CONTRIBUTION OF THE MESOLIMBIC PATHWAY TO DISTRACTION FROM ONGOING CONSUMMATORY BEHAVIOUR: IMPLICATIONS FOR A MODEL OF SCHIZOPHRENIA

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ABSTRACT

Schizophrenia is a severe and debilitating condition affecting 1% of the population. Cognitive dysfunction in schizophrenia is poorly understood and there are no effective treatments. Current animal models, especially those characterising cognitive deficits, may not fully represent the deficits seen in patients. Disturbances in attention and increased distractibility are features of schizophrenia and increased distractibility may underlie deficits in the novel object recognition (NOR) task, which are seen in the subchronic phencyclidine (PCP) animal model. Here we developed a novel assay for distraction based on rats' self-paced licking for saccharin. We used this assay in the subchronic PCP model to investigate distraction. In addition, as dopamine is implicated in the schizophrenic deficit and key dopaminergic areas are involved in consumption and distraction, we used fibre photometry to assess activity of mesolimbic circuits during this task. Our data demonstrate that rats pause ongoing licking in response to distracting stimuli but that these responses habituate quickly. Moreover, amphetamine increases distraction implicating the involvement of catecholamine neurotransmission. However, interestingly we found no differences in PCP treated rats although this may be due to limitations of this pharmacological model. Fibre photometry recordings revealed increases in ventral tegmental area (VTA) neural activity in response to licking and distracting stimuli, with greater increases in activity on trials where rats where distracted vs. non-distracted. Finally, we evaluated different strategies to specifically target dopamine neurons using a Cre-expressing tyrosine hydroxylase specific virus and using a transgenic Cre-expressing rat. In conclusion, we did not find clear differences in distraction within the PCP model, however, we characterised VTA responses associated with distractors and consumption in this paradigm. These results highlight the complex role of dopamine in maintaining ongoing appetitive and consummatory behaviours whilst also monitoring the environment for salient stimuli.

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List of abbreviations

5-HT – 5-hydroxytryptamine / serotonin
AAV – Adeno-Associated Virus
ADHD – Attention Deficit Hyperactivity Disorders
ANOVA – Analysis of Variance
AMY – Amygdala
CaM – Calmodulin
CIAS – Cognitive impairments associated with schizophrenia
CPG – Central Pattern Generator
CPu – Dorsal striatum/Caudate Putamen nuclei
DI – Distraction Index
DR – Dorsal Raphe nucleus
EEG – Electroencephalogram
GABA – Gamma-Aminobutyric Acid
GECIs – Genetically Encoded Calcium Indicators
GFP – Green Fluorescent Protein
ICI – Inter Cluster Interval
ILI – Inter Licks Interval
ITI – Inter Trial Interval
i.p. – intraperitoneal injection
LH – Lateral Hypothalamus
MCH – Melanin Concentrating Hormone
MMN – Mismatch Negativity
NAc/NAcSh – Nucleus Accumbens / Nucleus Accumbens Shell
NMDA – N-methyl-D-aspartic acid
NOR – Novel Object Recognition
PCP – Phencyclidine
PDP – Post Distractor Pause
PFA – Paraformaldehyde
PFC/mPFC – Prefrontal Cortex / medial Prefrontal Cortex
PPTg – Pedunculopontine tegmentum
RRF – Retrorubral Field
RPE – Reward Prediction Error
SAL – Saline
SC – Superior Colliculus
SEM – Standard Error to the Mean
SK channels – Small conductance calcium-activated potassium channels
SNc – Substancia Nigra pars compacta
STh – Subthalamic nucleus
TH – Tyrosine Hydroxylase
TRIO – Tracing Relationships between Inputs and Outputs
VGLUT – Vesicular Glutamate Transporter
VTA – Ventral Tegmental Area

CHAPTER 1

General Introduction

1. GENERAL INTRODUCTION

1.1 Background

A long-standing question in neuroscience is how the brain encodes external stimuli and integrates this information with internal need states. Understanding how these signals are used to guide adaptive behaviour is of central importance in modern neuroscience. Decades of research have investigated the role of specific brain areas and transmitters in motivated behaviour in both health and disease. Among the earliest work in this field, pioneering experiments by Olds and Milner (1954) were the first to show that specific brain circuits were involved in reward. Their seminal studies demonstrated that rats would press a lever to receive targeted electrical stimulation to the midbrain and other areas, especially the medial forebrain bundle. These studies became the foundation of an entire field of neuroscience investigating the role of these areas in reward and motivation (Wise, 2002; Wise, 2005).

In parallel to these early behavioural neuroscience studies was the discovery of the neurotransmitter dopamine in the mammalian brain. Despite being first synthesised in 1910 (Barger and Ewins, 1910; Mannich and Jacobsohn, 1910) up until the 1950's dopamine was seen as simply an intermediary in the synthesis of noradrenaline and was seen to have only mild vasopressive effects (Blaschko, 1939). In the 1950's dopamine was located in the mammalian brain in especially high concentrations within the striatum, an area known at the time to contain little noradrenaline (Bertler & Rosengren, 1959). This and other observations led a Swedish research group to consider the potential role of dopamine as a transmitter in its own right (Carlsson et al., 1958). It was Arvid Carlsson who first revealed the significance of dopamine as a neurotransmitter. His work was amongst the first to reveal that depletions of dopamine were catastrophic for movement – his experiments in rabbits demonstrated that such depletion resulted in profound akinesia. The Swedish group's work was in fact so ground-breaking that it earned Carlsson and colleagues the Nobel Prize. These findings, in turn, led to landmark works that implicated dopamine cell

death in Parkinson's disease (Ehringer & Hornykiewicz, 1960) and to the discovery of L-DOPA as a treatment to restore movement in patients with the condition (Cotzias et al., 1967)

Following on from its discovery, the role of dopamine has been extensively studied not only in the context of movement and movement disorders, but in the context of psychiatric conditions including addiction and schizophrenia and in motivated behaviours, learning and attention. The identification of dopamine receptors, discovery of its synthesis pathway and investigations of pharmacological antagonists have been crucial to understanding how this transmitter contributes to these conditions and what it encodes in the intact brain (Seeman et al., 1976). An especially important development was the discovery and classification of the two subtypes of dopamine receptors (Kebabian & Calne, 1979). During the 1970's there was an explosion of research into the dopamine system. In particular, the search began for an 'antipsychotic receptor' which could account for the effects of early neuroleptics in alleviating symptoms of schizophrenia.

Seeman et al., (1976) determined that there were sites in the brain that bound both dopamine and haloperidol (the antipsychotic which was effective in treating positive symptoms in schizophrenia). The discovery of these receptors was then further refined by the identification of two distinct subtypes, the D1-like and D2-like subtypes (Kebabian & Calne, 1979). The distinction being their opposing effects on adenylyl cyclase, with D1 receptors stimulating and D2 receptors – the target of haloperidol – inhibiting the actions of adenylyl cyclase.

From its initial discovery as an intermediate in the synthesis of adrenaline and noradrenaline, to the realisation of its importance in movement and the classification of the dopamine receptors and their involvement in psychiatric conditions, dopamine has become one of the most well studied neurotransmitters. Current research is now concerned with the function of dopamine, not only in disorders of motivation such as addiction, schizophrenia and ADHD, but also in the healthy brain. There is considerable research showing that this once overlooked brain chemical may subserve a range of

crucial and complex functions from reward processing to motivation and action selection. The decades of research that have followed these initial discoveries have provided insights into some of the functions of dopamine, particularly dopamine arising from the midbrain. However, the fundamental roles of dopamine circuits are still not fully understood and vigorous debate remains as to the specific roles of dopamine in the brain (Berke, 2018; Berridge, Venier & Robinson, 1989; Salamone & Correa, 2012; Schultz, 1986; Schultz, 2007). The advent of new and increasingly specific tracing techniques, immunohistochemistry, neural recording methods and calcium imaging combined with cell specific viral targeting strategies and improved imaging methods have increased the precision and power of investigations into the dopamine system. We are now closer than ever to understanding the nuanced, complex and vital roles of these circuits in normal and abnormal motivated behaviours.

1.2 Midbrain dopamine pathways

During the 1960's pioneering new methods were developed to visualise dopamine neurons in the brain. Before modern immunohistochemistry utilising fluorescent antibodies, the Falck-Hillarp formaldehyde fluorescence technique involved exposing freeze-dried brain tissue to formaldehyde vapour which induced the conversion of dopamine to iso-quinoline molecules that emitted yellow-green fluorescence, which could be imaged (Falck and Torp 1962). As both dopamine and noradrenaline show the same fluorescence they could be separated by the presence of dopamine beta-hydroxylase, which converts dopamine to noradrenaline. This fundamental development allowed dopamine neurons to be visualised in the brain and localised for the first time. The basic description of the anatomical organisation of these neurons originally described by Dahlstrom and Fuxe (1964) is still used today despite improvements in immunohistochemistry methods.

Dopaminergic neurons in the midbrain are clustered into three groupings, cells of the retrorubral field (RRF or area A8), those of the substantia nigra pars

compacta (SNc, area A9) and those of the ventral tegmental area (VTA, area A10). RRF and SNc neurons project to the caudate and putamen forming the nigrostriatal system which is critical in movement and which is fundamentally perturbed in Parkinson's disease. VTA neurons project predominantly to the nucleus accumbens (NAc) and amygdala, forming the mesolimbic pathway, as well as to cortical areas forming the mesocortical pathway (Swanson, 1982). **Figure 1.1** shows the midbrain dopamine neurons with their basic projections in the rat brain.

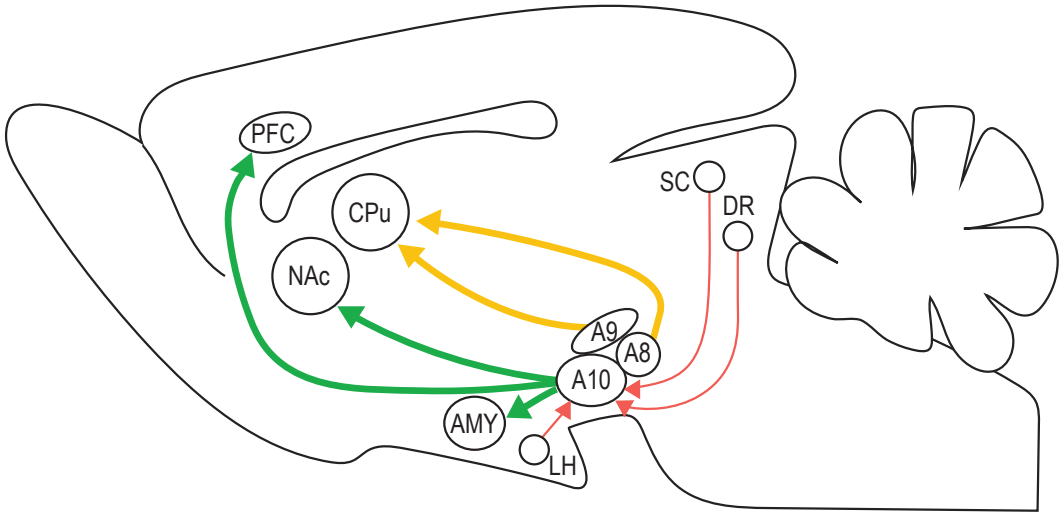


Figure 1.1 – *Key projections in the rat midbrain*

Green projections show the mesolimbic and mesocortical dopamine projections from A10 neurons in the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc), amygdala (AMY) and prefrontal cortex (PFC). Yellow projections show the nigrostriatal dopamine projections from A9 neurons in the substantial nigra pars compacts (SNc) to the striatum/caudate putamen (CPu). In red are several key inputs to the VTA, excitatory input from the lateral hypothalamus (LH) and superior colliculus (SC) as well as the modulatory input from the dorsal raphe (DR).

1.3 Neuronal types in the VTA

Before discussing the inputs and outputs to the VTA, it is important to consider the local circuitry within this area. The VTA does not solely consist of discretely connected dopamine neurons with single inputs and output target regions. The area is much more complex and contains a rich local circuitry of different cell types (dopamine, GABA, glutamate and combinatorial neurons releasing various combinations of these) which project to other VTA neurons as well as to long range targets (Morales and Margolis, 2017). In vitro studies have shown the ability of midbrain dopamine neurons to show glutamaterigic signalling components (Sulzer at al., 1989, Dal Bo et al., 2004) and co-transmission has been demonstrated. There is expression of both glutamate and TH markers in a population of VTA neurons (Kawano et al., 2006; Yamaguchi et al., 2007; Yamaguchi et al., 2011; Yamaguchi et al., 2015) and ex vivo slice experiments have shown glutamate and dopamine co-release occurs in a subset of VTA neurons (Sruber et al., 2010; Tecuapetla et al., 2010; Zhang et al., 2015). The mixed population of neurons highlights the heterogeneity of the VTA, which is an important feature of this brain structure.

1.4 Inputs to VTA neurons

Excitatory glutamate inputs can modulate the activity of the mesolimbic pathway quite profoundly. Retrograde labelling and in situ hybridisation to identify neurons positive for glutamate transporters (VGLUT1 and VGLUT2) have shown the VTA receives prominent excitatory inputs from diverse areas (Geisler & Zahm, 2005; 2006). The densest afferents originate from the prefrontal cortex (PFC), particularly the prelimbic area (Sesack and Pickel, 1992; Carr and Sesack, 2000), lateral hypothalamus (LH) (Geisler, Derst, Veh & Zahm, 2007), pedunculopontine tegmental nuclei (Charara et al., 1996; Floresco et al., 2003; Lodge & Grace, 2006), bed nucleus of the stria terminalis (Georges & Aston-Jones, 2001, 2002) and the superior colliculus (SC) (Coizet at al., 2003).

Watabe-Uchida and colleagues (2012) have used newer, molecular targeting strategies to selectively assess inputs to VTA. They selectively targeted only dopaminergic neurons within the VTA and mapped all of their monosynaptic inputs throughout the whole brain using a sophisticated rabies tracing method combined with Cre-loxP system (Watabe-Uchida et al., 2012). They identified many of the same inputs as previous studies but highlight the importance of LH and subthalamic nuclei (STh) inputs as having important modulatory roles. These two inputs in particular may provide competing excitatory influences to the VTA and these different inputs may encode different information. For example, LH neurons have been implicated in reward and the LH is modulated by internal states such as hunger (Ono et al., 1986; Burton, Rolls & Mora, 1976; Lammel et al. 2008). On the other hand, the subthalamic nucleus (STh) is more involved in encoding the salience of external stimuli (Lammel et al., 2008). The integration of internal states with external sensory information is crucial in reward learning and behaviour and the diverse inputs from different regions to the VTA provide information to help drive adaptive behaviours.

As described, glutamatergic afferents, which synapse directly onto VTA dopamine neurons, provide excitatory input. Increasing glutamate in the VTA increases the firing rate of dopamine neurons (Grace and Bunney, 1984) and stimulation of the PFC increases VTA activity (Carr and Sesack, 2000). Conversely, blocking glutamate (through ionotropic receptor antagonists) reduces burst activity in these neurons (Chergui et al., 1993). However, it is important to note that these inputs do not only target dopaminergic neurons. Those neurons projecting from the LH and paraventricular thalamus do seem to synapse preferentially onto dopamine neurons. However, those originating from the anterior cingulate cortex and central amygdala more often synapse onto GABA neurons in the VTA (Watabe-Uchida et al., 2012)

However, despite some differences in target neuron cell types, many of these excitatory inputs send synapses to both dopamine and GABA neurons within the VTA and there is considerable overlap in their inputs (Beier et al, 2015). This makes their modulatory effect on VTA activity more complex than direct excitation of dopamine neurons. In addition, the VTA receives several inhibitory

influences from local GABAergic interneurons and long-range GABAergic projections. There are also peptidergic inputs from neurons releasing substances such as orexin (Borgland et al., 2006), ghrelin (Abizaid et al., 2006) and melanin concentrating hormone (MCH) (Domingos et al., 2013). Other modulatory inputs arise from serotoninergic neurons (particularly those of the dorsal raphe nucleus, Luo et al., 2015) and noradrenaline neurons from the locus coeruleus (LC) as well as acetylcholine neurons from multiple sites.

The complexity of VTA inputs allows for a vast array of possible combinations of modulatory influences. Such diverse information input may be used to combine information from a wide variety of areas about environmental stimuli and to integrate these with internal need states to drive adaptive behavioural choices. It is clear that the VTA is a heterogeneous structure formed of multiple cell types with diverse inputs, multiple excitatory, inhibitory and modulatory input as well as complex local circuitry capable of directly modulating VTA activity.

1.5 Outputs of VTA neurons

The VTA projects to several areas including the nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and amygdala (AMY). Perhaps the most extensively studied of these is the NAc, which has been implicated in the reinforcing or rewarding properties of common drugs of abuse, in other aspects of motivation and in goal directed behaviours (Kringlebach & Berridge, 2016). Recent advances in circuit mapping particularly new viral strategies have provided insights into the more nuanced details of the anatomical organisation of midbrain circuits. In particular the methods of TRIO and cTRIO – which stand for Tracing Relationships between Inputs and Outputs and in the case of cTRIO ‘c’ refers to cell specific – where targeted viral strategies can be used to investigate the outputs from given neurons based on their inputs. In the cell specific version of TRIO, not only can the inputs and outputs be investigated, but this is done in a cell specific manner.

Such approaches have revealed that there is a topographic organization of VTA dopamine neurons based on their projection targets (Lammel et al., 2008). A particularly heavily studied projection target is the NAc. There is a division along the medial-lateral axis, with neurons projecting to the lateral part of the NAc originating in the lateral VTA and those to the more medial parts of the NAc originating from closer to the medial VTA. Beier et al., (2015) investigated the inputs to VTA neurons based on the outputs to four key regions of the VTA: The lateral NAc, medial NAc, medial PFC and the amygdala (AMY). Based on their outputs, dopamine neurons projecting from the VTA to the lateral NAc received most inputs from the anterior cortex, dorsal striatum and the core and lateral NAc shell. In comparison, those VTA neurons projecting to the medial NAc showed greater inputs from the medial NAc shell and the dorsal raphe nucleus. Based on their outputs, these different populations of VTA dopamine neurons receive differential inputs, mainly differing on the inputs from striatal and accumbal regions.

There are also direct monosynaptic synapses from mPFC neurons onto NAc-projecting dopamine neurons (Beier et al., 2015) (an observation which was previously thought not to be the case (Carr and Sesack, 2000; Beier et al 2015). Neuronal signals from the frontal cortex can directly influence activity in NAc-projecting (and specifically lateral NAc-projecting) dopamine neurons in the VTA. Furthermore, these authors demonstrate a functional role of these cortical connections. Activation of cortical input leads to dopamine release in the lateral NAc and is reinforcing (Beier et al., 2015). The direct, excitatory input from the cortex is an important site of regulation of VTA neurons and is functionally significant, directly contributing to motivated behaviours. These findings provide insights into the complex organisation of the VTA based on its inputs and output targets and start to provide possibilities for anatomically distinct circuits for different functional, behavioural output generated by the VTA or routed through the VTA. NAc-projecting neurons from the VTA are not a homogenous population.

Not only can VTA neurons be divided anatomically based on their inputs and outputs, but subpopulations of VTA cells may also show distinct molecular

markers and physiological properties, which can further differentiate their functional roles. In fact, it has been suggested that the basic VTA/SNc division may be too simplistic. Bjorklund and Dunnet (2007) have shown that VTA and SNc neurons are not as neatly segregated as previously thought. There appears to be another level of anatomical organisation along the dorsal / ventral axis in both areas. Bjorklund and Dunnet (2007) show that there is a tightly packed ventral layer of dense angular cells – with a bias in projections to sensorimotor areas – whereas the dorsally located cells are less dense and project preferentially to the ventral striatum, limbic and cortical and some parts of dorsal striatum specifically matrix compartment (Lammel et al., 2008).

Furthermore, Bjorklund and Dunnet (2007) find differences between dorsal and ventral neurons in both VTA and SNc in terms of cellular markers, potassium channel expression and morphology. This heterogeneity may have important functional consequences for our understanding of midbrain circuits. It is now becoming increasingly clear that dopamine (and non-dopaminergic) neurons in the VTA are not a homogenous group. The heterogeneity of these different VTA subpopulations may underlie different and diverse behavioural states (Marinelli & McCutcheon, 2014). The vast input modulation, output projection targets, local microcircuitry and heterogeneity of the VTA makes this area particularly well-placed to integrate complex disparate information and perform a wide variety of behavioural functions.

1.6 Features of dopamine neurons

Dopamine neurons have a distinct “functional fingerprint” with unique firing characteristics (Roeper, 2013). The way in which neurons within the NAc and limbic-cortical circuitry encode information is crucial in understanding the function of dopamine in these areas. Dopamine neurons have two distinct modes; tonic and phasic firing patterns. Tonic activity in dopamine neurons involves spontaneous single spike activity, which is driven by membrane currents (Grace and Onn, 1989). Tonic firing is slow (1-5 Hz) and pacemaker like (Dreyer et al., 2010; Grace, 1991). It provides dopamine tone in accumbal

terminal regions (Keef, Zigmond & Abercrombie, 1993) by acting on extrasynaptic D2 receptors in their high affinity state (Dreyer et al., 2010; Grace, 1991; Richfield, Penney & Young, 1989). Extracellular dopamine tone is determined by the number of dopamine neurons spontaneously firing tonic spikes (Floresco et al., 2003) and is mainly modulated by GABAergic inhibition (Grace and Bunney 1979). Most dopamine neurons show pacemaker firing even when all inputs are removed and in vitro slice experiments have demonstrated that regular pacemaker activity is autonomously generated (Grace, 1991). This activity originates from calcium dependent membrane potential oscillations (Grace, 1991, Grace & Onn, 1989) and is modulated by several ion channel conductances.

