, however, point to a broader role of the OPFC in enabling behavioural flexibility

which is especially important in complex and ambiguous environments. These results suggest that the OPFC`s role in determining the relevance of cues in conjunction with animals past experiences together afford behavioural flexibility which subsequently leads to more advantageous outcomes for the animal. After OPFC inactivation, rats exhibit generalized responses during the testing, but not the preference portion of the context discrimination tasks, implicating the OPFC in navigating uncertainty. During testing, several factors increase uncertainty as compared to those experienced during preference. For example, the presence of the middle alley provides an additional safe cue, as shocks were never received when the middle alley was present. Further, because all assessment occurred within the safe room, within the shock context there is a conflict between the foreground fear cue (shock context) and the safe background cues (safe room). Also, during preference, rats are given direct access to both chambers, thus all the cues composing both context chambers are within their immediate sensory surroundings. Therefore, I show that as the task becomes more uncertain the role of the OPFC increases.

Novelty and complexity are two contrasting situations that result in uncertainty when learning about predictors for biologically significant events. The two conditions represent different aspects of uncertainty, the latter when information is lacking and the former when there is a surplus of information. The present results represent a role for the OPFC in guiding behaviour during complex uncertainty. These results are in accordance with several groups suggesting the OPFC aids in discriminating between different valued predictors during complex uncertainty allowing for constrained responses (Farovik et al., 2015; Walton et al., 2015; Ward et al., 2015). Further, I suggest a role for the OPFC in facilitates flexible generalization during novel uncertainty.

The most common understanding is that generalization processes follow a gradient, such that generalized responses increase with the similarity between the original conditioned stimulus and the encountered stimulus (Lissek, Bradford, et al., 2013; Onat & Büchel, 2015). However, more recently evidence for a flexible model is provided by results from a fear conditioning study wherein the authors conclude that rather than the likeness of stimuli dictating a passive generalization process, there is a mechanism that can actively broaden generalization processes (Onat & Büchel, 2015). At first glance, the gradient approach to stimulus generalization appears to be appropriate given that perceptually similar stimuli are likely to signal similar outcomes, but the more dissimilar a stimulus becomes, the less advantageous it would be to respond similarly. However, it would also be advantageous for this gradient to be more relaxed in increasingly novel situations, such that more generalization should occur when situations are highly uncertain. Simply put, more generalization should occur in an entirely novel (highly uncertain) environment. I suggest that the OPFC may function to more deliberately control generalization processes during uncertainty to more flexibly respond to complex and changing environments (Mushtaq, Bland, & Schaefer, 2011).

Research has demonstrated that the OPFC facilitates the integration of previously learned associations to determine likely outcomes in a novel situation (Jones et al., 2012; Takahashi et al., 2013). Therefore, the OPFC integrates or discriminates between information according to its relevance to generate expected outcomes. During certain conditions when simple associative information provides adequate information for task solution then outcome expectancy information generated in the OPFC is not essential for constrained responses. However, when uncertain conditions create a situation where appropriate responding requires the

integration of multiple forms of information or requires animals to discriminate between multiple predictive cues according to their relevance, then the OPFC becomes important.

### *Implications for generalized anxiety disorder*

Similar to the generalized fear exhibited by rats without a functional OPFC, patients with generalized anxiety disorder continue to respond to increasing generalized stimuli following training in a fear conditioning task (Lissek et al., 2014). Although generalization occurs when patients and rats are anticipating the likelihood of receiving a shock, both can and even excel at avoiding threatening situations. Here, OPFC inactivation resulted in significantly augmented freezing within the unpaired or neutral context in comparison to controls, however freezing levels exhibited within the paired context remained similar across groups. This resembles the differences between GAD patients and healthy controls. When presented with threatening or negative images, amygdala activation levels are similar between both groups, however in response to ambiguous neutral images, or in anticipation of viewing either ambiguous or negative stimuli GAD patients, but not controls exhibit amygdala hyperactivity (Blair et al., 2008; Hölzel et al., 2013; Lissek et al., 2005; Nitschke et al., 2009). Further, during attention tasks highlight GAD patients exhibit a bias for emotionally relevant stimuli however the largest impairments in attention occur when presented with contrasting emotional stimuli. The presentation of threat stimuli induces a slight response delay for GAD patients compared to controls. Conversely, controls exhibit no response delay following the presentation of ambiguous stimuli, whereas the same ambiguous stimuli induce large delays (comparable to the response delays induced by threat stimuli) in the

GAD subjects. Finally, GAD patients show a deficit in utilizing outcomes on a trial to trial basis to gain a task advantage similar to impairments seen in subjects with OFC damage (Olatunji, Ciesielski, Armstrong, Zhao, & Zald, 2011). Therefore, GAD patients and subjects with OFC damage maintain the ability to identify properly and respond to direct threats but are impaired when anticipating the likelihood of future threats, or handling uncertainty. This could be because other brain regions can "take over" responses when faced with an immediate or unambiguous threat, however, anticipating the likelihood of threat requires weighing the options. Further, when anticipating there is always some uncertainty requiring that the most likely outcomes be determined or chosen from an array of potential outcomes. In order to determine most likely outcomes, all the different potential outcomes must be compared and contrasted which requires the ability to integrate abstract potential outcomes to determine the most likely outcome.

### *Conclusion*

These results demonstrate a role for the OPFC in supporting associative functions during uncertain conditions because of this region's role in determining the relevance of cues. I show that after OPFC inactivation rats exhibit generalized responses on an appetitive and aversive context discrimination tasks. Further, I show that without input from this brain region rats favor responding according to spatial rather than cue information in a competition water task. Therefore, the hippocampus and the orbitofrontal cortex may function together to disambiguate uncertain conditions. Specifically, these results support that during complexity the OPFC functions to constrain responses to the most relevant stimuli. Further, previous work suggests that in situations that are novel, or lack ample information,

the OPFC facilitates the integration of relevant memories to infer likely outcomes. Therefore, according to the current situational demands and the determined relevance of cues, the OPFC facilitates between discrimination and generalization to facilitate appropriate responses during uncertainty.

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| Experiment | Task | Group | Assessments completed | Infusion before |
|---|---|---|---|---|
| **1** | DFCTC (aversive) | A | Test block 1, Test block 2, Preference | Test block 1 |
| | | B | Test block, Preference 1, Preference 2 | Preference 1 |
| **2** | CPP (appetitive) | A | Activity level test block 1, Activity level test block 2 | Activity level test block 1 |
| | | B | Preference 1, Preference 2 | Preference 1 |