In contrast, high frequency (>15 Hz) rapid bursts of synchronised spike activity are characteristic of dopamine neurons in phasic mode (Grace, 1991). This activity depends primarily upon the glutamatergic excitation from areas such as the pedunculopontine tegmentum (PPTg) (Floresco et al., 2003). Application of N-methyl-D-aspartic acid (NMDA) induces bursting in dopamine neurons and is particularly important for this activity (Deister et al., 2009). Burst discharges produce rapid, transient increases in extracellular dopamine within terminal regions which occupy low affinity D1 receptors in NAc (Dreyer et al., 2010). These phasic dopamine release events termed transients (Sombers et al., 2009) are short lived (<100 ms). Dopamine is removed rapidly via dopamine transporters at pre-synaptic terminals (Chergui, Suaudchagny & Gonon, 1994). In addition, somatodendritic dopamine D2 autoreceptors are activated and have an inhibitory effect playing a critical role in terminating bursts (White and Wang, 1984; Bannon & Roth, 1983). There is considerable evidence that these transient increases encode important information about environmental stimuli with motivational relevance to the animal such as rewarding and aversive stimuli.

Mesolimbic VTA neurons projecting mainly to the NAc have been the subject of many studies investigating their role in a vast array of behaviours including reward, learning and motivation (Wise 2004; Shultz, 2002). Despite considerable research using electrophysiology, microdialysis, fast scan cyclic

voltammetry and behavioural paradigms the precise role of dopamine in limbic-cortical circuitry has not been fully characterised. There is substantial debate surrounding the way in which these circuits mediate complex behaviours. There is particular controversy over VTA and NAc responses to aversive stimuli, and substantial differences in experimental findings using different techniques (McCutcheon et al., 2012). Furthermore, dopaminergic dysfunction within these pathways has been implicated in schizophrenia and addiction. However, the contribution of dopamine dysfunctions in the mesolimbic and mesocortical pathways to psychiatric conditions, in particular schizophrenia, remains to be elucidated.

1.7 The midbrain dopamine system and reward

Much research has been undertaken to determine the information that mesolimbic burst activity encodes. Early reports suggested dopamine was an important mediator of the hedonic or rewarding aspects of stimuli including food, water, sex and common drugs of abuse (Salamone et al., 1994; Yoshida et al., 1992; Young, Joseph & Gray, 1992; Mermelstein & Becker, 1995; Bozarth & Wise, 1981). Facilitating dopamine transmission through electrical stimulation of the mesolimbic pathway is itself rewarding. Stimulation of the medial forebrain bundle – the principal fibre tract connecting VTA and NAc regions – produces transient dopamine increases in the NAc and acts as a powerful reinforcer. It has been robustly reported that rats will readily lever press for self-stimulation of this dopamine pathway (Wise & Rompre, 1989; Phillips, Mora & Rolls, 1979). Furthermore, microinjections of dopamine agonists into the NAc potentiate self-stimulation and antagonists attenuate it (Colle & Wise, 1988; Stellar & Corbett 1989). In addition, drugs that increase extracellular dopamine – such as nomifensine and amphetamine – are readily self-administered directly into the NAc by rodents (Hoebel et al., 1983; Phillips, Robins & Everitt, 1994 ; Carlezon, Devine & Wise, 1995). Finally, conditioned place preference experiments show a reinforcing effect of both direct and indirect dopamine agonists, with rats preferring a context that has been previously paired with dopamine enhancing drugs (Carr & White, 1983).

Further evidence for a role of NAc dopamine as a reward mediator can be seen in the case of addictive drugs. Virtually all drugs of abuse have a common consequence of increasing dopamine release in the NAc (DiChiara, & Imperato, 1998; Pierce & Kumaresan, 2006). Although drugs of abuse have different primary pharmacological actions, they all lead to an eventual increase in NAc dopamine. For example, in the case of opioids; although they act on endogenous opioid receptors, this results in inhibition of GABA interneurons in the VTA, which in turn disinhibits dopamine neurons leading to an increase in firing and dopamine transmission in the NAc (Johnson & North, 1992). Wise (1996) and others have suggested a role for dopamine in reward-related processing. Elevated dopamine transmission in NAc appears to be involved in the reinforcing properties of both natural and artificial rewards. Despite considerable evidence of dopamine release and VTA responses to reward stimuli, these neurons likely do not only subserve reward processing. It is now widely accepted that burst activity in the mesolimbic pathway encodes more than simple responses to reward stimuli and they have a role in a vast array of behaviours, as detailed below.

1.8 Dopamine and incentive salience

Robinson and Berridge (1993; 2008) expand the reward theory to include learning and attentional mechanisms. They argue that the dopamine system does not respond to reward per se, but rather dopamine signals the incentive value or salience of rewards. They argue that dopamine responses imbue rewards and cues which predict rewards with “incentive salience”. This increases the amount of attention apportioned to stimuli and makes them “wanted”, which drives approach and appetitive behaviours. In addiction, when this system becomes sensitized by artificial drug reinforcers, excessive attribution of incentive salience to drugs and drug-related cues causes craving. Crucially, they propose that wanting is dissociable from liking; the hedonic effects of rewards are separate from the drive to acquire them. Incentive salience attribution signals the motivational importance of behaviourally relevant

stimuli and makes it easier for such stimuli to enter into learned associations. Robinson and Berridge (1993) do not define the neural locus of these dopamine responses. However, given other evidence the NAc would be a convincing candidate for such a substrate. For example, Parkinson et al., (2002) found that dopamine antagonists delivered to NAc impaired responding to reward-related cues but did not affect the consumption of reward. In this way, NAc dopamine abolition seems to affect the motivational aspects of reward rather than processing the reward itself.

1.9 Reward prediction error and reinforcement learning

One of the most influential theories to describe the function of dopamine arises from the seminal work of Wolfram Schultz and colleagues (Hollerman & Schultz, 1998; Schultz, 2016; Mirenowicz & Schultz 1994). These studies found that phasic dopamine signals do not just report the occurrence of reward or signal the motivational salience of stimuli. They argue that the timing of dopamine neuron burst firing has an important role in the expectation of reward and in reinforcement learning. In their conceptualisation, dopamine acts as a teaching signal to provide information about the prediction of reward occurrence. This is the basis of the reward prediction error hypothesis (Schultz, 1998).

In their recording of VTA dopamine neurons in the monkey during reward related learning tasks, they observed transient increases in burst activity following unpredicted/unexpected liquid rewards (Mirenowicz & Schultz 1994; Shultz, Dayan & Montague, 1997). Unexpected omissions (which represent an outcome less than predicted) were followed by depressions or pauses in firing at the time of the usual occurrence of reward (Shultz, Apicella & Ljungberg, 1993). If a reward is fully predicted there is no response to its occurrence (Shultz, Dayan & Montague, 1997). Shultz's work shows precise encoding of reward occurrence in line with learned predictions for the timing of reward delivery. Once a reward becomes predicted by a conditioned stimulus such as

light or auditory cue the dopamine response shifts to that predictor (Mirenowicz & Schultz 1994). Dopamine neuron activity in the VTA encodes a temporal reward prediction error (RPE), which is a difference between expected and delivered rewards. This RPE acts to alert the animal to mismatches in prediction and forms a reinforcement and teaching signal for learning, allowing the animal to modify their expectations and inform their behavioural actions to maximise reward (Schultz, 2016).

The reinforcement learning account posits that larger, unexpected rewards and their predictive cues will evoke larger dopamine responses (as the error incurred is greater). However, recent work by Berke (2018) and Hamid et al., 2015) has offered a different interpretation of the reward prediction error findings. Berke seats the phasic changes in dopamine within a temporal discounting framework. Instead of signalling the reward prediction error – that is, the difference in predicted reward and actual reward – he posits that these neurons actually have a different role in monitoring the ongoing value of reward. In temporal discounting there is more value in being closer in time to rewards, to have a certainty around receiving such rewards and there is, of course, more value if a reward is larger. 'Discount curves' represent the value of rewards in a given state. The value of reward at any given time is the state value, this is represented by the proximity to reward, the future reward minus the current distance from it. In this way the value of reward is constantly changing with reward history. It is this 'value' that Berke (2018) argues is encoded in the dopaminergic signal.

It is possible that dopamine can encode both RPE and monitor reward value but on different time scales. There may be two modes of dopamine activity tracking or encoding both reward value (slow, tonic) and reward prediction error (faster, phasic responses). One-minute microdialysis experiments show that the concentration of dopamine in the NAc directly correlates with the density of rewards received during behavioural tasks (Hamid et al., 2015). That is, dopamine concentration tracks relative rate of reward on this slow scale, supporting the idea that slow changes in dopamine can monitor the density of rewards. In addition, dopamine seems to ramp up as rats physically approach

rewards in a two arm maze task (Howe et al., 2013) but not during their consumption, which is still consistent with both RPE account and the value encoding proposed by Berke. Berke has shown that dopamine release not only tracks the reward history and value, but this activity also seems to be involved in motivation and vigour. During reward choice tasks the animals' latency to initiate a trial (their vigour to approach the port to start a trial) is shorter if they have received a high density of rewards in preceding trials. The vigour, drive or motivation to engage in a trial is directly affected by the recent reward

1.10 Dopamine responses to aversive stimuli

Mesolimbic dopamine signalling is an important neural substrate for reward and plays a role in learning as well as in calculating or keeping track of the value of rewards to inform adaptive behaviour. However, there is considerable debate over whether the mesolimbic system also encodes or responds to non-reward related stimuli. There appears to be a more complicated relationship between dopamine and various stimuli than just responses to stimuli with positive valence.

The study of dopamine neuron activity in response to aversive stimuli has yielded inconsistent and often conflicting results. A number of electrophysiological studies find inhibitory responses to aversive stimuli – as hypothesised by the reward prediction error theory (Mirenowicz and Schultz, 1996; Ungless, Magill & Bolam, 2004; Cohen et al., 2012). However, others report excitation in response to aversive stimuli (Horvitz 2000; Brischoux et al., 2009; Matsumoto & Hikosaka, 2009). According to Romo and Schultz (1990) around 75% of VTA neurons respond to primary rewards and around 55-70% respond to conditioned stimuli predictive of food reward (Shultz 1998). In contrast, only 14% show phasic activation to primary aversive stimuli (Mirenowitcz & Schultz 1996) and 11% to conditioned aversive stimuli (auditory or visual cue predicting aversive stimulus) (Mirenowitcz & Schultz 1996). Schultz and colleagues argue that the mesolimbic dopamine system responds

to appetitive or rewarding stimuli preferentially (Schultz, 1998; Schultz, 2000; Mirenowicz & Schultz, 1996).

Proponents of the reward-only view assert that few dopamine neurons respond to aversive stimuli and those neurons that do are not dopaminergic. In fact, the identification of VTA neurons has proven somewhat controversial (Margolis et al., 2006; Ungless and Grace, 2012). There is evidence of a sub-population of VTA neurons, which may be misidentified, and these putative dopamine neurons have slightly narrower action potentials than true dopamine neurons (Cameron et al., 1997; Chiodo et al., 1980). Ungless (2004) used juxtacellular labelling to identify TH+ neurons whilst performing electrophysiological recordings during aversive foot pinch. Those neurons activated by aversive stimuli were found to be non-dopaminergic. TH+ dopamine neurons were uniformly inhibited by aversive stimuli – showing consistently reduced firing rate and burst activity (Ungless, 2004). This evidence supports a selective role for dopamine neurons originating in VTA in reward stimuli. In line with the reward prediction error hypothesis, dopamine neurons are depressed by aversive stimuli in the same way as omission of reward (Schultz et al., 1993). However, one important factor may be the use of anaesthetic in these studies as this may considerably dampen aversive responses and should be considered when interpreting these results.

Using similarly strict dopamine neuron identification methods, investigators from the same laboratory (Brischoux et al., 2009) performed recordings from VTA neurons in rats in response to noxious foot shock. They found two distinct populations of VTA cells which were anatomically separate. Dorsal VTA neurons were inhibited by the aversive stimulus, whereas a more ventral population were activated by foot shock. Lammel et al. (2011) suggest further anatomical segregation based on the projection targets of VTA subpopulations. Their findings show reward stimuli activate the NAc medial shell whereas aversive stimuli affect those neurons projecting to the medial prefrontal cortex. They propose a third circuit, those neurons projecting to the NAc lateral shell, which appears to be involved in both reward and aversion – they suggest this circuit is involved in the processing of general saliency. Recordings in several

anatomically segregated and functionally distinct populations within the heterogeneous area that is the VTA may go some way to explain the contradictory findings in aversive responses.

There is evidence of increases in phasic dopamine signalling, not only in response to rewards, but to neutral, novel and aversive stimuli as well as conditioned stimuli predictive of both rewarding and aversive events (Joseph, Datla & Young, 2003; Ikemoto & Panksepp, 1999). Microdialysis studies in rats have shown increases in dopamine release in response to aversive stimuli including foot shock (Young, Joseph & Gray., 1993) tail shock (Abercrombie et al., 1989) and physical restraint (Imperato et al., 1992). Fast scan cyclic voltammetry experiments have also shown dopamine responses to a variety of aversive stimuli including, quinine and aversive tastants in conditioned taste aversion paradigms (Roitman et al., 2008; McCutcheon et al., 2012), tail pinch (Budygin et al., 2012) and social defeat (Anstrom, Miczek, and Budygin, 2009).

There are a number of methodological considerations when assessing findings from electrophysiological, microdialysis and voltammetry experiments, especially in the case of aversive stimuli where there are some conflicting findings reported. There is considerable variation in the types of stimuli used. For example, Schultz used air puffs to the paw and hypertonic saline drops into the mouth of passive monkeys. These stimuli promote avoidance behaviours but would be considered only mildly aversive. More salient aversive stimuli – such as foot or tail shocks – may evoke dopamine responses. There may also be species differences between monkeys (primarily used in electrophysiological recordings) and rats (microdialysis, voltammetry) which could contribute to the sometimes conflicting findings observed.

A potential issue of microdialysis is its poor temporal resolution of several minutes. During behavioural experiments where aversive stimuli are presented there are multiple stimulus presentations and importantly multiple terminations within the recording period. These presentations and terminations are intermixed in dialysate samples and it is not possible to separate out the effects of aversive stimulus and the relief of its termination. Relief is presumably

rewarding and may well lead to an independent release of dopamine not related to the application of the aversive stimulus itself. In fact, Imperato et al. (1991; 1992) have found that restraint causes NAc dopamine elevation and removal from restraint is accompanied by a second, separate, dopamine increase in the NAc and others have shown that dopamine increase is linked to stimulus onset rather than offset (Young et al., 2004).

In addition, it is difficult to directly compare responses to aversive and appetitive stimuli; reward stimuli such as food are transduced by different sensory pathways compared to foot shock or tail pinch (McCutcheon et al., 2012). In an elegant set of experiments, McCutcheon et al. (2012) measured phasic dopamine changes in the NAc shell in response to intra-oral delivery of sucrose, in animals with different learning histories. Sucrose was paired with lithium chloride in a conditioned taste aversion paradigm in half of the rats. Sucrose had opposing effect on phasic dopamine release in the NAc shell depending on the conditioning history of the animal. Those in which sucrose had been paired with illness showed conditioned aversion (taste reactivity paradigm, negative orofacial movements). This behavioural aversion was accompanied by decreased frequency of dopamine transients in NAc shell. This dopamine inhibition was similar to that seen with the aversive tastant quinine, and opposite to the transient increase observed with sucrose in unpaired rats (McCutcheon et al. 2012). This experiment shows that the same stimulus can elicit opposite dopamine responses based on prior conditioning. In the appetitive case dopamine transients are increased, in the aversive case they are inhibited. This highlights the multiplicity of dopamine responses and provides evidence for the complex and multiple roles these pathways play in motivated behaviours.

1.11 Responses to neutral and sensory stimuli

As well as responding to reward related and some aversive stimuli, the mesolimbic pathway seems to show some sensory responses to seemingly neutral stimuli. In sensory pre-conditioning, dopamine release appears to occur even when there is no motivational stimulus involved (appetitive or aversive) to neutral stimuli (Young et al., 1998). It is possible that neutral stimuli may have some innate rewarding properties or these responses are to the novelty of such stimuli. Reed, Mitchell and Nokes (1996) have found that animals prefer a reward lever if it is coupled with a cue such as a stimulus light. However, the sensory pre-conditioning study does suggest a role for dopamine in attention or associative learning more generally rather than within situations specifically relating to reward.

These kinds of responses were originally referred to as ‘sensory salience’ (Redgrave et al., 1999), and as such, dopamine responses to neutral but salient stimuli could provide a means for orienting attention to potentially important stimuli (Joseph et al., 2003; Redgrave & Gurney, 2006). These responses likely originate in the cortex and are routed to dopamine neurons in the midbrain via the superior colliculus (Bertram et al., 2014). This circuit allows for fast dopamine responses to sensory stimuli driven by the cortex (Bertram et al., 2014). Overton and colleagues argue that such responses provide an ‘alerting’ role to allow new stimuli to be learnt and to enable behavioural switching (Overton et al., 2014). Direct projections from the superior colliculus to the VTA innervate both dopamine and non dopaminergic neurons (Comoli et al., 2003) and are the primary source of short-latency visual (and possibly other modalities) input to the VTA (Overton et al., 2014). Short auditory stimuli and light flashes result in burst firing in VTA dopamine neurons (Horvitz et al., 1997) and stimulation of the visual cortex – which projects to the SC produced elevations in dopamine via the VTA (Bertram et al., 2014). These very short latency activations arising via the SC are related to stimulus intensity (Bromberg-Martin et al., 2010, Fiorillo et al., 2013). This evidence suggests there are circuits involving primitive structures including the SC which can provide signals to represent stimulus salience. These circuits may inform

midbrain areas that an important stimulus or event has occurred and help to guide adaptive motivated behaviours in the face of new information. A mechanism by which sensitivity to external stimuli is increased could allow for engagement with previously unattended or ignored stimuli (Redgrave et al., 2011)

In fact, Joseph et al. (2000, 2003) suggest that NAc dopamine release may act to broaden attention to previously devalued or unattended stimuli during latent inhibition conditioning. Dopamine elevations increase the salience of stimuli that would otherwise be ignored which allows them to enter into new learned associations. This hypothesis is particularly interesting when one considers the deficits in attention seen in schizophrenia. An inability of patients to ignore irrelevant stimuli and marked deficits in latent inhibition may be linked to mesolimbic dopamine dysfunction which has been found in patients and to disturbances in the collicular or cortical inputs to the VTA.

Given considerable data showing dopamine responses to non-reward stimuli Ikemoto and Panskep (1999) propose a broader role for NAc dopamine in motivation. They theorise that NAc dopamine release acts to alert the animal of important behaviourally relevant stimuli and orient attentional resources. In turn, this acts to stimulate flexible approach or avoidance behaviours when an animal faced with multiple salient stimuli. Horvitz (2000; 2002) has also formalised a more general prediction error hypothesis. He asserts that the NAc dopamine signal is more concerned with unexpected salience rather than unexpected reward. Salient stimuli of any valence can activate dopamine signalling and draw attention meaning that this account can accommodate findings of NAc dopamine increases in response to both rewarding and aversive stimuli. The neural circuitry underlying such responses may provide some explanation for how the VTA and its inputs and outputs are organised in a way that allows for the processing of diverse and often contradictory information to inform motivated adaptive behaviours within complex environments. The aforementioned responses to salient and unpredicted stimuli could be crucial for behavioural switching and flexibility and for deciding which stimuli to interact with or ignore.

The ability to selectively attend to important, relevant behavioural stimuli within the environment is essential. Although traditionally these functions are thought to be subserved by the cortex, direct inputs from cortex to the VTA and NAc may contribute significantly to the direction of attention. Attentional functions involve the constant monitoring of salient stimuli. There is an important contribution of VTA dopaminergic inputs to cortical areas and vice versa which subserve attention, driving attentional switching and behavioural flexibility (Totah, Kim & Moghaddam, 2013). In addition, the aforementioned cortical inputs to the VTA, both direct but also indirectly via the superior colliculus providing fast sensory information which can guide attentional resources. VTA projections provide real-time information during learning about the state of environmental cues.

Aberrant activity in dopaminergic neurons from the VTA projecting to accumbal and cortical regions could lead to the excessive allocation of attention to irrelevant distractors biasing cortical and accumbal responses to attribute importance and processing resources to non-relevant stimuli (Ishiwari et al 2004; Salamone et al., 2009). The importance of the VTA in reward processing and in providing reward prediction error learning signals (Schultz, 2002; Schultz, 2016) and its contribution to organising behaviour is crucial to investigate further in both normal and disease models, especially in models of schizophrenia as well as under baseline conditions in the intact animal.

1.12 Dopamine and action initiation

There is a well establish role for dopamine in the dorsal striatum in movement, and it's reduction is central in the movement disorder Parkinson's Disease. In the dopamine and reward prediction error literature, dopamine responses to reward predicting cues are often confounded by the initiation of movement to retrieve a reward or make a response. Syed et al. (2015) have attempted to disentangle the role of dopamine signalling in reward and in action initiation in a series of voltammetry experiments.

Syed et al (2015) investigated the role of dopamine in action initiation as well as in response to reward cues. They used a go/no-go task, where auditory cues signal that a rat must either ‘go left’ or ‘go right’ and respond with lever presses, or ‘no go’ and remain in the nose poke. Using this task with voltammetry recordings in the NAc, they found dopamine responses to auditory cues signalling go left or go right following correct trials (where the animal responded appropriately). However, strikingly, no such increases were observed on no go trials (where reward predicting cues have the same value but action initiation is not required). Furthermore, when they aligned voltammetry signals to the action of exiting the nose poke (in both go and no go trials) dopamine responses were seen in both trials types. This shows that it is the response requirement of the trial which determines whether dopamine is increased. These authors show that dopamine release in the NAc is not only involved in reward prediction, but is fundamentally linked to action initiation and movement. Dopamine responses to reward cues are dampened unless movement is initiated (Syed et al., 2015). NAc dopamine may have a key role in not only reward prediction, but also in the promotion of reward seeking by movement (Syed et al., 2015). NAc responses may be linked to the vigor of action initiation (Jin & Costa, 2010; Nicola, 2010; Roitman et al., 2004)

1.13 Aims of the current thesis

Dopamine has been implicated in multiple behaviours. Although dopamine has been linked to a wide range of rewards both natural (e.g. food, social interaction) and artificial (e.g. drugs of abuse), this thesis is primarily concerned with dopamine’s contribution to feeding, in particular the role of dopamine in the consumption of saccharin and the possibility of a role for dopamine in the choice to engage with consumatory behaviour in the face of distracting stimuli.

We assess the important question of how, when faced with multiple options of various competing stimuli of different modalities, does an animal or human

prioritise which actions to perform and where to allocate valuable and finite attentional resources.