Figure 1: Representation of the procedures carried out in chapter 2 experiments. Subjects included in experiment 1 were trained and tested on the aversive context discrimination task whereas those included in experiment 2 completed the appetitive version of the context discrimination task. Within the two experimental groups subjects were segregated into two groups that completed the same training procedures, but were tested using different assessment measures. The assessments completed for each group are noted in the fourth column. Subjects received infusions prior the assessment measure noted in the fifth column.
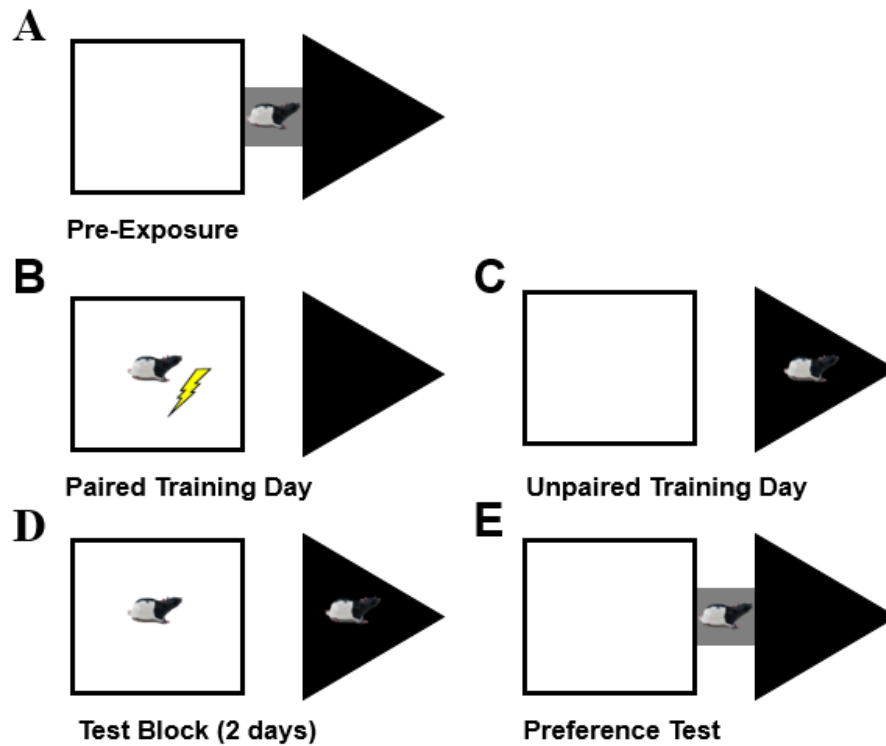
Figure 2: Pictorial representation of each phase of the discriminative fear conditioning to context task. A. Pre-Exposure. Contexts were connected by an alley allowing the animals to explore both contexts freely for 10 minutes. B. Paired context training. Foot-shocks were administered within the paired context. C. Unpaired context training. No foot-shocks occurred in the unpaired context. Over 8 training days, rats were exposed to the contexts on alternating days. D. Test Block. Two assessment sessions occurred, one in the paired and the other in the unpaired context during which time spent freezing was measured. E. Preference. The connecting alley was replaced, allowing animals to move freely between contexts.
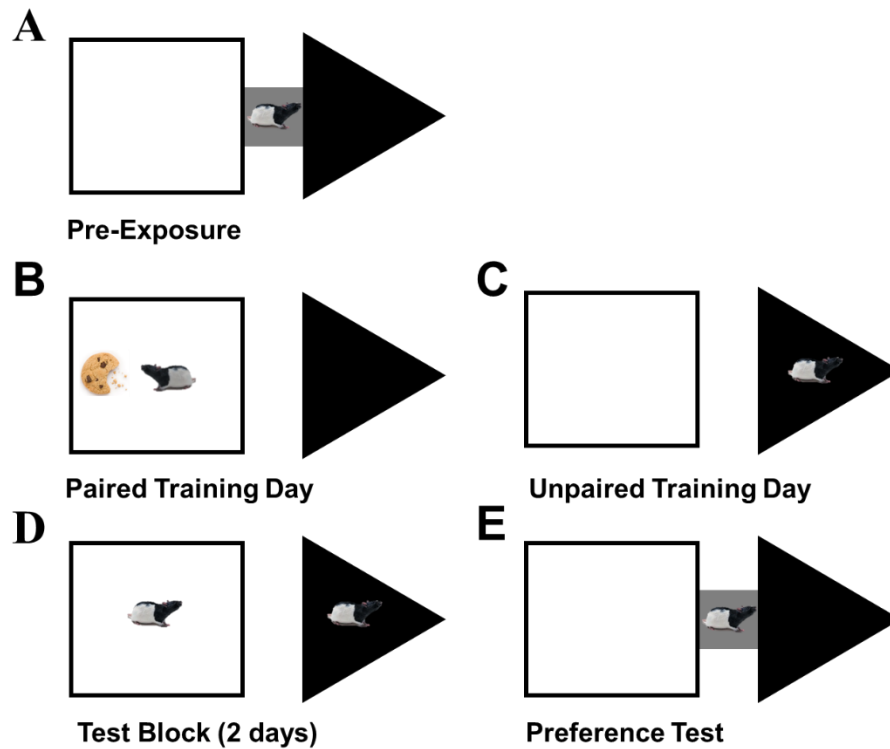
Figure 3: Pictorial representation of each phase of the conditioned place preference task A. Pre-Exposure. Contexts were connected by an alley allowing the animals to explore both contexts freely for 20 minutes. B. Paired context training. Chocolate chip cookies were given within the paired context. C. Unpaired context training. No chocolate chip cookies were given in the unpaired context. Over 8 training days, rats were exposed to the contexts on alternating days. D. Activity Level Testing Block. Two assessment sessions occurred, one in the paired and the other in the unpaired