Taken together, previous investigations of the role of dopamine in the mesolimbic pathway strongly suggest that the responses of VTA circuits are not only related to rewarding events but also more generally to salient environmental stimuli to help to guide motivated behaviour, a process altered in some neuropsychiatric disorders such as schizophrenia.

The overarching aim of this thesis is to describe the contribution of the VTA to ongoing consumption in the face of distracting stimuli. In this thesis I explored this by using a novel behavioural paradigm in which rats are exposed to distracting stimuli during saccharin consumption (chapter 2). I then tested the utility of this paradigm in assessing behavioural differences in distractibility in a model of schizophrenia (chapter 3). In chapters 4 and 5 I have used fibre photometry to assess calcium changes in the VTA during this behaviour and to investigate responses in cell bodies and NAc terminals of the VTA neurons. Finally, I discuss possible strategies for dopamine cell specific recording during this task using different viral strategies with fibre photometry.

CHAPTER 2

Validation of distraction protocol in rats – disruption of ongoing saccharin consumption by distracting stimuli

2.1 INTRODUCTION

2.1.1 Licking microstructure

When ingesting palatable solutions, rats and mice show rhythmic and predictable patterns of licking. Licks are separated by short intervals – inter lick intervals – of less than 100 ms. These periods of high frequency licks form bursts which are each separated by short pauses (250-500 ms). Bursts of licking are further classified into clusters when there are pauses of over 500 ms (Davis and Smith, 1992). The overall frequency of licking within a burst is constrained by a central pattern generator (CPG) located in the brainstem (Wiesenfeld et al. 1977), this limits the speed of licking to the asymptotic rate of licking which is approximately 6-7 Hz (Spector et al., 1998). **Figure 2.1** shows the grouping of lick patterns into bursts and clusters using the criteria outlined by Davis and Smith (1992).

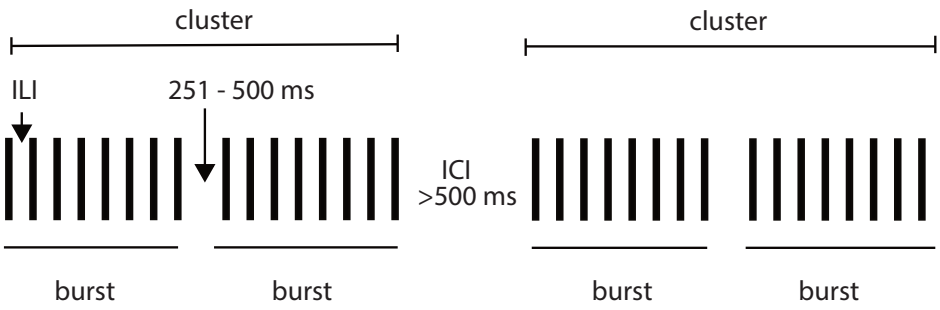


Figure 2.1 – *Licking microstructure schematic*

Black lines show individual licks, these are grouped into bursts and clusters. ILI – inter lick interval, ICI – inter cluster interval. Bursts are separated by ILIs of between 251 and 500 ms and clusters by ILIs of over 500 ms.

Changes in the pattern of licking are related to the palatability of the solution being consumed (Davis, 1973). Longer clusters (more licks within a cluster or longer duration of cluster) are indicative of increased palatability. As such, cluster size increases with increasing concentrations of sucrose solution (Davis and Smith, 1992; Davis and Perez, 1993; Spector et al., 1998) and conversely, smaller clusters are observed when normally palatable solutions are adulterated with bitter quinine in a concentration dependent manner (Hsiao and Fan, 1993). The cluster size and other variables can reflect the incentive motivational properties of the solution being consumed and the motivation to engage in consummatory behaviour can be assessed by the number of initiations of bursts and clusters (Paolo, 2010).

The assessment of licking microstructure can be done using relatively low cost, simple equipment and provides a large amount of data on a precise timescale during consummatory behavior. Not only do internal need states such as hunger and ingestive factors such as palatability influence lick microstructure. There are also important influences on these parameters by external stimuli. Long interruptions in licking (over 500 ms between clusters, the inter cluster intervals) are often due to engagement in competing activities such as grooming (Davis and Smith 1992), or responses to salient environmental stimuli, which attract attention.

2.1.2 Developing distraction assay

Using lick microstructure measures, O'Connor and colleagues (2015) assessed the role of a GABAergic pathway from the nucleus accumbens shell (NAcSh) to the lateral hypothalamus (LH) in controlling feeding. They monitored consumption using lickometers during circuit manipulations including optogenetic inhibition of this pathway. They used self-paced licking behaviour in mice to trigger brief distractor stimuli when the mice initiated bouts of feeding. They assessed the persistence of feeding in the face of external stimuli. Distractors were triggered following 3 consecutive licks occurring in less than 1

second. They found control mice consistently stopped licking in response to these stimuli, and there were significantly more occurrences of bursts of only three licks long. Inhibition of GABA neurons in the NAcSh → LH pathway produced prolonged feeding and, interestingly, this was sustained even in the face of distracting stimuli.

The current pilot experiment builds on this task to develop a measure of distraction and to probe the responses to distractors specifically. The aim was to measure both licking microstructure and to quantify distraction during consumption of saccharin. The following pilot study set out to test this assay, to divide trials into distracted and not distracted based on the length of pauses following distractor stimuli. From this we calculated the percent distracted for each session / rat. The following chapter details the task design and pilot results demonstrating the behavioural responses of rats in this assay.

Following this pilot, after we had characterised responses in a small cohort of rats and determined the key parameters of the task, we applied this assay to investigate distraction in a model of schizophrenia (**Chapter 3**) and then investigated the neural circuitry involved in distraction from ongoing saccharin consumption using fibre photometry (**Chapters 4,5 and 6**).

2.2 AIMS, OBJECTIVES, HYPOTHESES

2.2.1 Aims

1) Develop a behavioural assay based on self-paced licking for saccharin to measure distraction/distractibility
2) Determine baseline levels of distractibility in rodents under control conditions
3) Assess the effect of increased dopamine transmission on distractibility using amphetamine
4) Devise analysis methods using Python and determine measures of lick microstructure

2.2.2 Objectives

1) Develop and test licking based distractibility assay with pilot experiment
2) Determine the best parameters for the test, define distraction
3) Compare licking days lick microstructure to days with distracting stimuli and assess behavioural changes, responses to external stimuli, and impact on licking patterns
4) Write Python scripts for analysing licking patterns, classifying distraction, and exploring data

2.2.3 Hypotheses

1) Rats will pause licking in response to salient auditory and visual stimuli
2) The percent distracted will be significantly higher in the presence of real versus modelled distractors
3) Dopaminergic drugs will increase susceptibility to distraction, seen by a significant increase in the percent distracted measure

2.3 METHOD

2.3.1 Animals

A pilot experiment was conducted using 8 male Sprague Dawley rats purchased from Charles River UK and weighing 250 g – 300 g at the beginning of the experiment. Rats were housed in groups of 4 in individually ventilated cages under temperature controlled conditions (21°C ± 2°C; 40-50% humidity) and kept under a 12 hour light/dark cycle, with lights on at 7am. Rats had access to food and water ad libitum, except for a period of overnight food restriction before testing and during experimental sessions where only saccharin (0.2% in water) was available (1 hr per day). All procedures were carried out under the appropriate license authority in accordance with the Animals (Scientific Procedures) Act (1986). Project licence (PPL): 70/8069, personal individual licence (PIL) I9202C6A3.

2.3.2 Behavioural apparatus

All behavioural experiments were carried out in operant behaviour chambers (25 cm X 32 cm X 25.5 cm) housed inside sound attenuating chambers with inbuilt ventilation fans (Med Associates). Operant chambers consisted of a rear panel and door made of clear polycarbonate with two side walls consisting of three aluminium channels where various modular components were inserted. For this experiment, the left wall contained a house light (100 mA), the right wall had two panels with holes for sippers to be inserted and two cue lights, one above each sipper. During training and testing only a single bottle was used with the sipper closest to the door. Each chamber had a grid floor consisting of 19 steel rods (4.8 mm) spaced 1.6 mm apart. Contact lickometers were used to record individual licks as the animal made contact with the grid floor and metal spout. All programmes were written in MED-PC-IV, stimuli were computer controlled and licks as well as distractions were recorded using MED-PC software.

2.3.3 Lick training

For lick training days, rats were placed in the operant chamber with the house light turned on and given access to a spout with an attached lickometer and a bottle filled with saccharin solution (0.2% in UV treated drinking water). Rats could freely lick for saccharin during a 60 minute session with constant access to the sipper. The contact lickometer recorded individual licks, which were registered using MEDPC software as a TTL pulse for each lick. Before the first licking session only, rats were food restricted overnight (given 10 g per rat 24 hrs before testing) food was returned immediately following the lick training session and rats remained non-restricted for the entire remaining duration of the experiment. Rats were trained to lick for saccharin for 7 days until they reached a set criterion of 1000 licks within 60 minutes. No rats were excluded according to this criterion in this experiment.

2.3.4 Distraction task

For distraction testing, rats were placed in the same test chamber as for lick training with access to a single lickometer spout and bottle containing 0.2% saccharin. On distraction days the house light was off to maximise the salience and impact of visual stimuli, making them as distracting as possible and to differentiate these days from lick training. The distraction programme was written so that following three consecutive licks within 1 second, the rat received a distractor stimulus. Distractor stimuli were; tone (5 kHz, 80 dB), white noise (flat 10 – 25 kHz, 80 dB), cue light and house light with six possible combinations as follows: (1) white noise + house light, (2) white noise + cue light, (3) tone + house light, (4) tone + cue light, (5) white noise + tone + house light, (6) white noise + tone + cue light. Distractors were presented pseudorandomly (for each presentation a distractor was chosen randomly from the list of 6 combinations, this was randomised without replacement so that only once each distractor was presented once would the list start again). The duration of all distractors was 0.5 seconds.

If the animal paused licking following the distractor for over 1 second they were deemed to be distracted; if they did not pause, they were considered not distracted. To avoid rats receiving distractors too frequently, i.e. during long trains of licks, after receiving a distractor there was always a 'reset' pause of over 1 second before another was presented. **Figure 2.2** shows a schematic of the distractor presentation protocol. Animals were repeatedly tested using this task, the first day is referred to as the distraction task and any subsequent tests as habituation days.

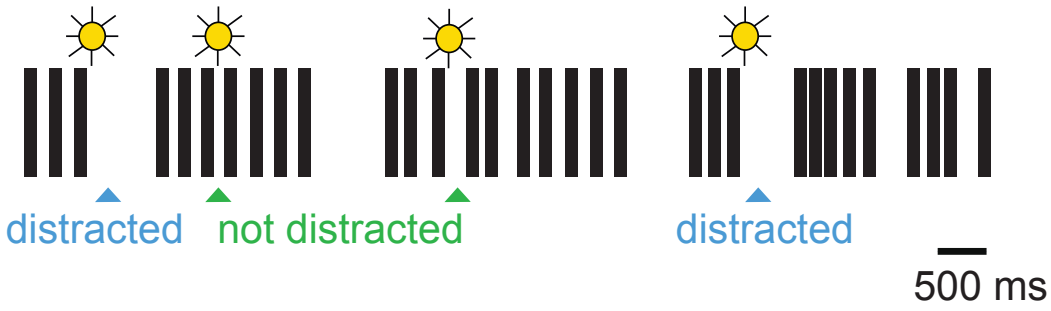


Figure 2.2 – *Schematic of distractor presentations*

Schematic to show lick bursts triggering distractors. Black lines show individual licks. Three consecutive licks occurring within 1 second triggers a distractor presentation. Yellow circles represent distractors. Distracted and non-distracted trials are labelled as such.

2.3.5 Experimental schedule

In this initial distraction pilot rats were lick trained up to criteria and then received three days of distraction testing. Following this the effects of amphetamine were assessed by administering i.p. amphetamine (1 mg/kg) 15 minutes before distraction testing. A control injection of saline was also given to rule out non-specific effects of injection stress on distraction levels, **Figure 2.3** shows this schedule. The injection ordering was not counterbalanced, all rats received saline injection before amphetamine three days following.

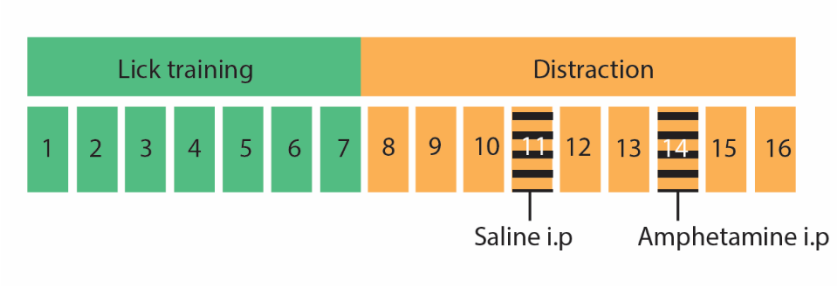


Figure 2.3 – *Experimental schedule for distraction pilot study*

Rats were trained to lick for saccharin for seven days (days 1 – 7) before a distraction test (day 8) which was repeated on days 9-16. Black lined areas shows days where rats received injections of saline (day 11) or amphetamine (day 14).

2.3.6 Data analysis

Licking data (times of licks, intervals between licks, bursts and clusters) and distraction data (times of distractors, number of distracted trials, and number of non-distracted trials) were extracted from MED PC data files and analysed using scripts written in Python. For statistical analyses all data were expressed as means and SPSS.24 was used to perform ANOVAs or t-tests, where appropriate. All assumptions of sphericity, homogeneity of variance and normality were satisfied unless otherwise stated. Alpha was set at $p < .05$ and all significance tests were two-tailed.

To confirm that the distraction task did not simply capture a normal pattern of licking (i.e., to verify that animals do not – independently of distractors – show patterns of 3 lick bursts with pauses), a distraction model was written in Python. This model was written to identify offline when the animal would receive a distractor and according to the ILI following this modelled distractor (post distractor pause), if they would have been classified as distracted or not. Modelled distractors on lick days were compared to real distractors on distraction days.

2.4 RESULTS

2.4.1 Licking microstructure

All animals were trained to lick for saccharin and reached criterion of 1000 licks within a 1 hour session after 7 days of lick training. **Figure 2.4** shows the mean number of licks on each day of training. On the final day of lick training (the day before distraction testing) the mean number of licks was 1358 (SEM = 160 licks). No animals were excluded for failing to meet criterion and several rats met criterion earlier than the 7th day.

Figure 2.5 shows licking microstructure measures in the 8 rats tested. The number of bursts, clusters as well as the mean number of licks within bursts and clusters are shown as well as the mean inter-burst and inter-cluster intervals. Rats showed a licking frequency of between 6 and 8 Hz (M = 7.66 Hz, SEM = 0.22).

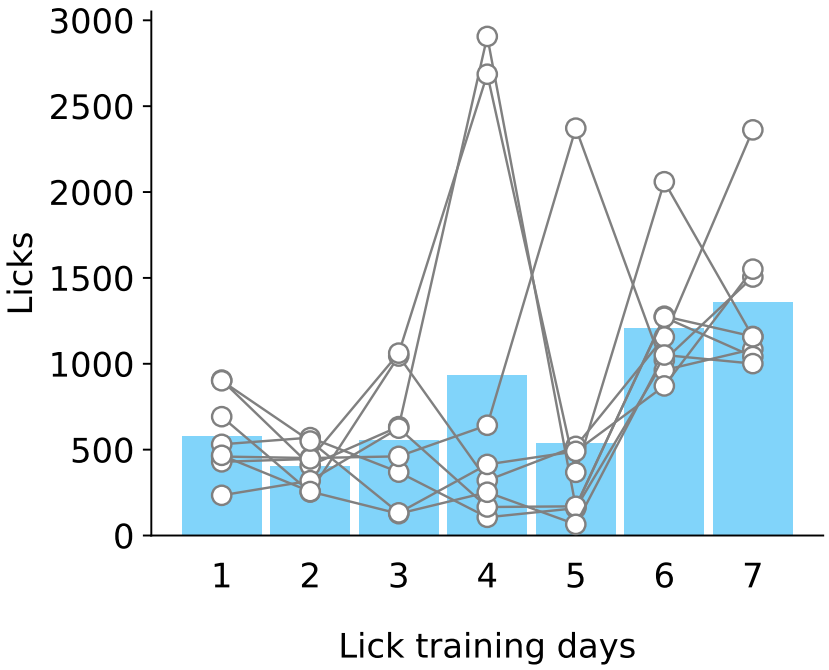


Figure 2.4 – *Licking across seven training days*

Blue bars show mean number of licks for saccharin solution (0.2%) within a 1 hour session across 7 licking training days. Circles indicate individual rat data with adjoining lines to show the same rats across 7 days. All rats reached 1000 lick criteria within 7 days of training.

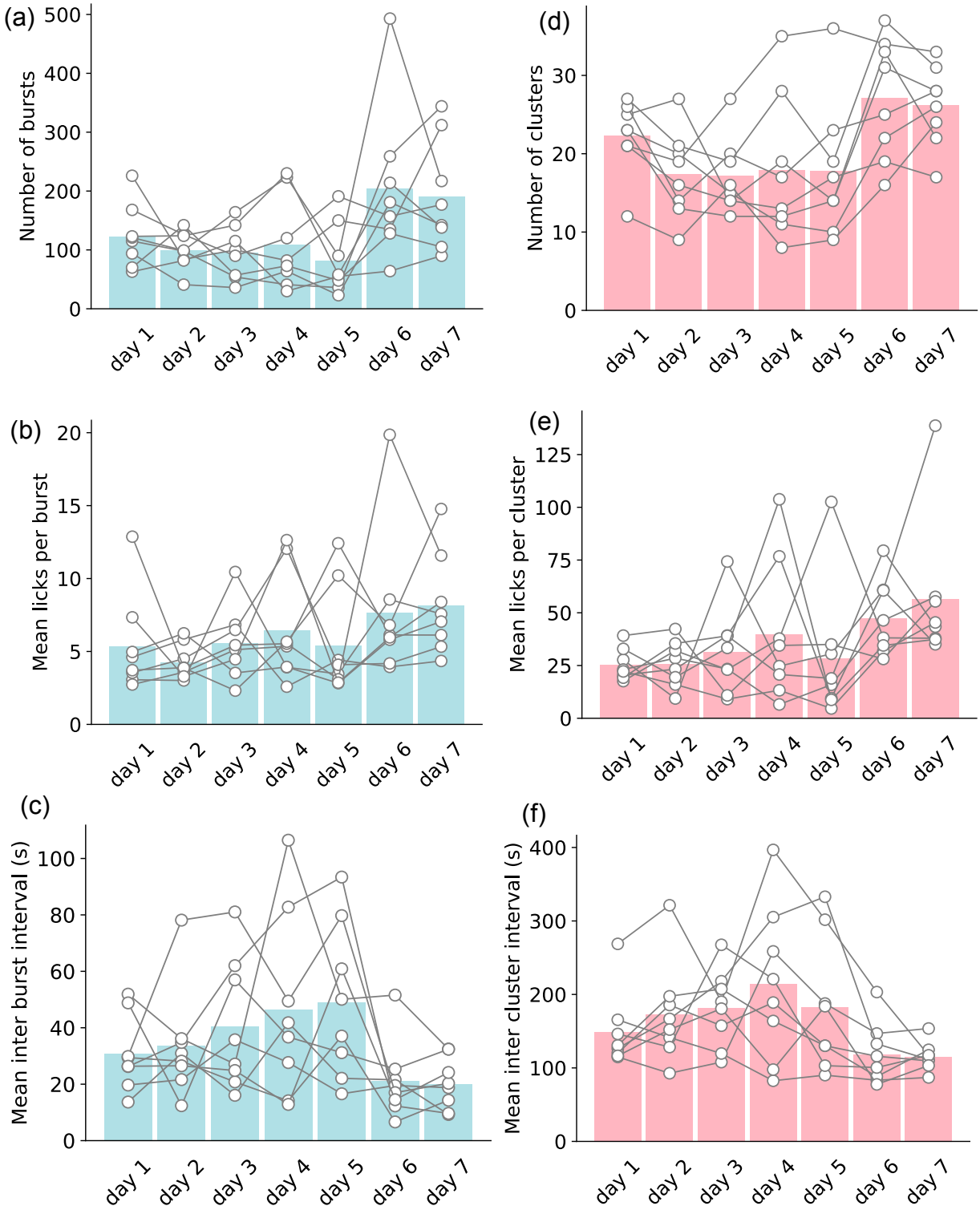


Figure 2.5 – *Microstructure measures*

Lick parameters for 8 rats across 7 days, light blue are burst measures and light pink clusters. **(a)** Number of bursts in the last lick training session, **(b)** Mean number of licks per burst within the session**, (c)** Mean inter burst interval**, (d)** Number of clusters**, (e)** Mean number of licks per cluster**, (f)** Mean inter cluster interval. Bars represent means and individual circles are individual rats.

2.4.2 Percent distracted across days

Distractor presentations were controlled by rats' licking; rats received between 22 and 166 distractors within the main distraction session (M = 62, SEM = 17) and between 32 and 101 on the first habituation day (M = 63, SEM = 9). Pauses in licking following distractors were determined with a post distractor pause of greater than 1 second and were classed as a distracted trial; pauses less than 1 second were classified as not distracted. Mean percent distracted was calculated from the total number of distracted trials and the total number of non-distracted trials. One way repeated measures ANOVA showed a significant main effect of test session (lick day, distraction session, habituation1, habituation2, saline IP, amphetamine IP) $F(5,35) = 16.11$, $p < .001$, **Figure 2.6** shows percent distracted across test sessions.

Bonferroni corrected post hoc comparisons showed that compared to the modelled distractors on a lick training day (M = 13%, SEM = 3%), rats showed significantly higher percentage distracted on the distraction test day (M = 51%, SEM = 6%, $p < .05$). Thus, rats do not show a distracted pattern of licking and pausing during lick training, in the absence of distractors.

There was a significant habituation effect following just one distraction session. On the second test day (habituation 1) rats were significantly less distracted (M = 15%, SEM = 2%) compared to the first distraction test (M = 51%, SEM = 6%, $p < .01$) and they remained at this lower level following habituation day 2 (M = 13%, SEM = 3%, $p < .01$). The percent distracted returned to the level of the modelled distraction day with no difference between the modelled day and either habituation day 1 ($p = 1.0$) or 2 ($p = 1.0$).