context during which time the number of line crosses made was measured to infer the animal's activity levels. E. Preference. The connecting alley was replaced, allowing animals to move freely between contexts.
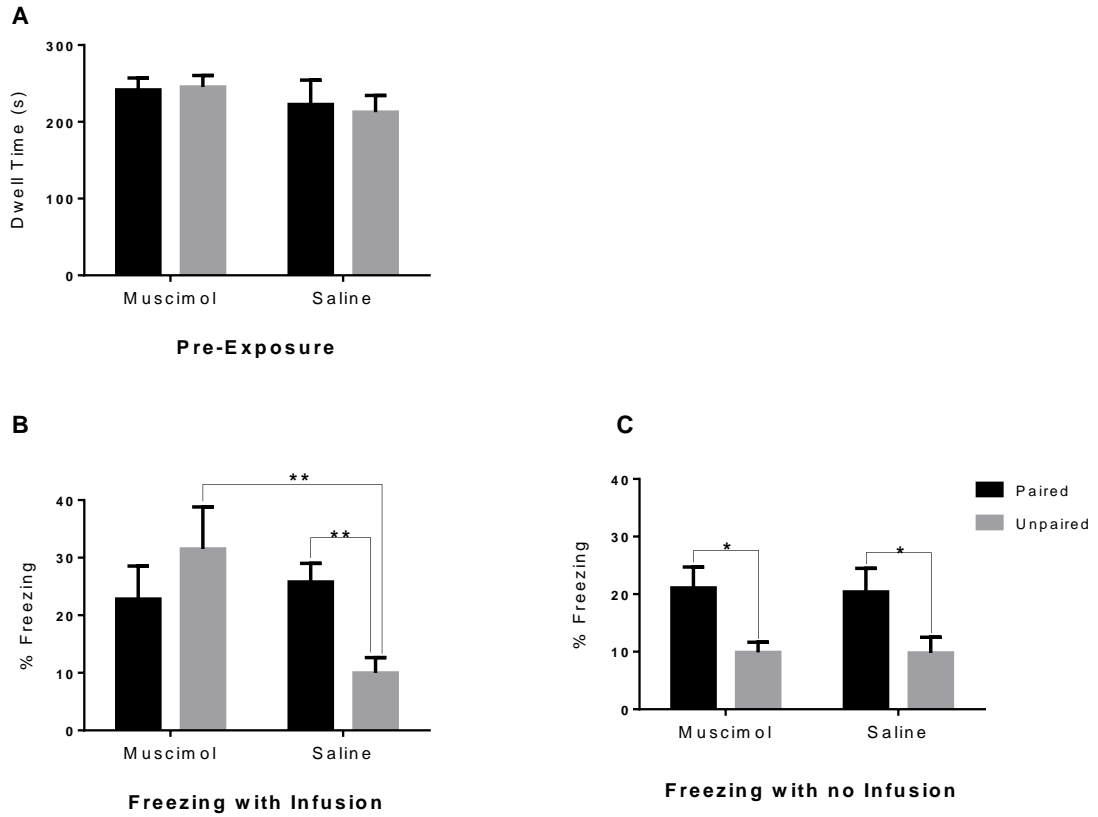
Figure 4: Experiment 1 group A results from the discriminative fear conditioning to context task. A. Dwell time in paired and unpaired context during pre-exposure. B. Effect of infusion of saline or muscimol into the OPFC on freezing. C. Time spent freezing without infusion.
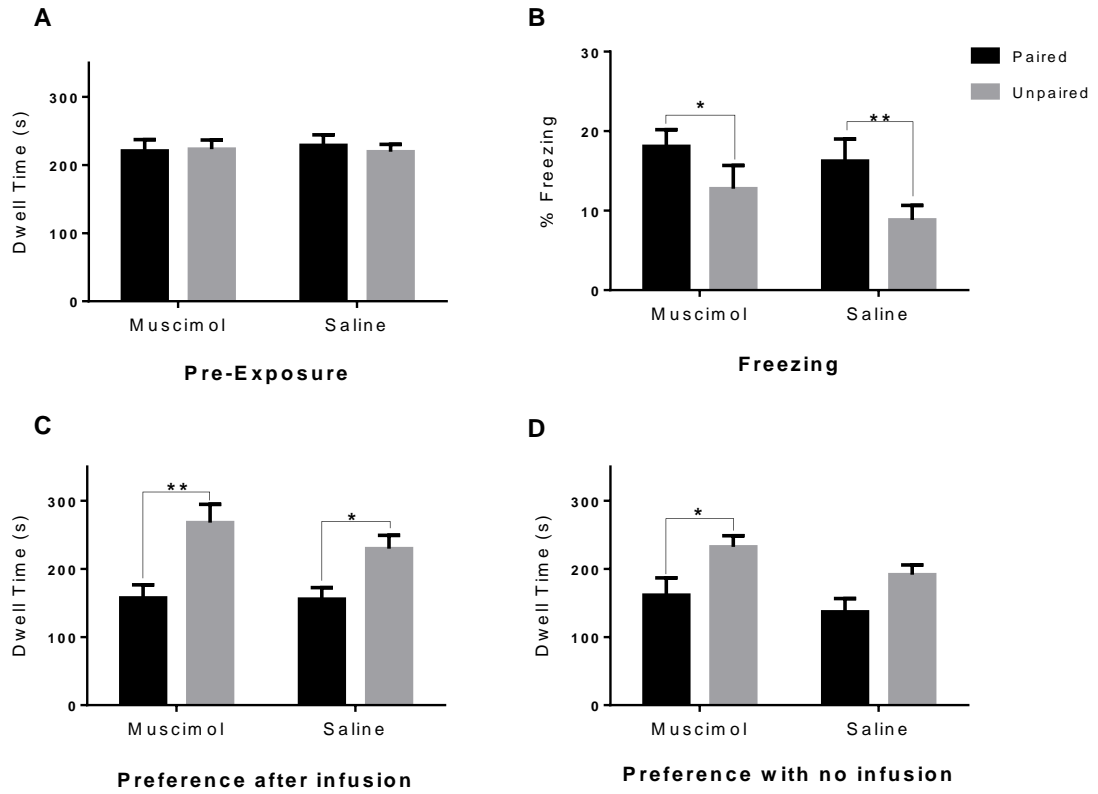
Figure 5: Experiment 1 group B results from the discriminative fear conditioning to context task. A. Dwell time in paired and unpaired context during pre-exposure. B. Comparison of time spent freezing in the paired and unpaired context. C. Effect of infusion of saline or muscimol into the OPFC on preference. C. Dwell time in each context with no infusion before the preference test.
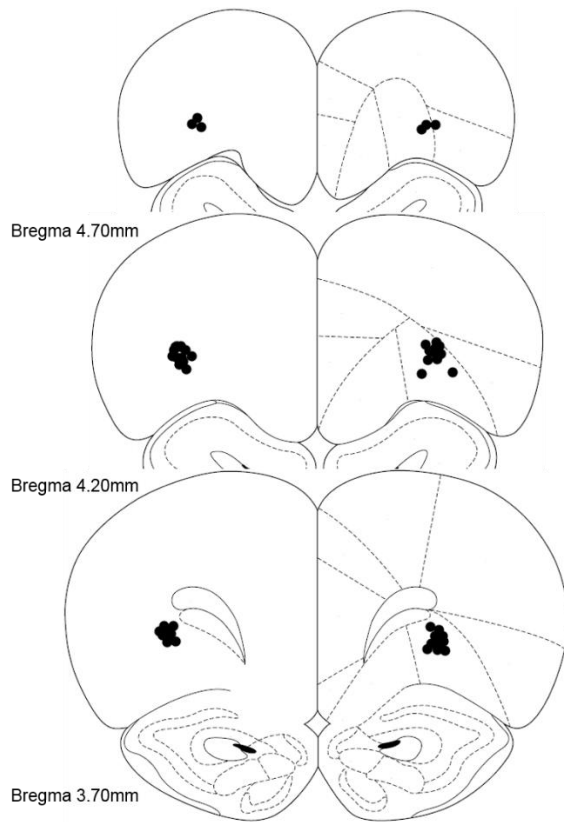
Bregma 4.70mm

Bregma 4.20mm

Bregma 3.70mm
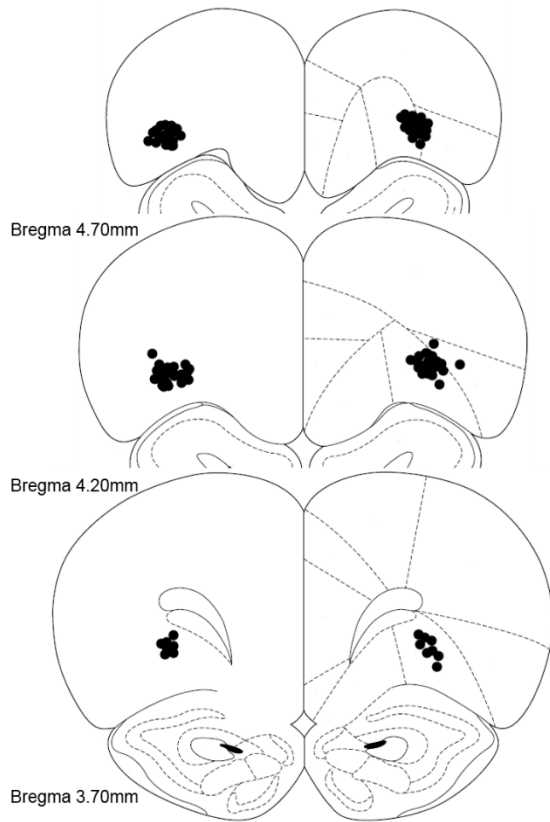
Figure 6: Solid dots in right and left hemispheres represent the approximate location of the infusion cannulae tips in the muscimol and saline animals included in Experiment 1 (Chapter 2). Modified from *The rat brain in stereotaxic coordinates*, 3rd ed. (Paxinos & Watson, 1997).
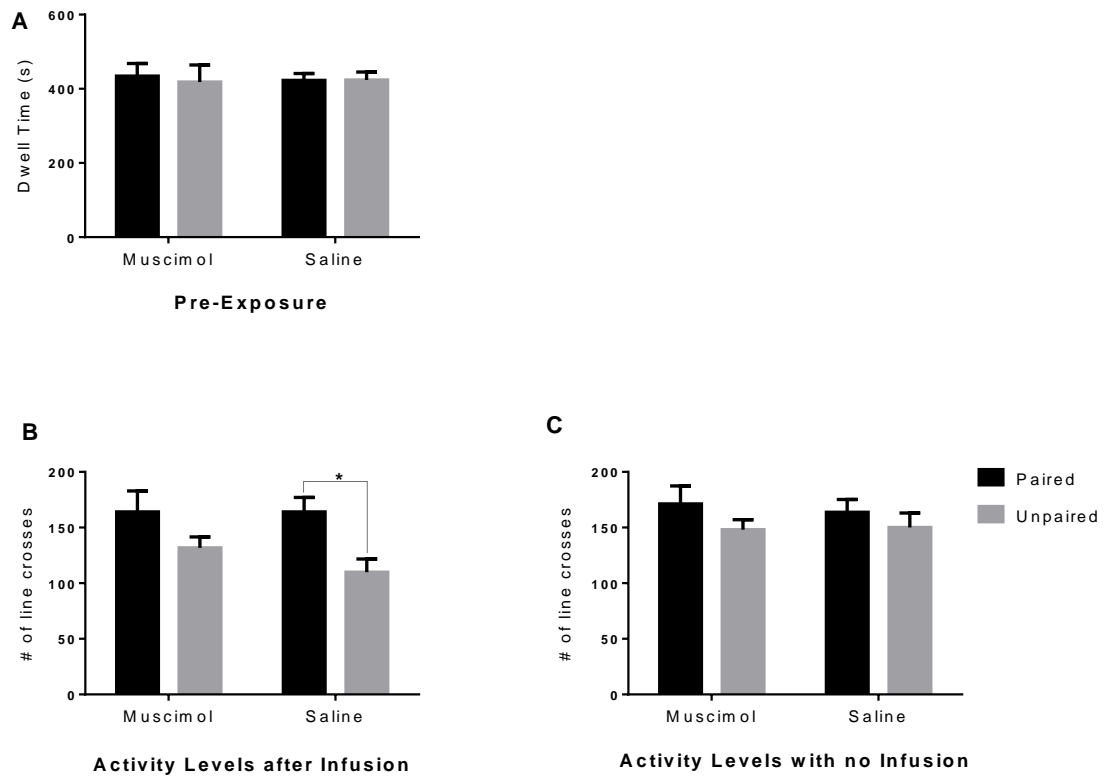
Figure 7: Experiment 2 Group A results from the appetitive contextual conditioning task. A. Dwell time in paired and unpaired context during pre-exposure. B. Effect of infusion of saline or muscimol into the OPFC on activity level. C. Comparison of activity level in the paired and unpaired context without infusion.
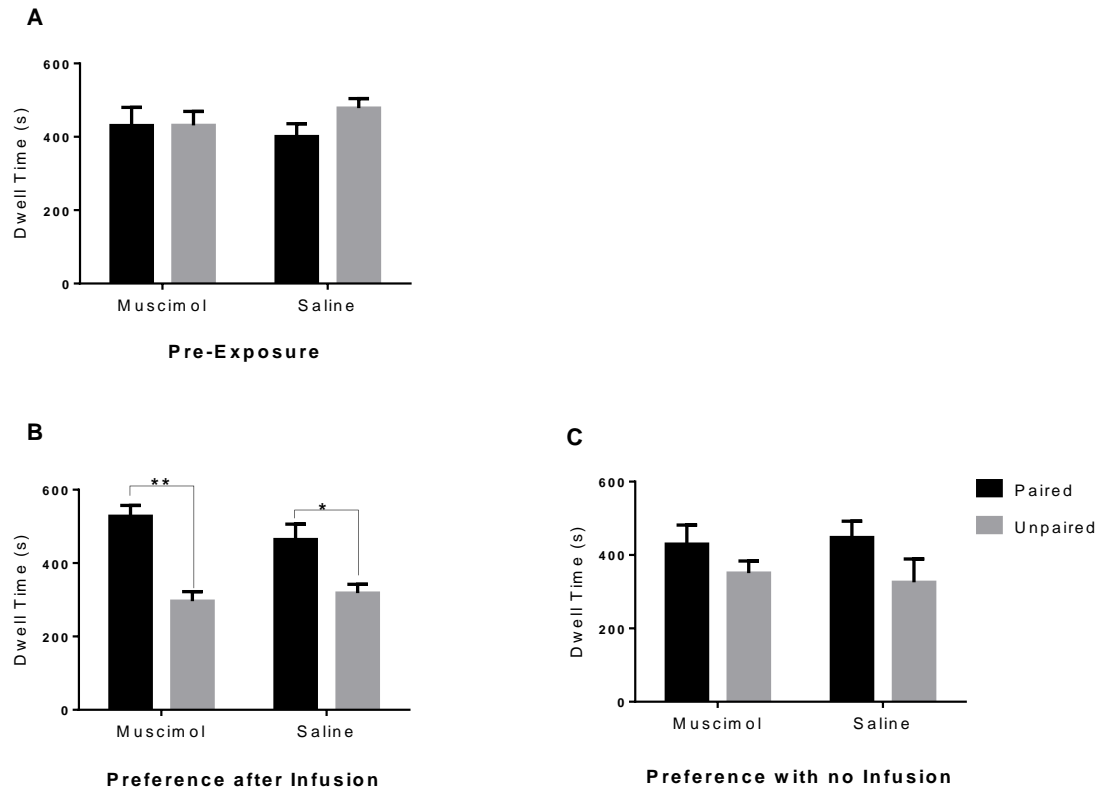
Figure 8: Experiment 2 group B results from the appetitive contextual conditioning task. A. Dwell time in paired and unpaired context during pre-exposure. B. Effect of infusion of saline or muscimol into the OPFC on preference. C. Comparison of dwell time in the paired and unpaired context without infusion on the second preference test.