To assess any possible non-specific effects of injection stress on percent distracted, this parameter was compared between habituation 2 and saline days. Saline injection did not increase percent distracted (M = 12%, SEM = 5%) above habituation levels on habituation day 2, M = 13%, SEM = 3% ($p = 1.0$), and was not significantly different to the modelled day ($p = 1.0$).

Amphetamine injection led to an increase in percent distracted, which was not significantly different from the main distraction day (p = .057). However, due to considerable variability in these data, the difference between the habituation day 2 and amphetamine day was not significant (p = 1.0). The percent distracted measure shows rats consistently pause following the presentation of distractors during the first test. This response to distractors habituates across days and may be reversed by amphetamine.

To assess possible motor and motivational effects of amphetamine, the total number of licks were compared for the distraction test day and the amphetamine distraction session using paired samples t-test. There was no significant difference in licks between distraction (M = 954, SEM = 206) and distraction with amphetamine injection (M = 665, SEM = 295, t(7) = 1.44, p = .193). **Figure 2.7** shows total licks across sessions and **Figure 2.8** shows licking microstructure across sessions.

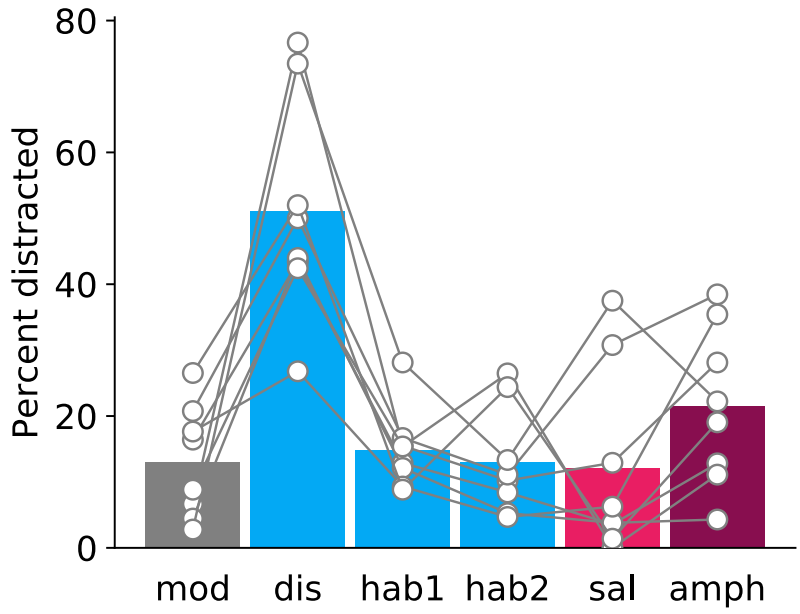


Figure 2.6 – *Percent distracted across experimental days*

Percent distracted across different sessions, grey bar = modelled distractors presented on licking day, blue bars show distraction test days with the main distraction test followed by two habituation days. Pink and dark purple bars show saline and amphetamine injections days, respectively, where the distraction test was repeated following these injections. Circles show individual rat data and bars show means.

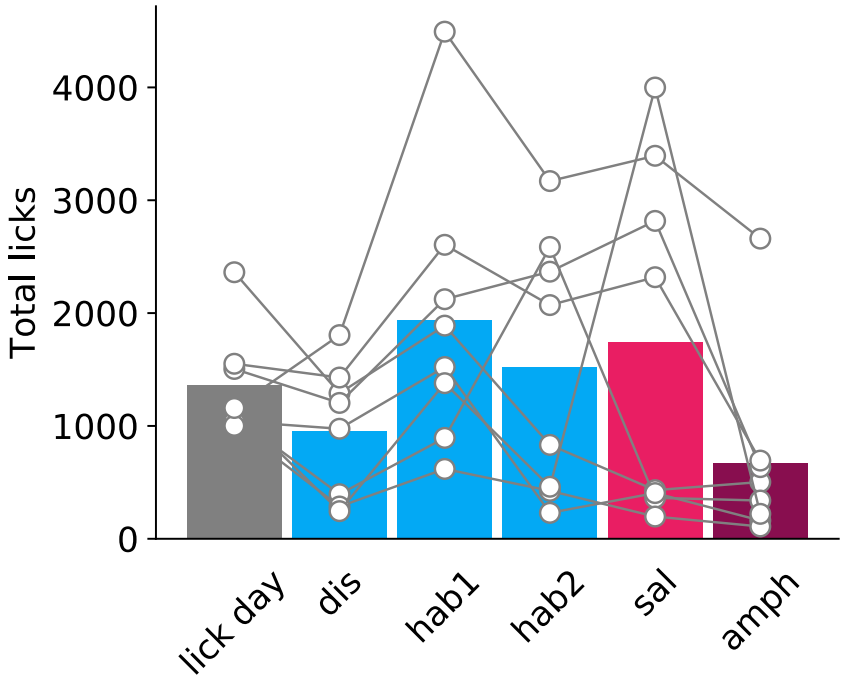


Figure 2.7 – *Total licks across experimental days*

Total number of licks within one hour sessions across test days, lick training day (grey), distraction days (blue), saline injection (pink) and amphetamine (purple). Circles show individual rat data and bars show means. Amphetamine injection did not significantly reduce licking compared to the main distraction test session.

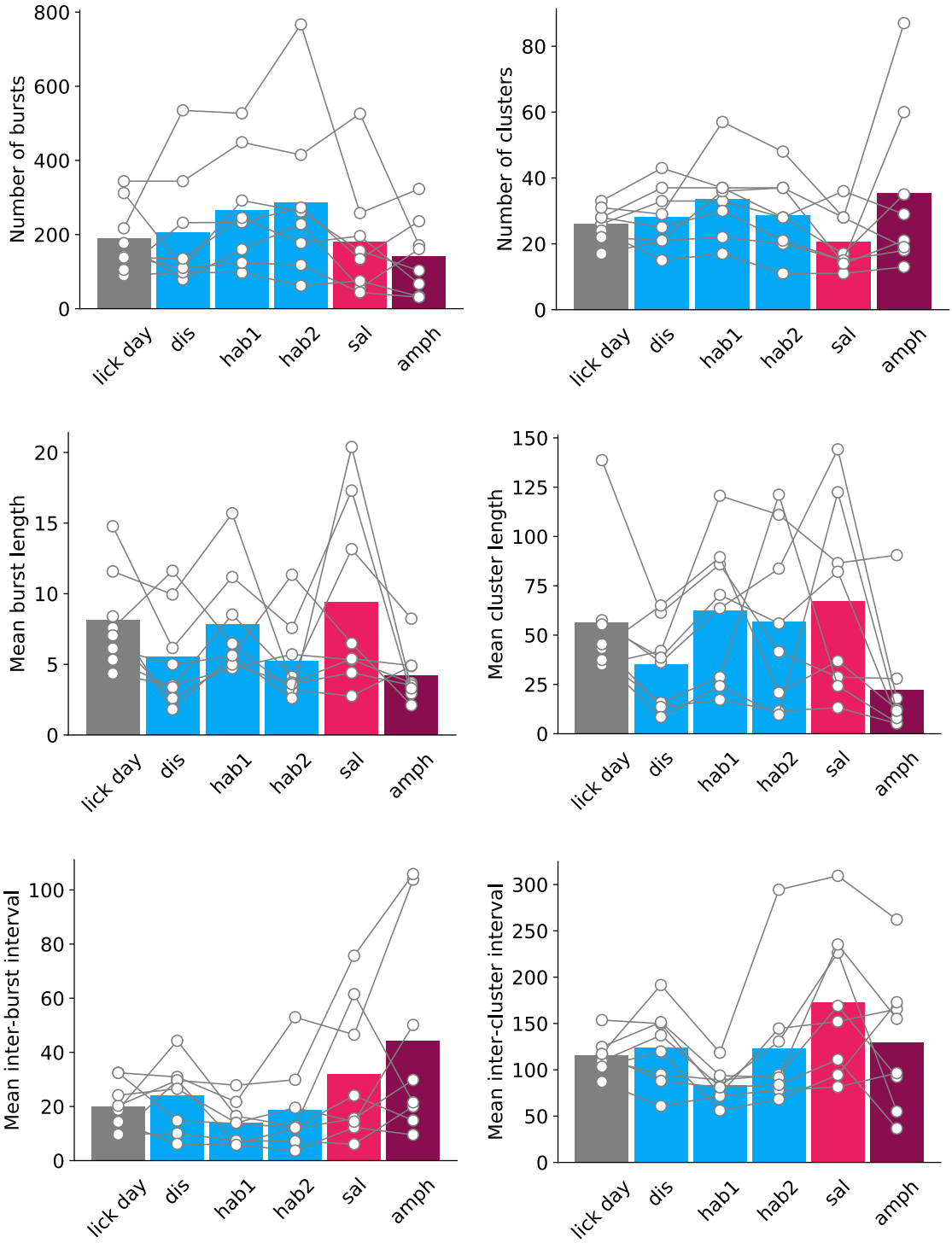


Figure 2.8 – *Lick microstructure measures across experimental sessions*

Total number of licks within one hour sessions across test days, lick training day (grey), distraction days (blue), saline injection (pink) and amphetamine (purple). Circles show individual rat data and bars show means. Amphetamine injection did not significantly reduce licking compared to the main distraction test session.

2.4.3 Characterising post distractor pauses

A second index of distraction was the post distractor pause (PDP), this is a continuous measure and was calculated as the pause, in seconds, from the onset of the distractor to the next lick. For each rat and session, PDPs were calculated. **Figure 2.9** shows cumulative plots of PDPs on modelled and distraction days. These plots show differences between lick training days and distraction days **(Figure 2.9 a-c)**. During lick training there is a steep slope reflecting many short pauses. The distraction test day cumulative plot shows a flatter curve with an inflection point, demonstrating increased numbers of longer pauses in the presence of distractors and showing a qualitative difference in behaviour during this day. **Figure 2.9d** shows PDPs split by distracted and not distracted.

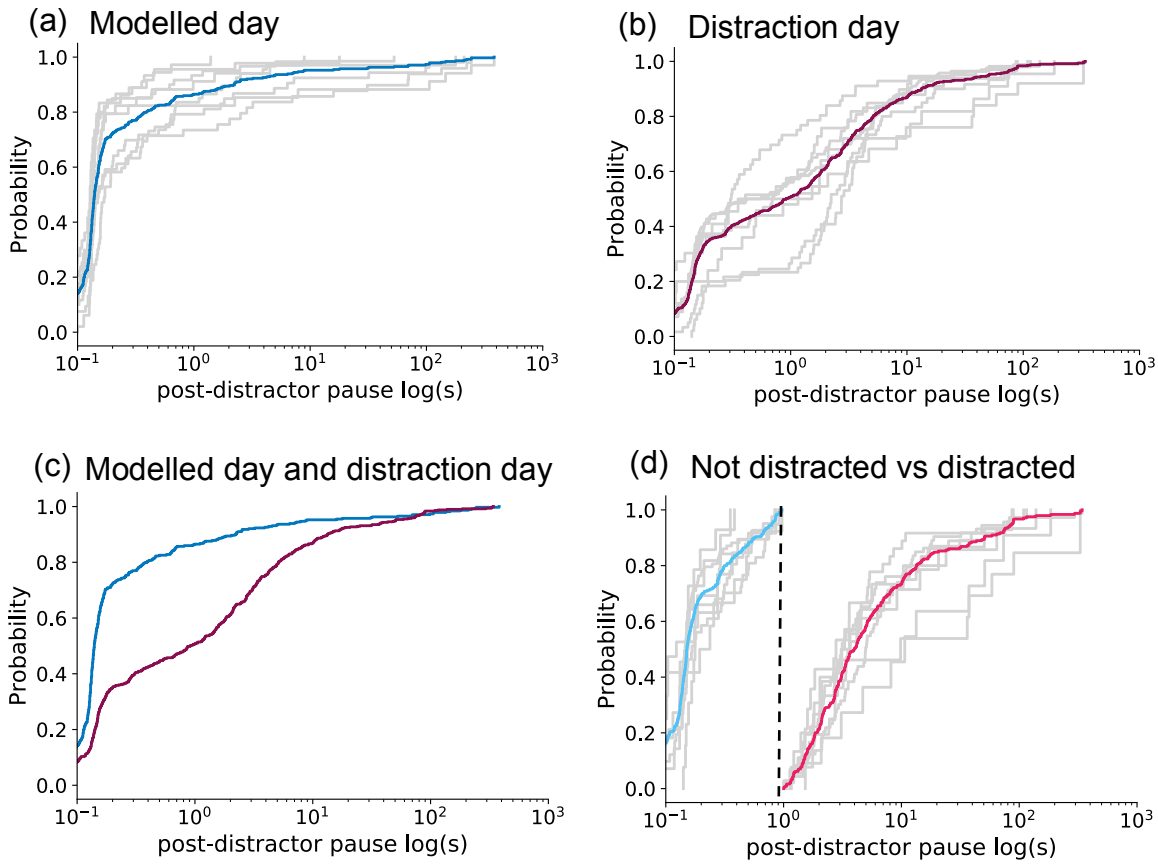


Figure 2.9 – *Cumulative plots for post distractor pauses*

Cumulative probability (or normalised cumulative frequency) of post-distractor pauses, plots are shown. Light grey lines on all plots show individual rats and darker lines represent the mean of all rats. (a) Modelled day, with calculated post distractor pauses (b) Distraction day, with real post distractor pauses (c) Modelled day and distraction day shown together, there are more short pauses observed on the modelled day (blue) than on distraction day (dark purple) and (d) Means plotted in blue for non-distracted trials and pink for distracted, dashed line shows the boundary of 1 second which classifies a pause as distracted.

2.4.4 Habituation effects on distraction day

To assess whether animals habituated to distractor stimuli within the main distraction test session, linear regressions were performed. There was no significant relationship between post distractor pause length and the time in session of the distractor (r^2 = 0.003, p = .23, **Figure 2.10a**).

There was a small but significant relationship between post distraction pause and the number of the distractor in session – that is the absolute number of the distractor presentation rather than when it occurs in the session (r^2 = 0.016, p < .01). With increasing presentations of distractors there is reduced distraction as measured by longer PDPs. However, this R^2 value is very low (around 1%) and represents a very small change in response over the session (**Figure 2.10b)**.

In addition, we assessed whether percent distracted changed across the session (ie. are rats less distracted towards the end rather than the beginning of the test session when they have received more distractors). Percent distracted was calculated for four time bins (15 minute periods across the 1 hour session, **Figure 2.11**). One way repeated measures ANOVA revealed no significant effect of time in session ($F(3,12) = 0.578$, p = .64). There appears to be little evidence of habituation in pausing responses to distractors within a single distraction test session.

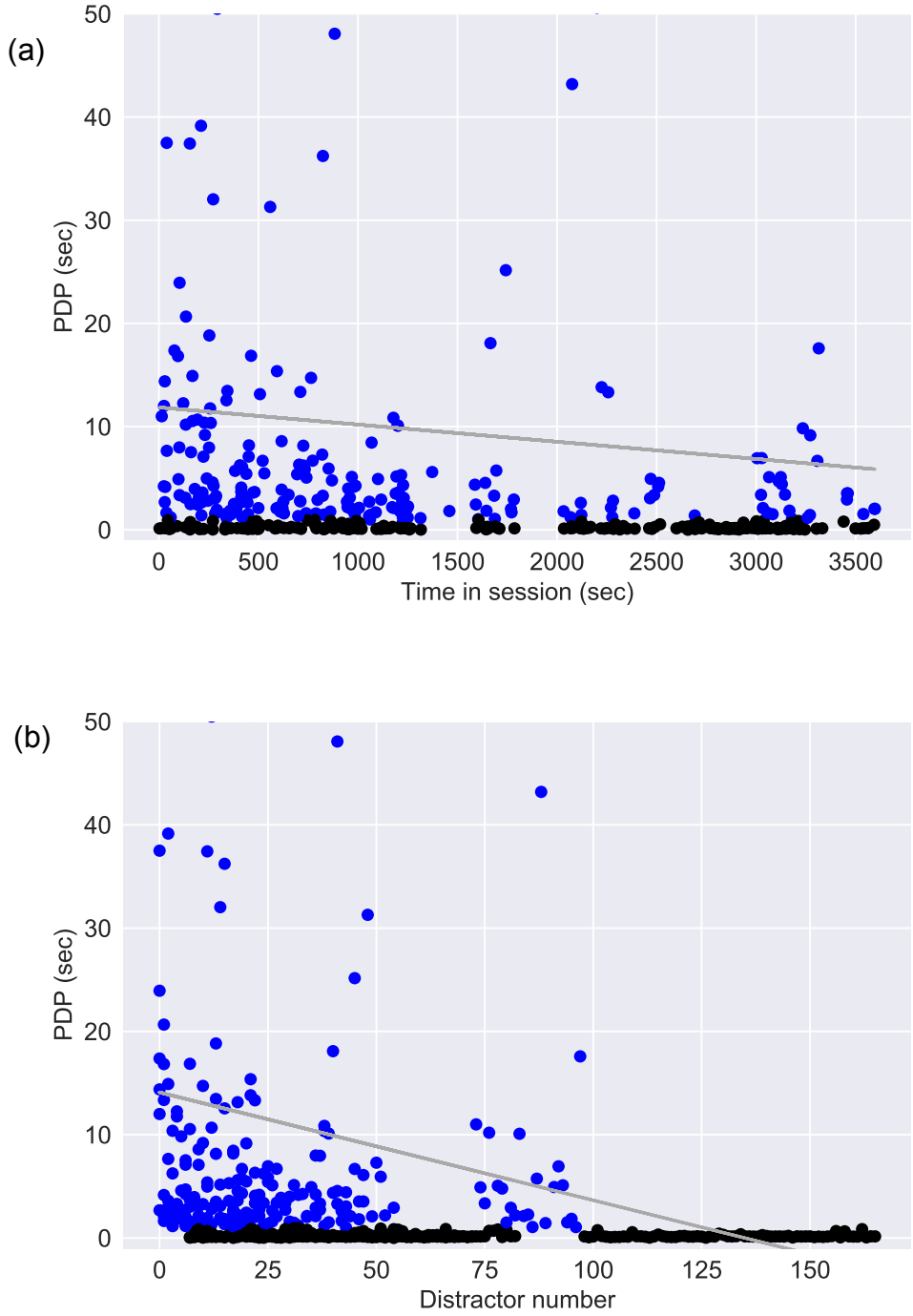


Figure 2.10 – *Linear regressions for post distractor pauses and distractor presentations*

(a) Scatter plot showing PDPs against time in session of distractor, line of fit is shown in grey with distracted trials as blue circles and not distracted in black **(b)** Scatter showing PDPs against distractor number, line of fit (grey), distracted trials (blue) and not distracted trials (black).

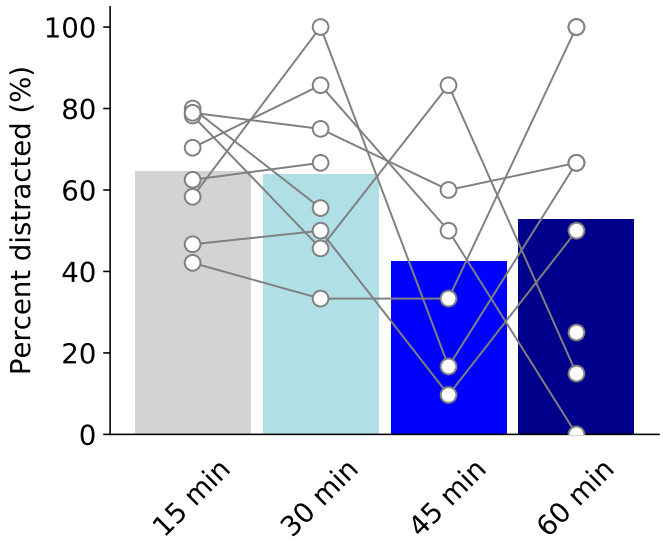


Figure 2.11 – *Percent distracted by time bin in the distraction test*

Percent distracted on the main distraction test day separated by time bins of 15 minutes. Bars show percent distracted in each 15 minute period, the first quarter of the session (grey), second quarter (light blue), third quarter (medium blue) and forth quarter (dark blue). For percent distracted measure there is no habituation effect during this session.

2.4.5 White noise containing distractors are more distracting

During behavioural testing it was noted that white noise seemed to be an especially effective distractor. There were six distractor types which were split into three groupings:

White noise = (1) white noise + house light, (2) white noise + cue light

Tone = (1) tone + cue light, (2) tone + house light

Combined = (1) white noise + tone + house light, (2) white noise + tone + cue

Percent distracted for the three different distractor groupings was assessed. One way ANOVA showed that there was a significant main effect of distractor type ($F(2,14) = 6.19$, $p < .05$). Different distractors were more or less distracting based on their modalities. A subsequent t-test comparing all distractors containing white noise (M = 57%, SEM = 6%) and those not containing white noise (M = 45.8%, SEM = 6%) showed a significant difference ($t(7) = 2.85$, $p < .05$). White noise appears to be more distracting than other distractor types.

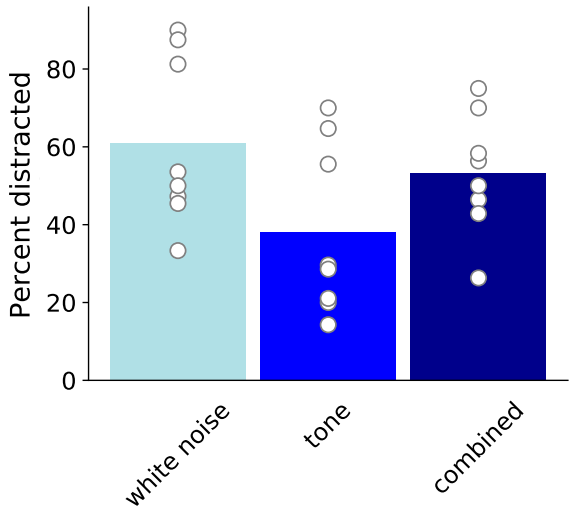


Figure 2.12 – *Percent distracted by distractor type*

Percent distracted on the main distraction test day separated by distractor type. Trials with white noise distractor (light blue), tone (blue) and combined (dark blue) are shown. Bars represent the mean of all rats and circles show individual rats.

2.5 DISCUSSION

This initial pilot experiment has demonstrated that it is possible to assess distraction in rats by exploiting self-paced licking for saccharin. All animals readily licked for saccharin and reached criteria of 1000 licks within a session in less than 7 days. Rats showed characteristic patterns of lick microstructure as described by Davis and Smith (1992), exhibiting bursts and clusters with a typical licking rate of between 6-7 Hz as expected.

Rats were robustly distracted by external stimuli, they paused following distractors on over half of trials. This pausing behaviour was not observed on the modelled day (where no real distractors were presented). Thus, distraction measured here (expressed as percent distracted) was not simply an artefact of normal licking behaviour. All rats showed initially high levels of distraction, there was little evidence of habituation within the first test session, however there was a habituation (decrease in distraction) over multiple test sessions. In most cases percent distracted returned to a baseline non-distracted level (similar to that seen on the modelled day) within two test sessions. Interestingly, we also observed that white noise proved to be more distracting than other stimuli. Perhaps this sharp burst of noise was more effective in capturing attention, it could be more salient than other distractors.

Cumulative PDP plots seem to show interesting differences in the lengths of post distractor pauses on distraction days when compared with lick training, with much longer pauses indicative of distraction, which parallels higher percentages distracted. In addition, the distribution becomes bimodal suggesting that the pause on distracted trials is fundamentally different from the pauses seen during regular lick runs. We observe similar licking microstructure and pausing in response to distractors that O'Connor et al (2015) found. However we additionally calculated the percent distracted measure and quantified distraction in this pilot study.

There was no effect of saline injection on percent distraction suggesting that non-specific effects of restraint or injection stress were not seen. Amphetamine

appears to increase percent distracted in this task however this effect was not statistically significant. It was predicted that increasing catecholamine transmission would lead to an increase in distraction as measured by a reversal of the habitation effect. However, in this initial pilot experiment with a small sample size this effect was not significant.

It is of note that there is considerable variability in the number of licks for saccharin between rats during lick training and in the overall percent distracted between rats. This variability may be related to the individual rat's preference for saccharin. Perhaps some rats find saccharin more palatable than others. It would be interesting to assess whether the palatability of saccharin correlates with the percent distracted. We might presume that it would be easier to distract an animal for whom the taste of saccharin is less reinforcing.

We chose saccharin as the solution for rats to consume in this task due to its sweet taste but lack of caloric value. Rats will consume saccharin freely, however, it does not have the same satiating effects as sugars and is perhaps less rewarding (Beeler et al., 2012). Further experiments could assess the effect of different tastants, particularly sucrose instead of saccharin. One might predict that sucrose would result in lower percent distracted as it has greater reinforcing properties and rats will be more motivated to continue licking in the face of distractors. In addition, we may see a change in the percent distracted across session as rats become sated with sucrose which would be interesting to investigate. We could also assess the effects of different internal need states by testing rats in sated and food restricted states. This could assess the competing drives of hunger and the need to attend to potentially important stimuli in the environment using this task. Finally, this paradigm could be used to assess the impact of anxiety or stress on attention to distractors during consumption. We could use brightly lit arenas or modify anxiety and assess the percent distracted.

Now that we have characterised licking microstructure and responses to distractors, the following chapter we will use this assay to investigate levels of distraction in a model of schizophrenia. We will use subchronic phencyclidine

treatment to model cognitive deficits in schizophrenia and assess distraction using this task. Key attentional impairments seen in this model may be underlined by increased distractibility. Chapter 3 will present data from both male and female rats which have undergone subchronic PCP treatment and which have been tested using the assay developed in this pilot study.

CHAPTER 3

Distraction from ongoing saccharin consumption in saline and phencyclidine pre-treated rats

3.1 INTRODUCTION

3.1.1 Schizophrenia background

Schizophrenia is a severe mental illness with a prevalence of between 0.5 and 1% (DSM-IV-TR, Bhugra, 2005). Despite being relatively uncommon it can be debilitating for patients and families and is one of the top 15 leading causes of disability worldwide (Vos et al., 2017). Half of those with a diagnosis of schizophrenia also have co-occurring mental health problems such as depression, suicidal thoughts and anxiety disorders and it is thus a significant burden to health. Schizophrenia is characterised by symptoms in three domains, positive, negative and cognitive. Positive symptoms include hallucinations (which are primarily auditory) and delusions, unusual perceptions and interpretations of events and surroundings. Negative symptoms manifest as flattened affect, social withdrawal and thought disorder. Finally, cognitive symptoms are expressed as significant impairments in working memory, attention, motivation and learning (Pearlson, 2000; Carter & Barch, 1999). Current antipsychotic drugs are reasonably effective in treating positive symptoms, although even then around 40% of patients do not respond well to treatment (Mletzer, 1997). These drugs are poor in alleviating negative symptoms and show no efficacy for cognitive symptoms, in some cases worsening these deficits (Keefe et al, 2007; Harvey and McClure, 2006). Even in those who do show improvement in symptoms, severe side effects of the drugs often reduce compliance.

3.1.2 Pharmacological treatments for schizophrenia

Pharmacological therapies for schizophrenia were first developed in the 1950's with the discovery that drugs, which we now know to be dopamine D2 receptor antagonists, alleviated symptoms in psychosis. These so called 'first generation' or 'typical antipsychotics' such as haloperidol and chlorpromazine are effective in treating positive symptoms but produce significant and severe side effects, with particularly debilitating motor impairments including extrapyramidal side effects caused by off target effects (Arana, 2000). The development of second

generation or atypical antipsychotics led to drugs such as quetiapine, olanzapine and aripriprazole which are now more commonly prescribed. These drugs have more complex pharmacology, acting at multiple sites beyond the dopamine D2 receptor. However, this does not equate to increased efficacy, in most cases these modern drugs are no more efficacious than typical antipsychotics (Stroup et al, 2000). They do, however show better side effect profiles which can greatly improve patient quality of life and treatment adherence (Stroup et al., 2000).

Despite considerable research effort, there have been no new drug treatments for psychosis and schizophrenia that have improved upon those currently available. Clozapine is one possible exception here. There is some evidence that clozapine is more effective than other drugs in treating cognitive symptoms (McEvoy et al., 2006). However, the use of clozapine is tightly regulated due to its potentially life threatening side effects of neutropenia, agranulocytosis and severe cardiac problems. It is administered in a clinic and requires overnight supervision and there is no long term, depot administration available. Because of this, clozapine is typically only prescribed in treatment resistant patients who have exhausted other options. Clozapine has a rich pharmacology, acting at a wide range of receptors including D2, D4, 5-HT and histamine. There have been several claims that it's efficacy comes from the high affinity for dopamine D4 receptors, however, there is little evidence that D4 antagonists are therapeutically relevant and treatments for cognitive symptoms in particular remain elusive.

3.1.3 Cognitive impairment associated with schizophrenia

Cognitive symptoms, also termed cognitive impairments associated with schizophrenia (CIAS) are ubiquitous, occurring in as many as 75% of those with a diagnosis of schizophrenia (O'Carroll, 2000). CIAS are arguably the most enduring and debilitating symptoms and persist, impacting patients' daily lives, even where positive symptoms are well managed (O'Carroll, 2000). Cognitive symptoms are central to diagnosis of schizophrenia and represent a substantial unmet clinical need (Goldberg, David & Gold, 1995). These symptoms may be

present before diagnosis and can precede the positive and negative symptoms of the disease: they are therefore an important area to investigate the pathophysiology of the condition (Tandon et al. 2009). Impairments in memory, attention and other aspects of cognition are also present in at high risk individuals and do not appear to be simply confounds of antipsychotic medication (Lewis, 2004; Whyte et al., 2006; Niemi et al, 2003) and in first-degree relatives of patients, without a diagnosis of schizophrenia themselves suggesting a potential genetic component (Snitz et al 2006, Keefe and Fenton, 2007).

Deficits in neurocognitive function are among the most important and significant predictors of functional outcomes (Green et al., 2004). Cognitive symptoms impact individuals in all domains, affecting school, work, daily life and the ability to maintain relationships with others. Greater instances of cognitive symptoms correlate with reduced quality of life, and long term disability (Addington et al, 2000; Insel, 2010). Cognitive symptoms often outlast positive and negative symptoms which can be managed with antipsychotic drugs to some degree. Modern atypical antipsychotics fail to consistently improve cognitive symptoms (Lieberman et al.,2005; Keefe et al.,2007) and our understanding of the neural underpinnings of these deficits is poor.

3.1.4 Attention and distraction in schizophrenia

Cognitive deficits take many forms: in schizophrenia there is evidence of significant attentional dysfunction in particular. Deficits in selective and sustained attention are consistently reported (Filbey et al., 2008; Mathalon et al., 2004; Morris et al., 2012). Those with schizophrenia also show an increased propensity to become distracted by salient but irrelevant stimuli in their environment (Leonard et al., 2014).

An example of increased distraction effects can be seen in visual search paradigms, where participants must locate a target shape on a computer screen in a field of other shapes. The task is made more difficult by the inclusion of an

attention grabbing, salient visual distractor. The detrimental effect of the visual distractors is greater in schizophrenia, profoundly slowing search times (Bacon and Egeth, 1994; Leonard et al., 2014). EEG studies have also found significant deficits in auditory processing in patients (Gaebler et al., 2015). Alterations in auditory discrimination are observed, with patients showing a deficit in mismatch negativity – a negative EEG component that is seen when mismatched tone is presented during a string of otherwise similar tones. This mismatch negativity (MMN) can function to inactivate visual areas during auditory tasks and is important in directing attention to the task. This reduced MMN may prevent the normal inactivation of visual brain areas during auditory tasks (Gaebler et al., 2015) and affect the processing of other environmental stimuli disrupting how attention is allocated (Gaebler et al., 2015; Javitt and Sweet, 2015). These multi-modality sensory deficits may explain the tendency to distraction seen in patients.

Processing of environmental stimuli is crucial in informing goal-directed behaviours. To respond appropriately to environmental cues we must attend to important and relevant stimuli while filtering out inconsequential and irrelevant distractions. An excess attention to irrelevant stimuli likely underlies some of the significant cognitive deficits and impairments in learning and memory seen in schizophrenia and is an important aspect of the disease to consider in animal models. A greater understanding of the neurochemical or biological basis of such abnormalities will help to improve therapies as research needs to identify a target for pharmacotherapies.

3.1.5 Dopamine and schizophrenia

Dopamine and schizophrenia have been extensively linked and dopamine dysfunction has become synonymous with schizophrenia. The dopamine theory was posited in the 70's (Carlsson, 1988; Carlsson, 1974) and remains centrally important to understanding symptoms of schizophrenia. However, the origin of dopaminergic changes in schizophrenia, their causal or symptomatic role and the different brain regions involved remains a topic of considerable research and debate

Evidence to support a key role of dopamine in schizophrenia comes from the observation that chlorpromazine produced surprisingly beneficial effects on symptoms in schizophrenia (Ban, 2007) and it was later discovered that this was due to its affinity for the dopamine D2 receptor (Howes & Kapur, 2009; Miyamoto et al., 2012). Carlsson and Lindqvit (1963) identified that antipsychotic drugs increase dopamine metabolism and this serendipitous discovery led to the development of antipsychotic medications based on dopamine D2 antagonism. The binding co-efficient of antipsychotic drugs for the D2 receptor is directly correlated with their clinical efficacy (Kapur & Mamo, 2003). Alongside the discovery of the therapeutic potential of D2 antagonist drugs the effects of psychostimulants, which potentiate dopamine transmission, also implicated dopamine in psychosis. Drugs in this class, such as amphetamine, have the ability to induce psychotic-like symptoms in healthy individuals and worsen symptoms in patients (Lieberman, Sheitman & Kinon,1997).

The ability of antipsychotic drugs to treat psychosis develops over repeated use and this may be due to the progressive inactivation of the mesolimbic dopamine neurons via depolarization block (Grace, Bunney, Moore & Todd, 1997). The poor efficacy of such drugs for cognitive and negative symptoms may be partly caused by reduced responsivity in forebrain dopamine systems which correlates with improvements in positive symptoms (Grace, 1992). Depolarization block not only alters phasic dopamine release but also affects tonic dopamine pools in the striatum which may worsen cognitive and negative symptoms (Moore, West & Grace., 1999).

Although the dopamine theory is well established, it is clear that it cannot account for the negative or cognitive symptoms of schizophrenia (Thaker & Carpenter, 2001). There is likely a complex interaction between dopamine systems in different brain regions, rather than a simple whole brain overabundance of dopamine. For example, human studies of dopamine metabolites show mixed results with some showing elevations in dopamine function and others showing no changes or even reductions (Howes & Kapur,

2009). Hypofrontality is seen, with decreased dopamine in the frontal cortex (Hill et al., 2004). Reduced dopamine activity in the cortex, which normally has an inhibitory action on subcortical areas, leads to disinhibition of dopaminergic transmission in the mesolimbic pathway and consequent elevated subcortical dopamine transmission (Howes & Kapur, 2009). There seems to be regionally specific prefrontal hypodopaminergia followed by a reactionary subcortical hyperdopaminergia. The origin of hypofrontality and its downstream consequences is unknown but likely involves multiple transmitter systems. Thus, current antipsychotic drugs may not be targeting the primary abnormality in schizophrenia, but rather they act on the downstream consequences of some other, fundamental dysfunction.

3.1.6 Glutamate and schizophrenia

In addition to the dopamine hypothesis there is an emerging understanding of the role of glutamate in the pathology of schizophrenia (Carlsson et al.,1997; Olney,1999). Evidence for glutamate involvement comes from the observed effects of dissociative anaesthetics such as phencyclidine (PCP) and ketamine. These non-competitive glutamate NMDA receptor antagonists have psychotomimetic properties (Luby et al., 1959). Both acute and repeat exposure induce psychosis remarkably similar to schizophrenia – mimicking symptoms in all three domains (Javitt & Zukin,1991) – as well as evoking neurochemical and metabolic changes consistent with schizophrenia (Morris, Cochran & Pratt, 2005). PCP also exacerbates psychosis in schizophrenic patients (Lahti, Roberts & Tamminga, 1995; Malhotra et al., 1997). These effects of NMDA antagonists suggest decreased glutamate function may be important in schizophrenia. In this context, it is notable that several identified risk factor genes for schizophrenia are implicated in dysfunctional glutamate transmission (Harrison and Owen 2003). There is also some suggestion that NMDA receptor agonists – such as glycine and D-serine – may alleviate symptoms of schizophrenia, however no large scale studies have confirmed this (Kantrowitz & Javitt, 2012).

Chronic PCP administration increases dopamine release in subcortical areas, particularly the NAc (Deutch et al.,1987; Jentsch, Elsworth, Redmond & Roth,1997). Increased mesolimbic dopaminergic activity produces sensitisation to the behavioural effects of NMDA receptor antagonists (Scalzo & Holson,1992), and dopamine agonists (Lannes et al.,1991). There is thus, evidence of reciprocal modulation of dopamine and glutamate systems. The decrease in NMDA activation may be the primary abnormality which leads dopamine systems to adapt to abnormal glutamatergic transmission.

3.1.7 The subchronic phencyclidine model

Due to its psychotomimetic properties, PCP is used as a research tool to model schizophrenia in rats. Acute administration increases locomotor activity and causes decreased social interaction, behaviours which are thought to model positive and negative symptoms, respectively (Nagai et al.,2003; Sams-Dodd,1996). PCP also produces cognitive dysfunctions including impairments in reversal learning (Idris, Repeto, Neill Large, 2005) and attentional set-shifting (Egerton et al., 2005) which can be reversed by typical and atypical antipsychotics (Freed, Bing Wyatt, 1984; Kitaichi et al., 1994). These effects, however, do not persist once the drug is removed from the system and do not reflect the often chronic course of schizophrenia.

Therefore, a sub-chronic PCP regime has been developed to overcome these limitations. Rats receive subthreshold doses of PCP for 5 days (2 mg/kg) which do not produce psychomotor effects as acute doses, twice a day, followed by a drug washout period. In the sub-chronic PCP model, many of the same deficits are produced. Importantly, the animal is drug free at the time of testing. The effects of the subchronic model reflect some neurochemical, conformational changes in the brain more similar to schizophrenia (Neill et al., 2010).

3.1.8 Modelling cognitive deficits – NOR assessing cognitive deficits

There is a pressing need to further our understanding of the neural underpinnings of cognitive disturbances to develop drug strategies for treating cognitive dysfunction. Several NIMH-sponsored initiatives have stressed the importance of developing more translational models of cognitive symptoms to improve treatment (Kerns, Nuechterlein, Braver & Barch, 2008; Barch et al.,2009). The CENTRICS (Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia) and MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) initiatives have identified key domains for pre-clinical animal research including processing speed, working memory, attention and vigilance (Nuechterlein et al., 2005).

One of the key tasks developed on the back of these meetings is the novel object recognition (NOR) task. This has been used to assess cognitive deficits in the subchronic PCP model. The NOR task measures visual recognition memory and exploits the innate drive of rats to explore a novel object in their environment. Once habituated to a test arena, rats are introduced to 2 objects they have not experienced before: they are allowed to explore the objects for 3 minutes. Then the animal is removed from the arena for a short inter-trial interval – typically 60 seconds. When they are placed back they are presented with one object they have seen before which is the familiar object and a new, novel object. Normal animals show a preference towards the novel object, exploring it for significantly longer than the familiar object. On the other hand, PCP treated animals show no preference, exploring both the novel and familiar objects to a similar extent. This is interpreted as showing impairment in the recognition memory of the familiar object (Neil et al., 2010). Deficits in NOR in the subchronic PCP model have been validated in multiple strains and sexes.

3.1.9 NOR and distraction

Within the NOR test, during the ITI between presentation of familiar objects and novel, the animal is handled to be removed from the test context. PCP treated animals are susceptible to the NOR deficits when handled during the ITI, by a change in context during the ITI and by the presentation of another unfamiliar (distracting) object during the ITI (Grayson et al., 2014). These manipulations during the ITI do not affect non-treated animals' ability to distinguish familiar from novel. Only if left totally undisturbed in the same arena during the ITI do PCP animals become able to discriminate the objects and show a diminished memory impairment. These manipulations demonstrate that memory acquisition in PCP is intact, however, PCP animals are easily distracted by external stimuli and they appear to encode irrelevant information during the ITI, which disrupts the labile memory of the familiar object. This raises the possibility that the subchronic PCP model may induce increased distractibility, which could underlie the deficits seen in NOR and potentially in other tasks of learning, memory and attention used to assess cognitive deficits. Since distraction is a key deficit seen in human patients, it is worthy of further study.

Despite the strive for better translational models of cognitive deficits, such tasks have yielded disappointing results in terms of the development of novel drug targets (Young & Geyer, 2015). The development of alternative tasks in rodents to measure cognitive deficits, and their component attentional, memory and learning related underpinnings, is essential to further our understanding of the neurobiological basis of these deficits in multiple disorders, not just schizophrenia. The contribution of distraction or distractibility to many cognitive functions which may be abnormal in multiple disorders of motivation including schizophrenia, ADHD and drug addiction makes this a particularly worthwhile concept to investigate further.

Taken together this literature provides rationale for the study of distraction in the subchronic PCP model. We aim to investigate whether an increased propensity to distraction is an underlying deficit, which may explain previous findings in the NOR literature. The following chapter assessed distraction within this model

using a novel assay (pilot study described in Chapter 2) to investigate potential differences in the pausing of ongoing licking in response to external stimuli. In addition, we use the novel object recognition (NOR) test to assess visual recognition memory. We present data from both male and female cohorts and discuss implications for models of schizophrenia.

3.2 AIMS, OBJECTIVES & HYPOTHESES

3.2.1 Aims

1) Characterise microstructure of licking in subchronic PCP treated and saline control rats
2) Assess the effects of subchronic PCP treatment on distractibility using a self-paced licking assay
3) Assess distraction in both male and female Sprague Dawley rats after subchronic PCP treatment

3.2.2 Objectives

1) Determine that PCP treatment does not impair licking and measure microstructure in PCP and saline treated rats
2) Use the distraction assay developed in pilot experiments to quantify and compare the levels of distraction in PCP and saline treated rats
3) Validate subchronic PCP treatment using NOR in male and female Sprague Dawley rats
4) Assess NOR and distraction in both male and female rats following PCP treatment to investigate possible sex differences

3.2.3 Hypotheses

1) Saline and PCP treated animals will show similar licking microstructure during lick training when tested after drug washout
2) Subchronic PCP treatment will significantly increase distractibility and PCP treated animals will show slower habituation to distractors
3) Subchronic PCP animals will show a deficit in NOR
4) PCP animals will be more sensitive to the effects of amphetamine on distraction

3.3 METHOD

3.3.1 Animals

Three cohorts of Sprague Dawley rats (250 – 300g) were used in experiments testing the effect of PCP on distraction. An initial cohort of 16 males was used and this experiment was then repeated using a second cohort of 16: these were combined to give a total group of 32, tested over two replicates. To assess potential sex differences a final, third cohort of 24 females were tested in a repeat of these experiments. All rats were supplied by Charles River, UK and were housed in groups of 4 in individually ventilated cages under temperature controlled conditions (21°C ± 2°C; 40-50% humidity). Animals were kept under 12 hour light/dark cycle with lights on at 7am. Rats had access to food and water ad libitum, except for a period of overnight food restriction before the first lick training session and during experimental sessions where only saccharin (0.2 %) was available. All procedures were carried out under the appropriate license authority in accordance with the Animals (Scientific Procedures) act (1986). Project licence for PCP experiments PPL: 60/4390, personal licence PLI: I9202C6A3.

3.3.2 Phencyclidine (PCP) pre-treatment

Rats were randomly allocated to either saline or PCP treatment groups. Rats were housed in groups of four for the entire duration of the pre-treatment and behavioural experiment, and in each cage 2 animals received saline vehicle injections and 2 animals received PCP treatment. All rats received intraperitoneal (i.p.) injections twice daily at 8 am and 4 pm for 5 consecutive days. Males were dosed with 5 mg/kg PCP dissolved in saline to 1 ml/kg or with saline vehicle (0.9% as 1 ml/kg). Females received a lower dose (2 mg/kg dissolved in saline to 1 ml/kg) or saline vehicle as males (0.9% as 1 mg/kg). PCP hydrochloride was purchased from Sigma Aldrich (UK) and doses were calculated using the molecular weight of the salt. Following 5 consecutive days of injections all rats were given at least 7 days washout, during which they received no drug or vehicle injections.