Figure 9: Solid dots in right and left hemispheres represent the approximate location of the infusion cannulae tips in the muscimol and saline animals included in Experiment 2. Modified from *The rat brain in stereotaxic coordinates*, 3rd ed. (Paxinos & Watson, 1997).
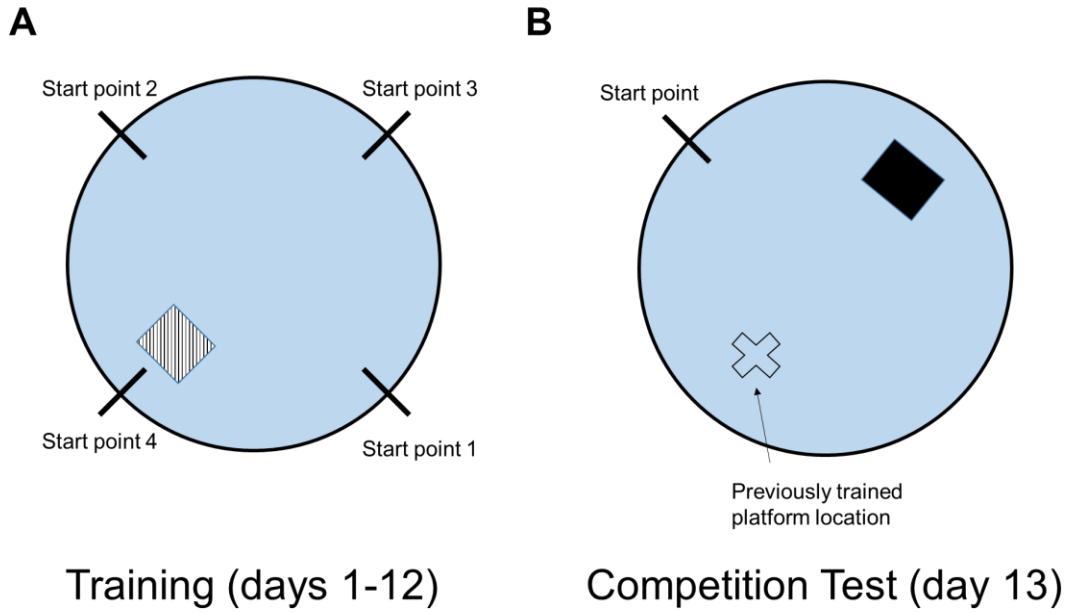
**A**

Start point 2    Start point 3

Start point 4    Start point 1

Training (days 1-12)

**B**

Start point

Previously trained
platform location

Competition Test (day 13)