3.3.3 Distraction behaviour testing

The distraction procedure is described in detail previously (**Chapter 2**). Briefly, following 5 day PCP or saline pre-treatment and 7 day washout rats were trained to lick for saccharin (0.2 %) daily for three days (1 hour session per day). Following saccharin training, rats were tested for distraction and the effects of repeated testing were assessed. **Figure 3.1** shows a schematic of the full experimental schedule. Rats were trained and tested in the same MedAssociates apparatus and licking and distraction were recorded. During distraction testing three licks bursts within 1 second triggered a distractor stimulus, as previously described. Following initial pilot experiments the MEDPC programme was modified to not only record all licks but to also record the onset and offset of each lick, this allowed for measurement of the length of each lick and helped to eliminate any anomalous measurements of licking caused by contact between the spout and the paws or shorting from liquid bridges. The distraction test was repeated for three days (distraction, habituation1, habituation2). To assess the effects of amphetamine, a further test session was carried out. Amphetamine was administered to all animals on this day, as a single i.p. injection (1 mg/kg as 1 mg/ml dissolved in saline vehicle 0.9%) 15 minutes prior to the session.

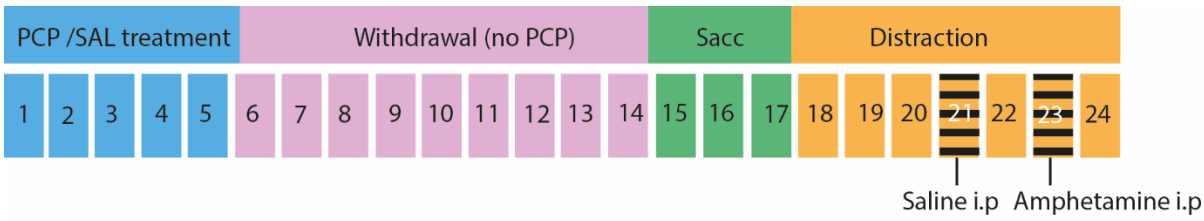


Figure 3.1 – *Schematic of experimental schedule*

Rats were pre-treated with either PCP (either 2 mg/kg in females or 5 mg/kg males) or saline vehicle (0.9 %) for 5 consecutive days followed by a drug free period (days 6-14). Saccharin training followed for three days (green) where rats were trained for 1 hour per day. The distraction test was then given on day 18, and repeated on consecutive days 19-24. Two drug injection days are included: on day 21, 15 minutes before the distraction test, animals received a i.p. saline injection; on day 23, 15 minutes prior to the distraction session, rats received amphetamine (1 mg/kg, i.p.).

3.3.4 Novel object recognition

Following the final distraction test session rats were habituated to the NOR test apparatus. They were placed in a large black Plexiglass arena (60 cm x 60 cm x 60 cm) for 20 minutes on two consecutive days to habituate them to the test context. Rats were placed in the arena in groups of 4 (by cage) and allowed to explore freely. On the following day NOR testing was conducted (**Figure 3.2**). The animals were placed in the arena individually, and tested as follows: 3 minute habituation period with no objects present, 1 minute inter-trial interval (ITI) in a second distinct box (a large yellow container, 40 cm x 40 cm x 40 cm), 3 minutes acquisition with two identical objects placed in the corners of the arena, 1 minute ITI and finally the retention test where the novel object was in one corner and a third identical familiar object was placed. Novel object placement was counterbalanced across animals. Familiar objects were standard sized tin cans (7.3 cm diameter, 10.6 cm height), the novel object was an upturned glass container (10 cm diameter, 6 cm height)

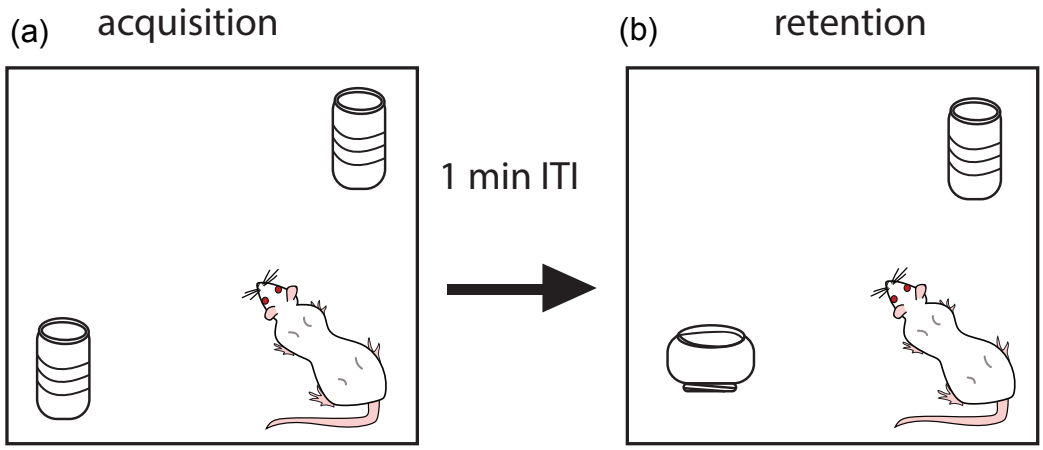


Figure 3.2 – *Novel object recognition procedure*

Following an initial habituation period of 3 mins where the rat is placed in the empty arena, there follows an acquisition trial **(a)** the rat is placed in one corner of an arena, during acquisition two identical objects are present (tin cans) the animal is allowed 3 mins to explore the objects before a 1 min ITI (the rat is removed and placed in a holding box). **(b)** Following acquisition and ITI the rat is placed back into the arena with a third identical object to the familiar objects (tin can) and a new, novel object which they have never experienced (upturned glass dish), again they are given 3 mins to explore the objects within the arena.

3.3.5 NOR scoring

During the NOR procedure acquisition and retention trials were video recorded (Logitech Webcam, C920) and all videos were manually scored by two independent experimenters blinded to the treatment conditions. Exploration times of each object were measured during acquisition (left and right) and retention (familiar and novel) using stopwatches and total exploration time was recorded. Exploration was classified as follows: Exploration of an object was defined as rats sniffing, licking or touching the objects with forepaws whilst sniffing at the object but not leaning against, or standing or sitting on the object (Grayson et al, 2007).

Discrimination index was calculated as follows (Grayson et al 2007):

$$DI = \frac{(\text{Time spent exploring novel object} - \text{Time spent exploring familiar object})}{(\text{Time spent exploring novel object} + \text{Time spent exploring familiar object})}$$

3.3.6 Data analysis

All data were initially analysed using Python 3.6 to identify licking timestamps and distractor presentations. Lick microstructure measures, percent distracted and post distraction pauses were calculated from these timestamps and modelled distractors were calculated (as per Chapter 2). Custom written functions in Python 3.6 were used to subset the data into drug treatment conditions and days and to calculate variables for statistical analysis. For NOR scoring videos were manually scored by two blinded experimenters and to assess locomotor activity ANYMAZE tracking software was used. For hypothesis testing, SPSS 24 (IBM) was used. Analysis of Variance (ANOVA) and t-tests were performed and all data are represented as means. Post hoc comparisons were Bonferroni corrected for multiple comparisons and adjusted p values are reported. Assumptions of normality, homogeneity of variance and sphericity were tested using Shapiro WIlks, Levene's, and Maulchly's tests, respectively. All assumptions were met unless otherwise stated. In the case of violated assumptions corrected tests or adjusted degrees of freedom were used

or if appropriate data were transformed where necessary: this is reported in individual results sections where applied. Alpha was set at .05 and tests were two tailed unless otherwise stated.

3.4 RESULTS

3.4.1 Effects of PCP pretreatment in male rats

3.4.1.1 No differences in lick microstructure between PCP and saline rats during saccharin training

First, to assess whether overall lick microstructure was affected by PCP pretreatment, licking for saccharin on lick training days was compared between saline and PCP-treated rats. **Figure 3.3** shows licking in the three days preceding the distraction test for saline and PCP treated rats. On the final day of lick training saline rats licked a means of 2318 licks (SEM = 275 licks) and PCP rats a mean of 1628 licks (SEM = 245 licks).

A 3 X 2 mixed ANOVA was used to assess whether saline and PCP treated rats showed different rates of licking for saccharin across three training days in male rats. The number of licks within a session was the dependent variable and this was compared across training days and between drug treatment groups. The within subjects factor was day (with three consecutive lick training days, expressed as days before distraction test, -3, -2, -1) and the between subjects factor was drug treatment (PCP or saline).

Licking across saccharin training days was similar in PCP and saline treated animals. There was a significant main effect of day ($F(2,60) = 13.864$, $p < .001$). Bonferroni corrected post-hoc t-tests showed that this difference was due to significantly more licking on day 2 (M = 1499 licks) compared to day 1 (M = 933 licks, $p < .01$). Rats also licked significantly more on day 3 (M = 1972), compared to day 1 ($p < .001$). There was no significant difference between licking on days 2 and 3 ($p = .119$). Licking steadily increased across the three training days reaching a stable rate by day 3, the day preceding distraction testing. There was no significant main effect of treatment ($F(1,30) = 2.164$, $p = .152$) and there was no significant interaction between day and treatment ($F(2,60) = 1.219$, $p = .303$).

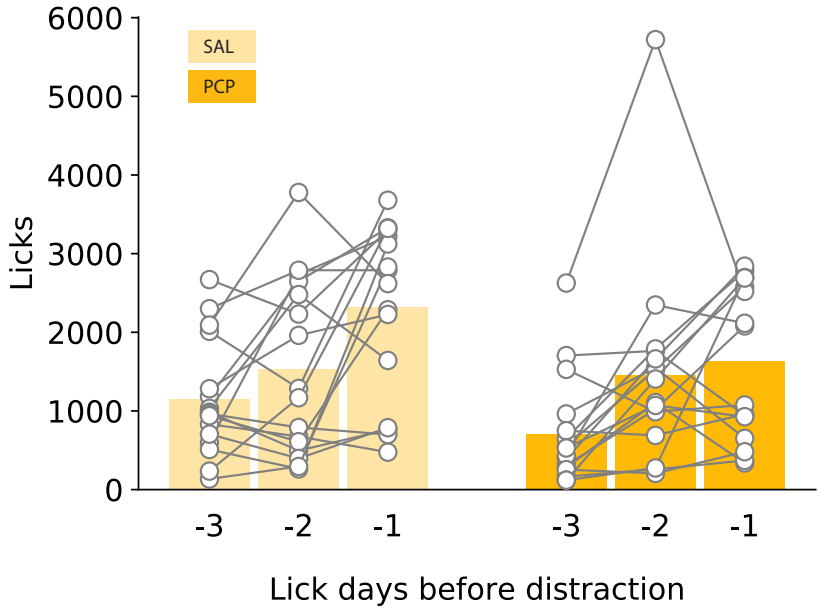


Figure 3.3 – *Number of licks within training sessions in saline and PCP treated male rats*

Figure shows the total number of licks across training days of rats licking for saccharin in 1 hour sessions. Three days preceding the distraction test day (days -3 to -1). Light bars show means for saline treated rats and darker bars means for PCP rats. Circle represent individual rats.

3.4.1.2 Licking microstructure is not significantly different between PCP and saline pretreated rats

To rule out differences in licking microstructure between saline and PCP treated male rats, a series of t-tests were performed on data from the last saccharin training day using three key measures of licking behaviour, namely, total number of licks; licks per cluster, and number of lick clusters (Davis and Smith, 1992). **Figure 3.4** shows means for licking (**Figure 3.4a**), burst length (**Figure 3.4.b**) and the total number of bursts (**Figure 3.4c**) in saline and PCP treated males.

Firstly, to compare consumption of saccharin the total number of licks on the last saccharin training day was compared for saline (M = 2318, SEM = 275) and PCP (M = 1627, SEM = 245) animals and no significant difference was found, $t(30) = 1.875$, $p = .071$. Secondly, to assess palatability of saccharin the mean number of licks per cluster was compared between saline (M = 9, SEM = 1.4) and PCP males (M = 11, SEM = 1.3): there was no significant difference, $t(30) = 0.817$, $p = .420$. Finally, to rule out any significant motor impairment in licking in PCP animals, the total number of bursts within the session was compared. There was no significant difference between saline (M = 283 bursts, SEM = 65.39) and PCP treated rats (M = 197 bursts, SEM = 29.91, $t(30) = 1.188$, $p = .244$). Taken together, these results show that PCP pretreatment has no effect on the overall drive to consume a sweet solution or on palatability of a sweet solution.

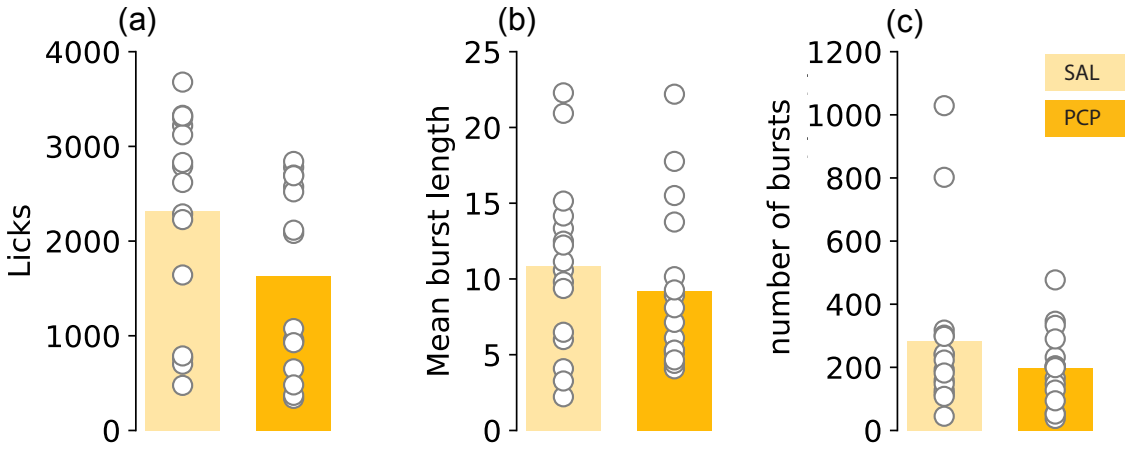


Figure 3.4 – *Lick microstructure in saline and PCP treated male rats*

Licking microstructure measures on the last lick training day, pale yellow bars represent means for saline rats and darker, orange bars show means for PCP pretreated rats. **(a)** Total licks on the final lick training day, saline and PCP animals. **(b)** Mean burst length, the average number of licks per burst, in saline and PCP males. **(c)** Number of bursts, the mean number of burst events within the last lick training session. White circles show individual rats and bars represent the mean

3.4.1.3 Percentage distracted SAL and PCP males

The percentage distracted (the number of trials where the animal paused licking in response to a distractor as a percentage of the total number of distractor presentations) was calculated on each day for each rat. Percentage distracted is shown in **Figure 3.5** for the five test days (modelled day, distraction, habituation1, habituation2 and amphetamine) for saline and PCP treated males.

To assess changes in percentage distraction across days and to investigate differences in saline and PCP treated animals, a 2 x 5 mixed ANOVA was performed. Experimental day was a within-subjects factor with five levels (modelled, distraction, habituation1, habituation2 and amphetamine), drug treatment was between-subjects (PCP or saline). There was no significant main effect of drug ($F(1,30) = 0.439$, $p = .51$). PCP pre-treatment did not significantly increase or reduce percentage distracted. There was a main effect of day ($F(2.7, 81.2) = 22.35$, $p < .001$), post hoc Bonferroni corrected t-tests were used investigate this effect of day further.

Compared to the modelled distraction day (no distractors, M= 9.13 %), rats were significantly more distracted on the distraction day (M = 35.3 %, $p < .001$) and on the amphetamine day (M = 21.18, $p < .05$). In addition, amphetamine day percent distracted was significantly lower than the main distraction test ($p < .05$) showing that although amphetamine reversed the habituation effect this did not reach the initial level of distraction in the first test day. Both habituation day 1 (M = 16.66 %) and habituation day 2 (M = 8.69 %) were not significantly different from the modelled day ($p = .12$ and $p = 1.0$ respectively). The percentage distracted on habituation days was similar to licking days without distractors. Due to a lack of drug effect there was no interaction between test day and drug pre-treatment ($F(2.7, 81.2) = .376$, $p = .75$).

PCP treatment did not alter percent distracted, all rats showed increased percent distracted compared to modelled baseline and, regardless of pretreatment, a substantial habituation effect was seen. There was no difference in the effects of amphetamine in PCP rats.

To assess whether saline treatment affected percent distracted in a way that was not seen in non-treated animals the two male cohorts (cohort 1 n = 8 and cohort 2 n = 8) were compared to the non-treated animals from the initial distraction pilot experiment (**Chapter 2**). One-way ANOVA revealed no significant difference in percent distracted between the three cohorts ($F(2,23) = 2.54$, $p = .103$). Percent distracted was similar in non-treated and saline cohorts.

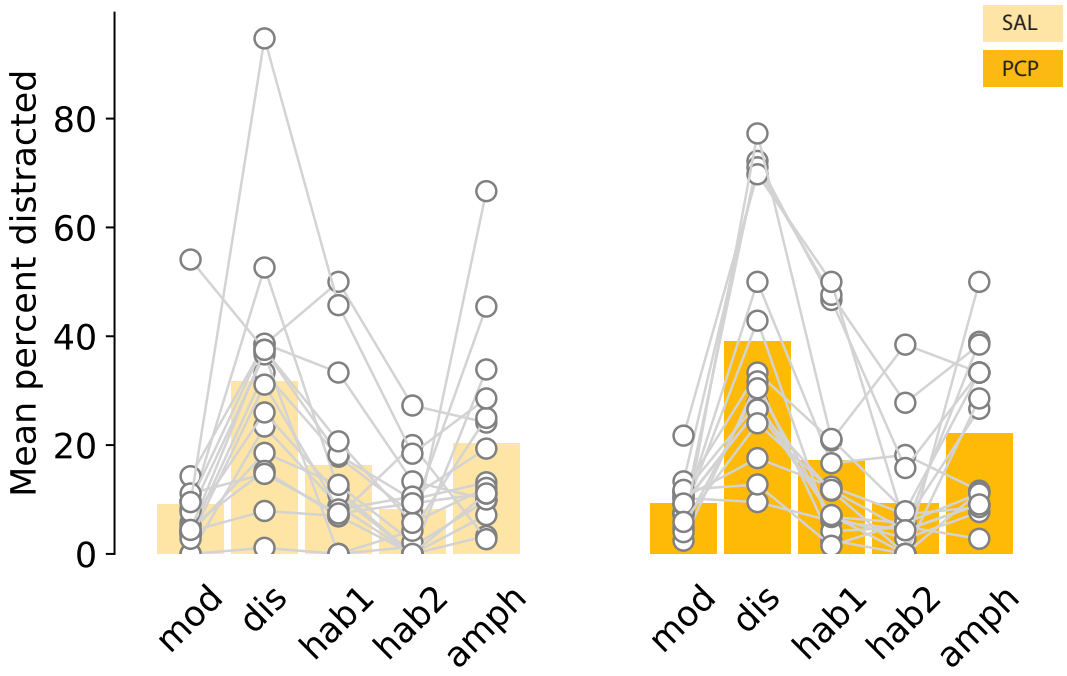


Figure 3.5 *Percent distracted across 5 days for saline and PCP treated males*

Percent distracted across test days in saline (light yellow) and PCP treated rats (orange). Percent distracted is shown for the modelled day (mod, where no distractor were presented), the main distraction test (dis), the two habituation days (hab1 and hab2) and the distraction test with amphetamine injection (amph). Bars represent means and circles show individual rat data.

3.4.1.4 Types of distractor

Preliminary experiments suggested that some distractors may be more effective in distracting animals from ongoing licking than others: in particular, those containing white noise (Chapter 2). Therefore, distractors were separated into white noise and non white noise containing and percentage distracted was calculated for these groups. **Figure 3.6** shows percent distracted for different distractor types in both saline and PCP treated rats.

To assess whether white noise is more distracting, whether this is different in PCP animals, and whether percent distracted habituates differently, a 3-way ANOVA (2 x 2 x 2) with three factors: distractor type (white noise vs non white noise), drug treatment (saline vs PCP) and day (distraction vs habituation 1). There was a significant main effect of distractor type, with white noise containing distractors causing rats to be more distracted (M = 33 %) than those not containing white noise (M = 22 %, $F(1,60) = 4.55$, $p < .05$). There was also a significant main effect of day, with percentage distracted higher on the distraction day (M = 38 %) than on the habituation day (M = 18 %, $F(1,60) = 55.95$, $p < .001$). There was no main effect of drug treatment ($F(1,60) = .443$, $p = .51$) and no interaction effects. Thus, white noise distractors are more distracting and regardless of distractor type rats are less distracted on habituation day. There is no modulation of the distractor type effect by PCP pre-treatment.

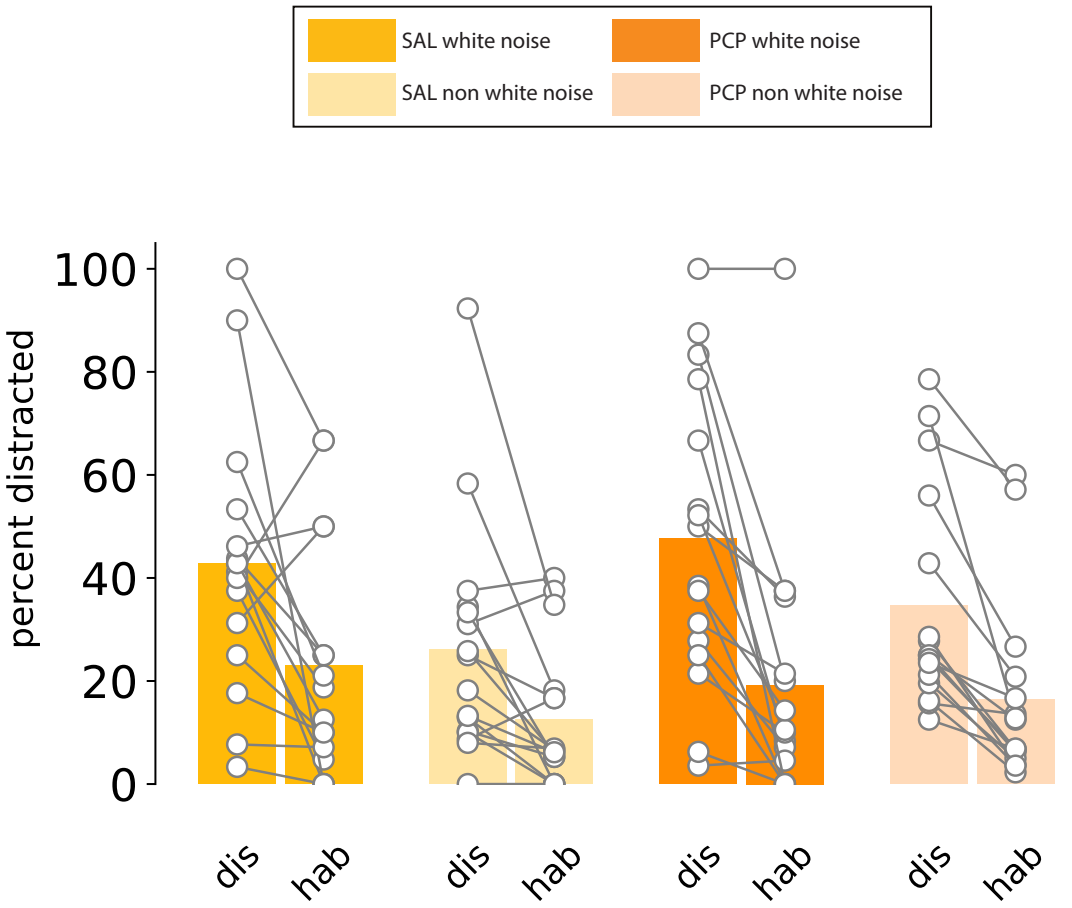


Figure 3.6 *Percent distracted on distraction and habituation days for white noise and non white noise distractors in both saline and PCP treated male rats*

Percent distracted in response to different types of distractor. White noise distractors are more effective in causing pauses in licking and this is not modulated by PCP pretreatment. Bars show mean percent distracted on the distraction day and habituation day in response to white noise distractors (gold bars show saline treated rats and orange bars PCP treated rats) and in response to non white noise distractors (pale yellow bars show saline rats and peach bars show PCP rats). Circles show individual rat data for percent distracted. White noise containing distractors (darker bars) show increased percent distracted

3.4.1.5 Post distractor pauses in saline and PCP males

Post distractor pauses (PDPs) were used as another index of distraction: that is the length of the pause following a distractor (regardless of whether the animals was distracted or not). **Figure 3.7** shows cumulative frequency plots of PDPs on the modelled and distraction days for saline and PCP treated rats. **Figure 3.7e** shows PDPs split by non distracted and distracted trials. These plots show qualitatively different pausing responses on licking days and during the distraction task.

For each rat the mean PDP was calculated and these were used to assess possible differences across days and between treatment groups statistically using 2 X 5 mixed ANOVA. Shapiro-Wilk tests of normality and visual inspection of the data showed that PDPs were non-normally distributed, and therefore to meet the assumptions of ANOVA, a log transformation was performed to correct this (all transformed data were statistically normally distributed). All data are thus expressed as log transformed values, **Figure 3.8** shows the mean PDP's across experimental session in saline and PCP treated males.

There was a significant main effect of day ($F(4,120) = 13.89$, $p < .001$). Bonferroni corrected t-tests showed PDPs were significantly longer on distraction day (M = 20.59 s, SEM = 3.71) compared to the modelled day (M = 6.10, SEM = 1.70, $p < .001$). In addition, as with percentage distracted, there was a significant effect of amphetamine with longer pauses on the amphetamine day (M = 16.97 s, SEM = 3,98) compared to the modelled day ($p < .001$). When amphetamine and distraction days are compared there was no difference ($p = 1.0$), showing similar PDPs. Both habituation day 1 (M = 12.93 s, SEM = 5.16) and habituation day 2 (M = 6.43, SEM = 2.92) were not significantly different from the modelled day ($p = 1.0$). Habituation days following initial distraction testing produced PDPs that were similar to the modelled day showing a clear habituation effect after a single distraction session. There was no significant main effect of drug ($F(1,30) = 0.922$, $p = .345$) and no drug/day interaction ($F(4,120) = 1.22$, $p = .31$). PDPs were different across days in a manner similar to percentage distracted (with the exception of

the distraction and amphetamine difference, which was not observed here). PCP pretreatment did not modulate the PDP differences across days.

In summary, as a measure of distraction PDPs were similar to percent distracted and showed broadly the same differences across days (with the exception of the distraction and amphetamine difference which was not observed). PCP pretreatment did not modulate the changes in PDP across days as with the percent distracted measure.

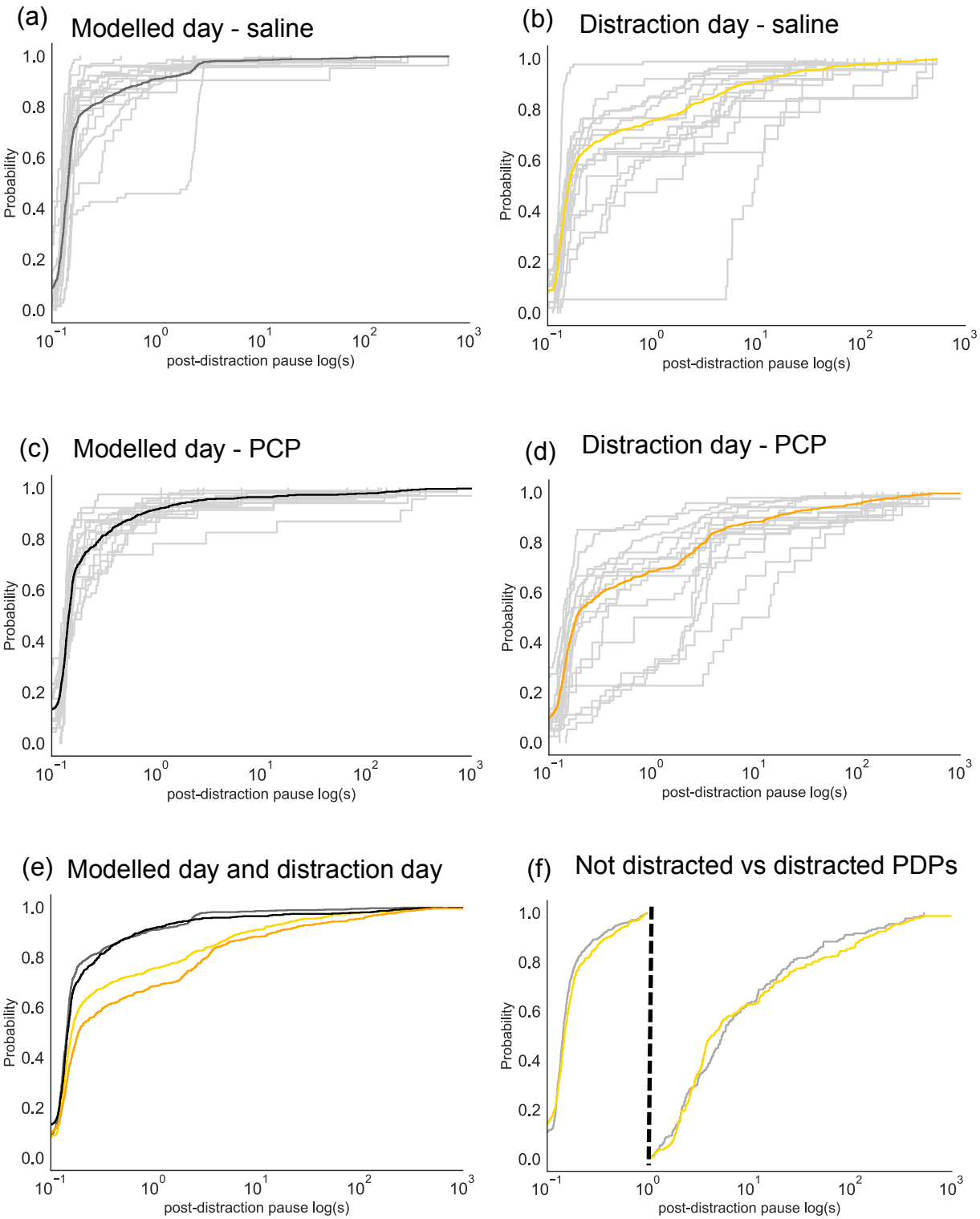

(a) Modelled day - saline
(b) Distraction day - saline
(c) Modelled day - PCP
(d) Distraction day - PCP
(e) Modelled day and distraction day
(f) Not distracted vs distracted PDPs
Probability
post-distraction pause log(s)

Figure 3.7 – *Cumulative plots for post distractor pauses in male rats*

Cumulative probability (or normalised cumulative frequency) of post-distractor pauses. Light grey lines on all plots show individual rats and darker lines represent the mean of all rats. **(a)** Modelled post distractor pauses on lick training day in saline rats, mean in dark grey. **(b)** Distraction day post distractor pauses in saline treated rats, mean in yellow. **(c)** Modelled post distractor pauses on lick training day in PCP treated rats, mean in black. **(d)** Distraction day post distractor pauses in PCP treated rats, mean in orange. **(e)** Mean PDPs for modelled day and distraction day for both saline and PCP treated rats shown together, there are more short pauses observed on the modelled day for both saline (black) and PCP (dark grey) treated rats compared to the distraction day, saline in yellow and PCP in orange. **(f)** Mean PDPs plotted separately for distracted (right) and not distracted trials (left) for both saline (dark grey) and PCP (yellow) rats. Dashed line shows the boundary of 1 second which classifies a pause as distracted.

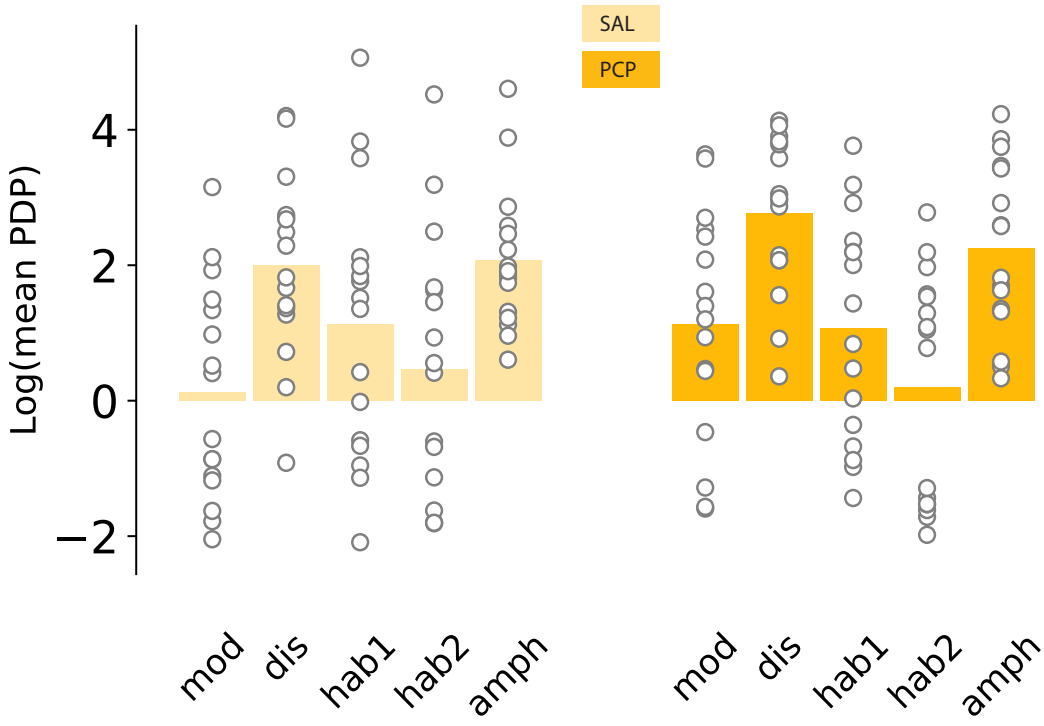


Figure 3.8 – *Post distractor pauses across days in saline and PCP males*

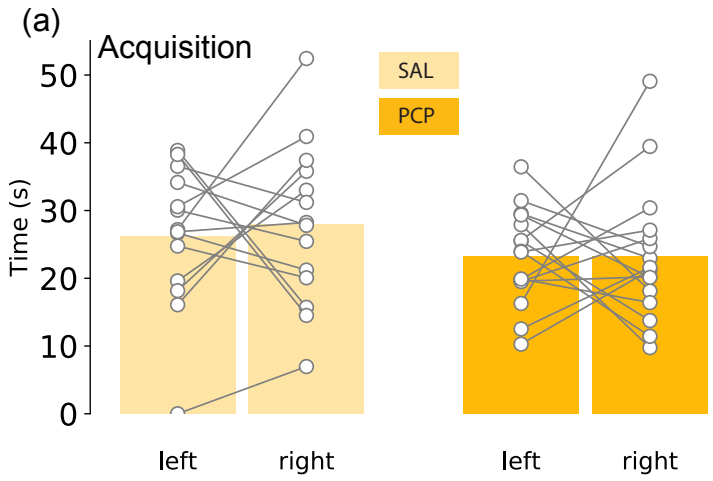
Mean PDPs are shown for the modelled, distraction, habituation1, habituation 2 and amphetamine days. Pale yellow bars show the group averages across days for saline rats and the orange bars show PCP treated rat averages. Circles show individual rat mean PDPs.

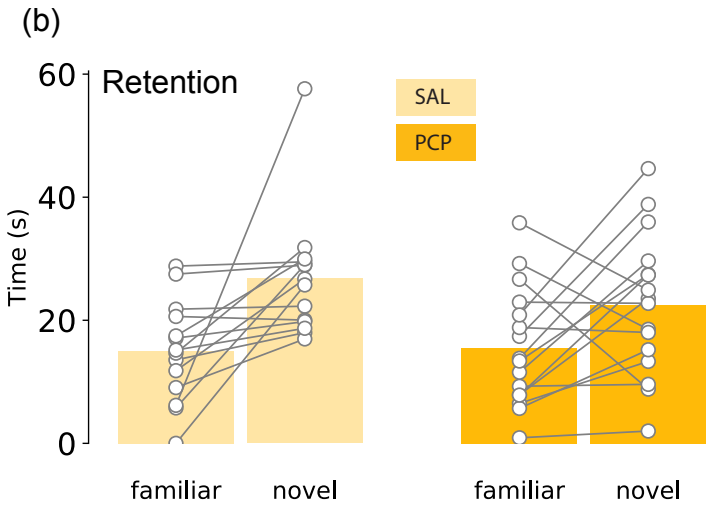
3.4.1.6 Novel object recognition in SAL and PCP males

To assess the efficacy of PCP pre-treatment, novel object recognition (NOR) was performed. A robust deficit is typically observed in PCP treated animals. To determine that rats do not show a side preference during acquisition a 2 x 2 ANOVA was used, exploration times were compared for left and right sides in saline and PCP treated animals at acquisition. There was no significant difference in object exploration times between left (M = 24.75 sec) and right (M = 25.59 sec) sides ($F(1, 28) = 0.10$, $p = .75$). No drug effect ($F(1,28) = 2.35$, $p = .14$) and no interaction between side and drug treatment ($F(1,28) = 0.09$, $p = .76$). No side preference was observed at acquisition (**Figure 3.9a**)

Exploration times during retention were measured and exploration time on the familiar and novel object was compared between saline and PCP animals using 2 X 2 ANOVA. Rats explored the novel object (M = 24.6 sec) significantly more than the familiar object (M = 15.2 sec, $F(1,28) = 14.13$, $p < .01$). There was no main effect of drug ($F(1,28) = 0.47$, $p = .50$) and no interaction effect ($F(1,28) = 0.93$, $p = .34$). T-test comparing discrimination index (DI) between treatment groups showed that DI was not significantly different between PCP (M = 0.20) and SAL (M = 0.29) animals ($t(28) = 0.78$, $p = .44$). Unexpectedly PCP pretreatment did not lead to a deficit in novel object exploration (**Figure 3.9b**)

One sample t-tests were also performed to show that rats had a discrimination significantly different from zero (no discrimination). Saline animals showed DI significantly different from zero ($t(13) = 3.44$, $p < .01$), as did PCP ($t(15) = 2.62$, $p < .05$), showing that both groups exhibited recognition of the novel object and discrimination (**Figure 3.9c**). This further supports a lack of PCP deficit.


(a)
Acquisition
SAL
PCP
Time (s)
50
40
30
20
10
0
left
right
left
right


(b)
Retention
SAL
PCP
Time (s)
60
40
20
0
familiar
novel
familiar
novel

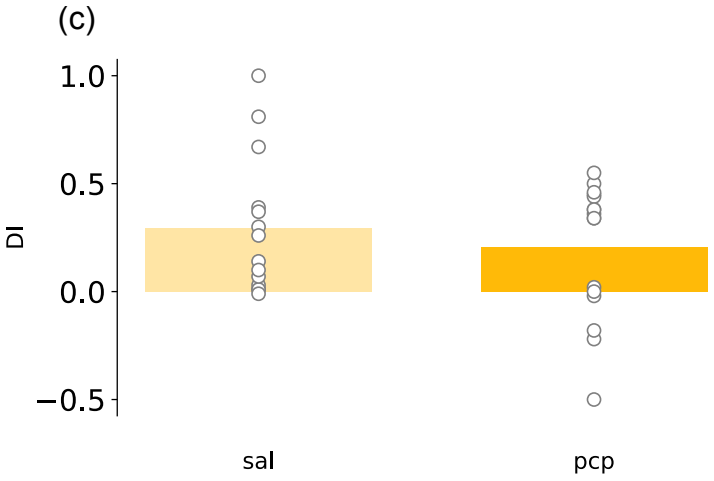

(c)
DI
1.0
0.5
0.0
−0.5
sal
pcp

Figure 3.9 *Novel object recognition in saline and PCP pretreated male rats*

Average exploration times during acquisition and retention trials of NOR as well as discrimination index in saline and PCP treated rats. **(a)** Acquisition – time spent exploring left and right object; there is no side preference in both groups **(b)** Retention – novel vs familiar object exploration; both saline and PCP treated rats show increased exploration of the novel object and there is no PCP deficit **(c)** Discrimination index – comparison of saline and PCP discrimination indices, both groups show significant discrimination of the novel object.

3.4.1.7 Individual differences in males

Due to the lack of drug effects and the large variability observed in percentage distracted, licking and NOR, individual differences were assessed to probe possible relationships between: (1) individual variability in the palatability for saccharin and percentage distracted; and (2) NOR scores and percentage distracted. Linear regressions were performed on saline and PCP data separately to assess this.

To assess a possible contribution of palatability to distraction – i.e. if rats that have an elevated preference for saccharin are less likely to be distracted, linear regression was performed. For saline animals, Linear regression was not significant for either saline treated animals ($r^2 = 0.08$. $p = .29$) or PCP pretreated animals ($r^2 = 0.0007$, $p = 0.92$), indicating that palatability did not significantly predict percentage distracted at test in either case (**Figure 3.10a**).

To assess relationship between individual NOR discrimination index scores and percent distracted simple linear regressions were performed for saline and PCP treated animals separately. Both saline treated animals ($r^2 = 0.00002$, $p = .99$) and PCP treated animals ($r^2 = 0.005$, $p = .80$) showed no significant relationship between DI and percent distracted (**Figure 3.10b**).

To assess the relationship between individual NOR discrimination index scores and licking on the lick training day, linear regressions were performed for saline and PCP treated animals. Both saline treated animals ($r^2 = 0.0004$, $p = .94$) and PCP treated animals ($r^2 = 0.085$, $p = .27$) showed no significant relationship between DI and percent distracted. (**Figure 3.11**).

Individual differences in palatability do not seem to influence the percent distracted seen at test. NOR scores are also not predicted by percent distracted. This is the case for both saline and PCP treated rats.