Figure 10: Pictorial representation of the cue/place water task. A. Location of the platform and 4 start points on days 1-12 of training. The platform is visible on days 1-3, 5-7, 9-11 and invisible on days 4, 8, and 12. B. Platform location and start point for the competition test day (day 13). The X serves as a reminder for the location of the platform throughout training, during the actual task no stimuli marked that location.
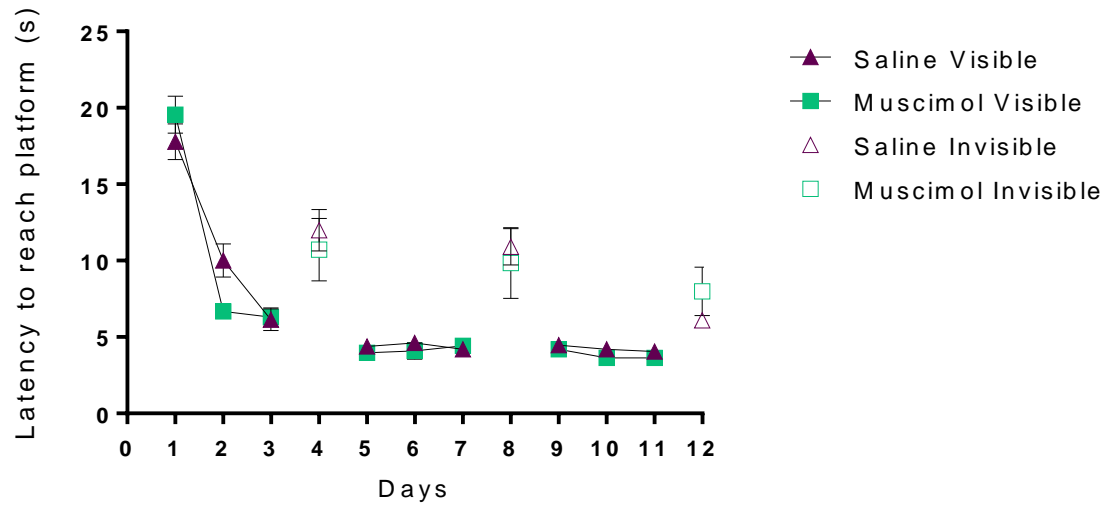
Figure 11: Average latency to reach platform across days during training of the cue/place water task.
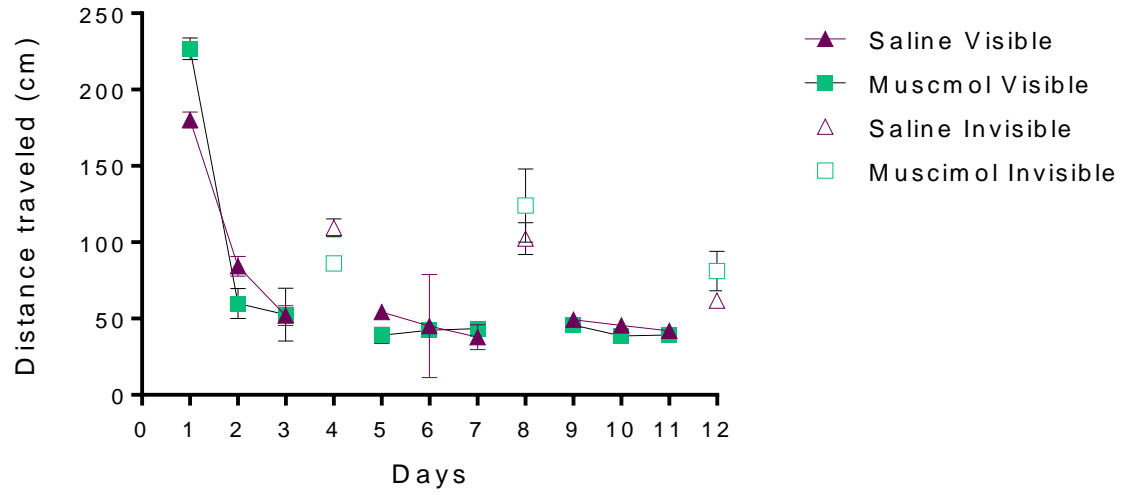
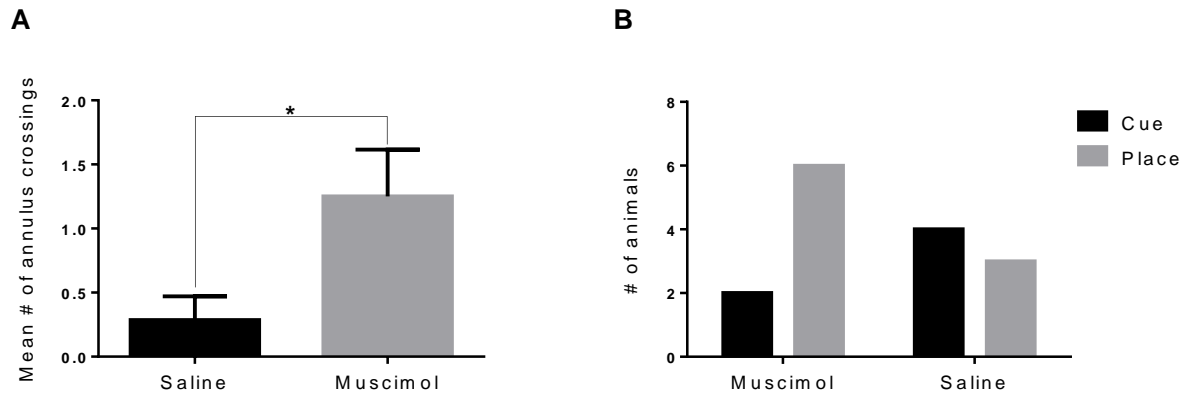Figure 12: Average distance travelled on each training day of the cue/place water task.

Figure 13: Results from the cue/place water task competition test. A. Effect of muscimol or saline infusion on the number of times the rats swam over the previously trained platform location. B. Effect of muscimol or saline infusion on the number of place or cue responses.