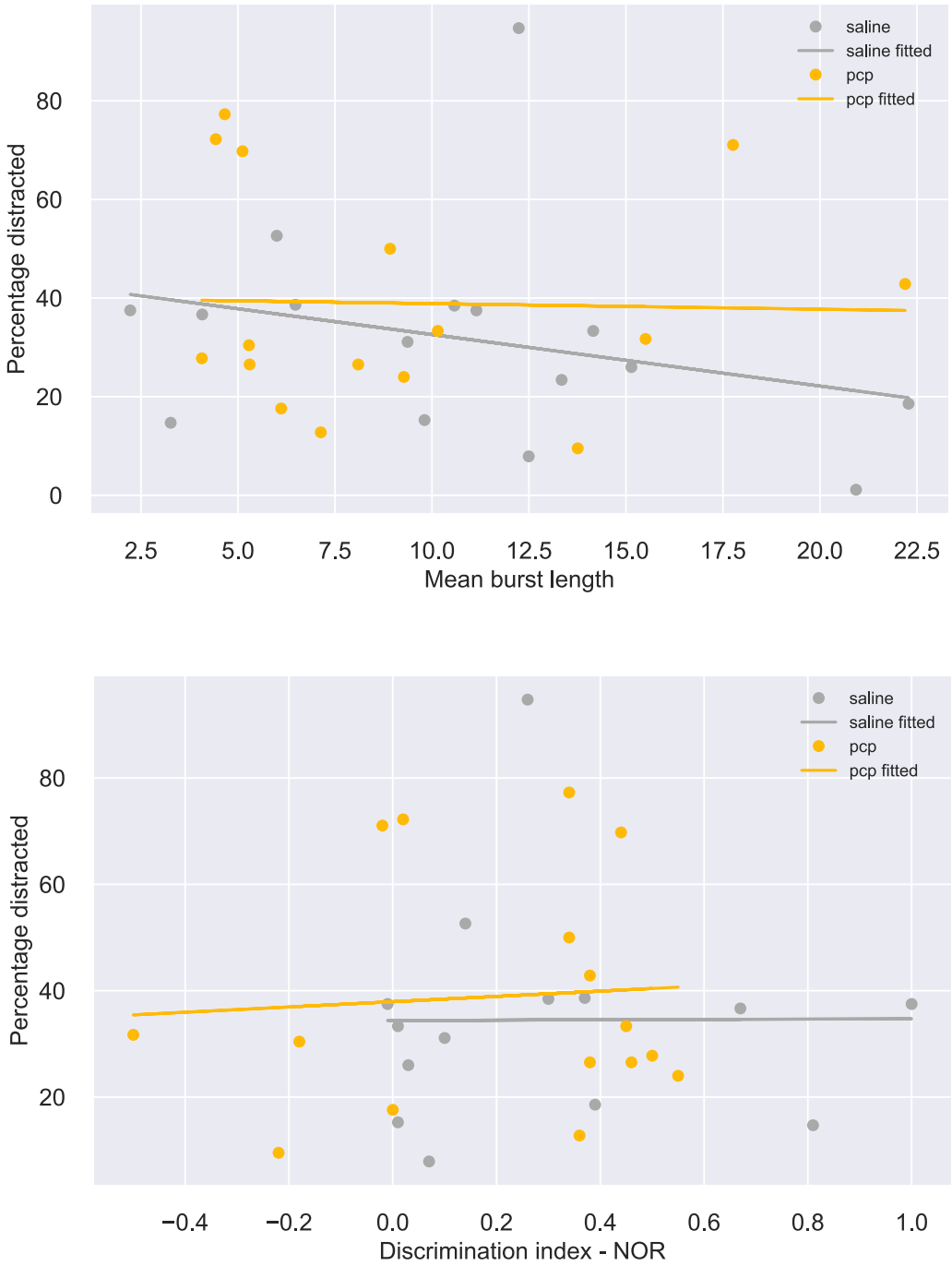


Figure 3.10 – *Linear regressions show no relationship between palatability of saccharin and percent distracted in saline or PCP male rats*

(a) Scatter plot showing percent distracted against burst length (a measure of palatability). Lines of fit for saline (grey) and PCP (yellow) show no significant relationship between the two variables in either group. **(b)** Scatter showing NOR discrimination index against percent distracted. Again, there is no relationship in either saline (grey) or PCP treated rats (yellow).

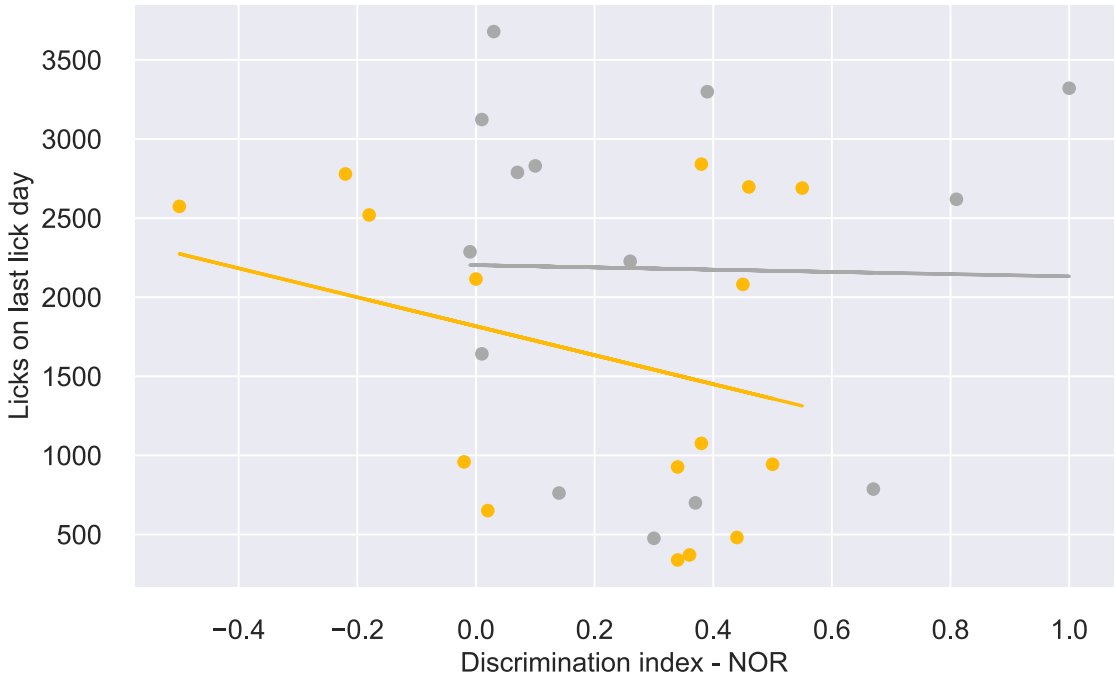


Figure 3.11 – *Linear regression show no relationship between discrimination index and licking in saline or PCP male rats*

Scatter showing NOR discrimination index against total licks on last lick training day. Data points are for individual rats with saline animals in grey and PCP treated in yellow. Lines of fit for saline (grey) and PCP (yellow) show no significant relationship between the two variables in either group.

3.4.2 Effects of PCP pretreatment in female rats

3.4.2.1 No differences in lick microstructure between PCP and saline rats during saccharin training

Figure 3.12 shows licking in the three days preceding the distraction test for saline and PCP treated female rats. On the final day of lick training saline rats licked a means of 2520 licks (SEM = 428 licks) and PCP rats a mean of 1821 licks (SEM = 379 licks). The standard error of the females in both groups are higher than the males showing increased variability in these data for licking.

A 3 X 2 mixed ANOVA was used to assess whether saline and PCP treated rats showed different rates of licking for saccharin across three training days in female rats. The number of total licks was compared across training days and between drug treatment groups. The within subjects factor was day (with three consecutive lick training days, expressed as days before distraction test, -3, -2, -1) and the between subjects factor was drug treatment (PCP or saline).

Analysis revealed that there was no main effect of day ($F(1.3, 27.7) = 1.21$, $p = .29$), no significant main effect of drug ($F(1,22) = 2.96$, $p = .099$), and no significant interaction between day and drug ($F(1.3, 27.7) = 0.48$, $p = .88$). The lack of effect of day was due to a high level of licking in females that was above criteria (1000 licks/day) from the first day (M = 1769 licks) and remained stable across three training days preceding distraction.

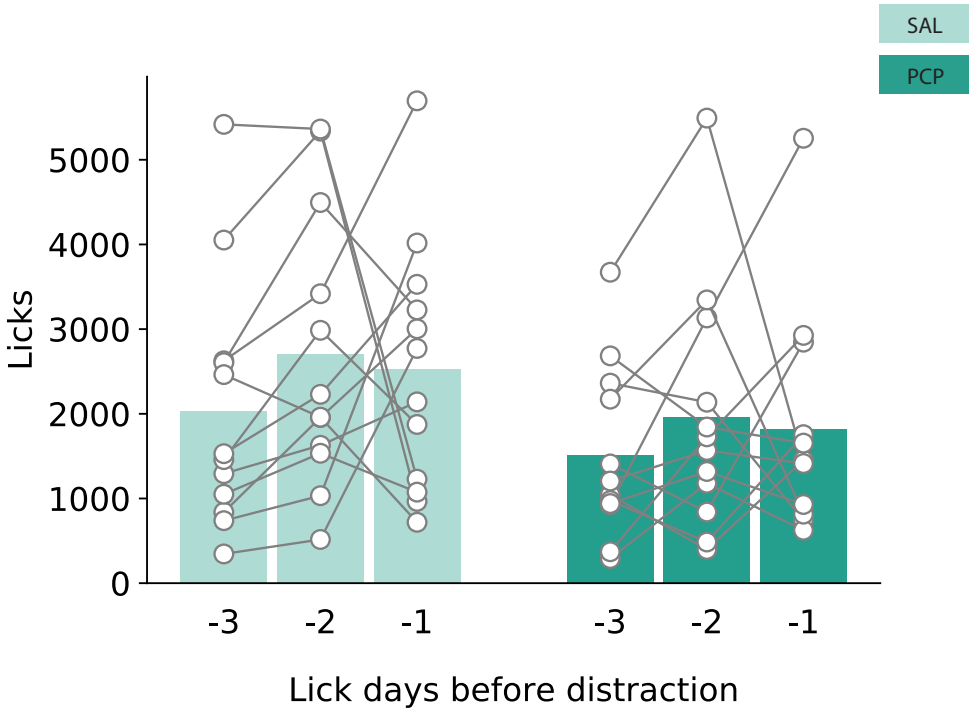


Figure 3.12 *Number of licks within training sessions in saline and PCP treated female rats*

Total number of licks across training days of rats licking for saccharin in 1 hour sessions. Three days preceding the distraction test day (days -3 to -1). Light bars show means for saline treated rats and darker bars means for PCP rats. Circles represent individual rats.

3.4.2.2 Licking microstructure was not significantly different between PCP and saline treated female rats

To rule out differences in licking microstructure between saline and PCP treated female rats a series of t-tests was performed. Total licks on the last saccharin training day were compared for saline (M = 2520 licks, SEM = 427) and PCP (M = 1821 licks, SEM = 379) animals: there was no significant difference, $t(22) = 1.22$, $p = .23$ (**Figure 3.13a**)

To assess palatability of saccharin the mean number of licks per cluster was compared between saline (M = 11, SEM = 1.7) and PCP females (M = 10, SEM = 1.5). Again, there was no significant difference ($t(22) = 0.494$, $p = .626$) (**Figure 3.13b**)

To rule out any significant motor impairment in licking in PCP animals, the total number of bursts within the session was compared. There was no significant difference between saline (M = 235 bursts, SEM = 25.17) and PCP treated rats (M = 184.92 bursts, SEM = 21.47, $t(22) = 1.534$, $p = .139$) (**Figure 3.13c**)

In summary, lick microstructure as measured by these three key variables was not significantly affected by PCP pretreatment in female rats. This suggests that PCP treatment in females does not alter the overall drive to consume a sweet solution or affect the palatability of a sweet solution and that there is no obvious motor impairment in these animals that precludes licking normally.

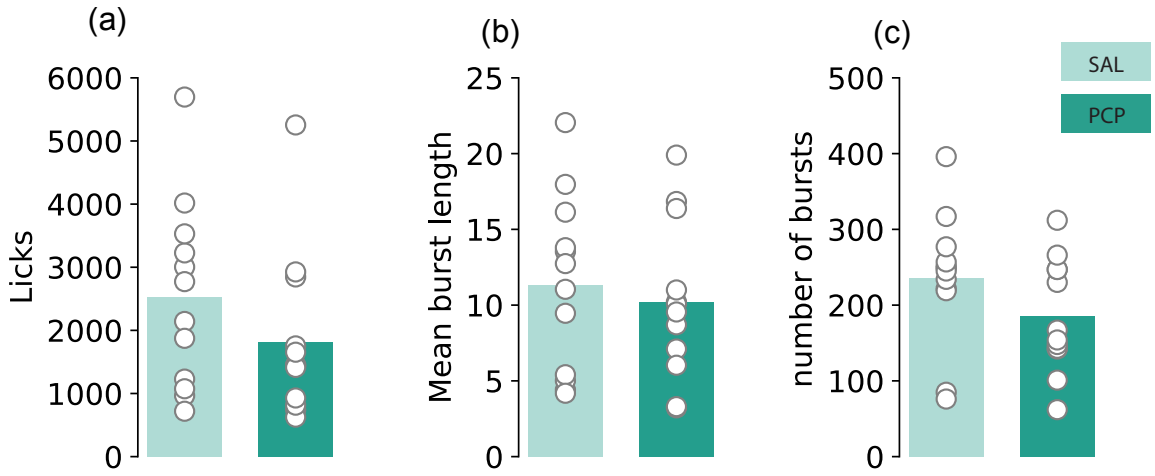


Figure 3.13 – *Lick microstructure in saline and PCP treated female rats*

Licking microstructure measures on the last lick training day, pale green bars represent means for saline rats and darker, teal bars show means for PCP pretreated rats. **(a)** Total licks on the final lick training day, saline and PCP animals. **(b)** Mean burst length, the average number of licks per burst, in saline and PCP males. **(c)** Number of bursts, the mean number of burst events within the last lick training session. White circles show individual rats and bars represent the mean

3.4.2.3 Percentage distracted SAL and PCP females

To assess changes in percentage distraction across days and to investigate differences in saline and PCP treated animals, a 2 x 5 mixed ANOVA was performed. Experimental day was a within-subjects factor with five levels (modelled, distraction, habituation1, habituation2 and amphetamine), drug treatment was between-subjects with two levels (PCP or saline)

There was no significant main effect of drug ($F(1,22) = 0.396$, $p = .54$). Data for percent distracted are shown in **Figure 3.14.** Analysis revealed that PCP pre-treatment did not significantly increase percentage distracted. There was a main effect of day ($F(2.8, 62.2) = 27.6$, $p < .001$), and Bonferroni corrected t-tests showed that compared to the modelled distraction day (M = 7 %), there was significantly higher percentage distracted on the distraction day (M = 36 %, $p < .001$), as well as on the first habituation day (M = 16.66 %, $p < .01$) and on the amphetamine day (M = 18.5 %, $p < .01$). The second habituation day (M = 11.9 %) was not significantly different from the modelled day ($p = .53$). Habituation appears by the second habituation day, unlike males where licking had returned to baseline on the first habituation day. Thus, the pattern of distraction was broadly similar in females as in males with the exception that females appeared to habituate more slowly with habituation appearing by the second habituation day, rather than the first habituation day.

In addition, percent distracted on the amphetamine day was significantly lower than the main distraction test ($p < .001$) showing that although amphetamine reversed the habituation effect, this did not reach the initial level of distraction in the first test day. PCP treatment did not alter percent distracted, affect the habituation to distractors or modulate the effects of amphetamine on distraction.

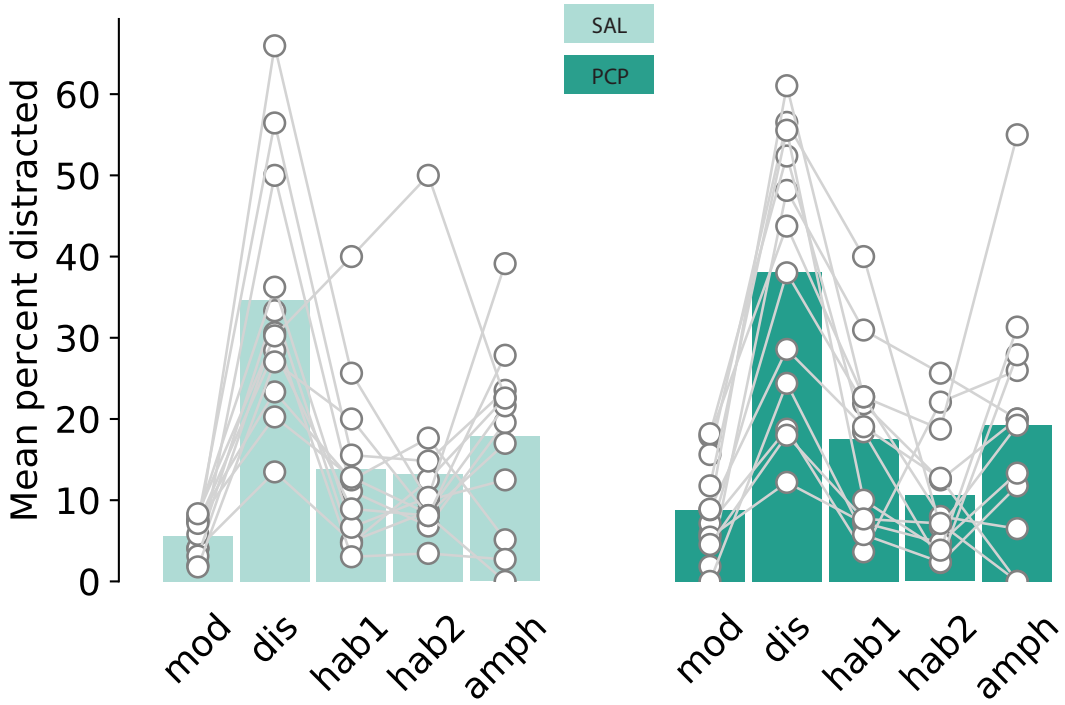


Figure 3.14 – *Percent distracted across 5 days for saline and PCP treated females*

Percent distracted across test days in saline (light green) and PCP treated rats (teal). Percent distracted is shown for the modelled day (mod, where no distractors were presented), the main distraction test (dis), the two habituation days (hab1 and hab2) and the distraction test with amphetamine injection (amph). Bars represent means and circles show individual rat data.

3.4.2.4 Percent distracted white noise – types

Distractors were separated into white noise and non white noise containing and percentage distracted was calculated for these groups. **Figure 3.15** shows percent distracted by distractor type in saline and PCP treated females. To assess whether white noise is more distracting, whether this is different in PCP animals and whether percent distracted habituates differently, a 2 X 2 X 2 ANOVA was performed to compare white noise and non white noise distractors in saline and PCP treated animals and on distraction vs habituation day 1. The three factors and levels were: distractor type (white noise vs nonwhitenoise), drug treatment (saline vs PCP) and day (distraction vs habituation 1).

There was no significant main effect of distractor type, although this result was approaching significance, so white noise containing distractors did not result in a higher percentage distracted trials (M = 31 %) than non white noise (M = 24 %, $F(1,44) = 3.85$, $p = .056$). There was a significant main effect of day, with percentage distracted higher on the distraction day (M = 38 %) than on the habituation day (M = 16 %, $F(1,44) = 59.23$, $p < .001$). There was no main effect of drug treatment ($F(1,44) = 1.75$, $p = .19$) and no interaction effects. White noise distractors are not more distracting in the case of females and, regardless of pretreatment, rats are less distracted on habituation day 1 than on the first distraction day.

In females, there is a level of variability that reduced statistical power and thus the observed increased distraction in response to white noise distractors is not statistically significant in females. As with males, PCP pretreatment did not modulate the response to distractors of different types.

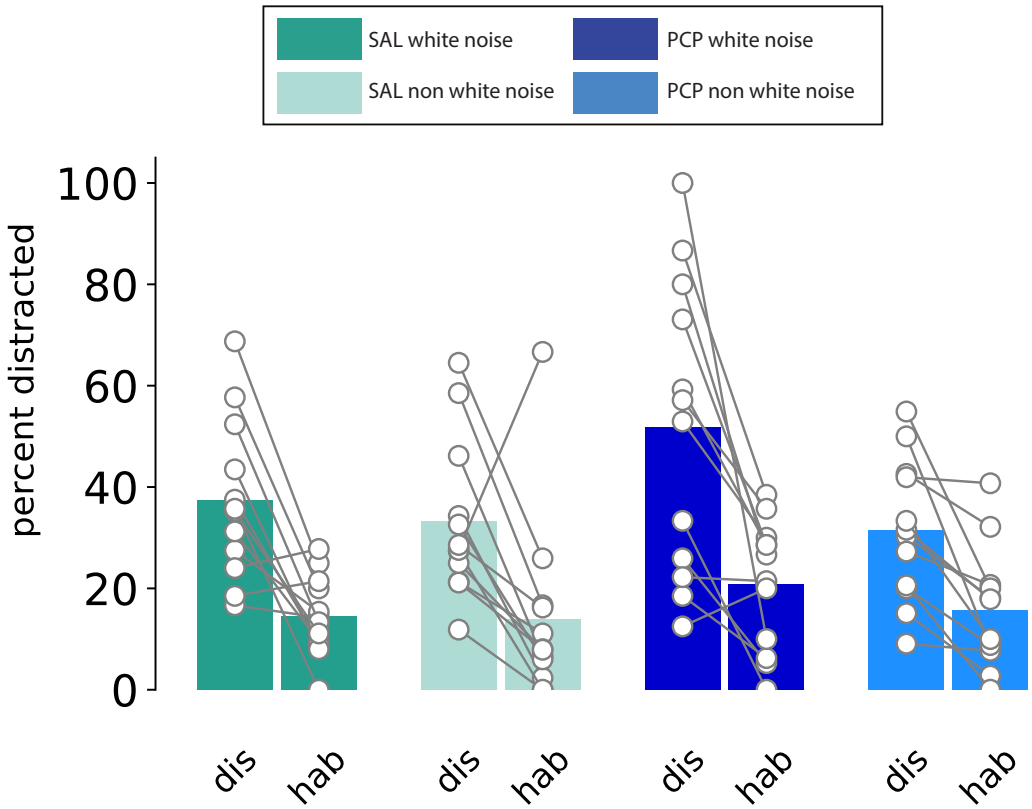


Figure 3.15 – *Percent distracted on distraction and habituation days for white noise and non white noise distractors in both saline and PCP treated female rats*

Percent distracted in response to different types of distractor. White noise distractors are more effective in causing pauses in licking and this is not modulated by PCP pretreatment. Bars show mean percent distracted on the distraction day and habituation day in response to white noise distractors and non white noise distractors. Circles show individual rat data for percent distracted.

3.4.2.5 Post distractor pauses saline and PCP females

Figure 3.16 shows cumulative frequency plots of PDPs on the modelled and distraction days for saline and PCP treated rats. **Figure 3.16e** shows PDPs split by non distracted and distracted trials. These plots show qualitatively different pausing responses on licking days and during the distraction task and demonstrate the differences in PDPs between distracted and not distracted trials.

For each rat the mean PDP was calculated and these were compared across days and between treatment groups using 2 X 5 mixed ANOVA. Shapiro-Wilk tests of normality and visual inspection of the data showed that PDPs were non-normally distributed log transformation was performed (all transformed data were statistically normally distributed). Therefore all data are expressed as log transformed values.

Unlike males, there was no significant main effect of day ($F(2.7,56.4) = 1.18$, $p = .11$). Post distractor pauses did not differ across test days, this was unexpected and does not reflect the results from percentage distracted measures. There was no significant main effect of drug ($F(1,21) = 0.025$, $p = .876$) and no drug/day interaction ($F(2.7,56.4) = 0.20$, $p = .87$ (**Figure 2.17**)

In female rats, PDPs do not vary with percent distracted in the way they do in the male rats. However, as with the males, PCP pretreatment did not modulate the changes in PDP across days as with the percent distracted measure.

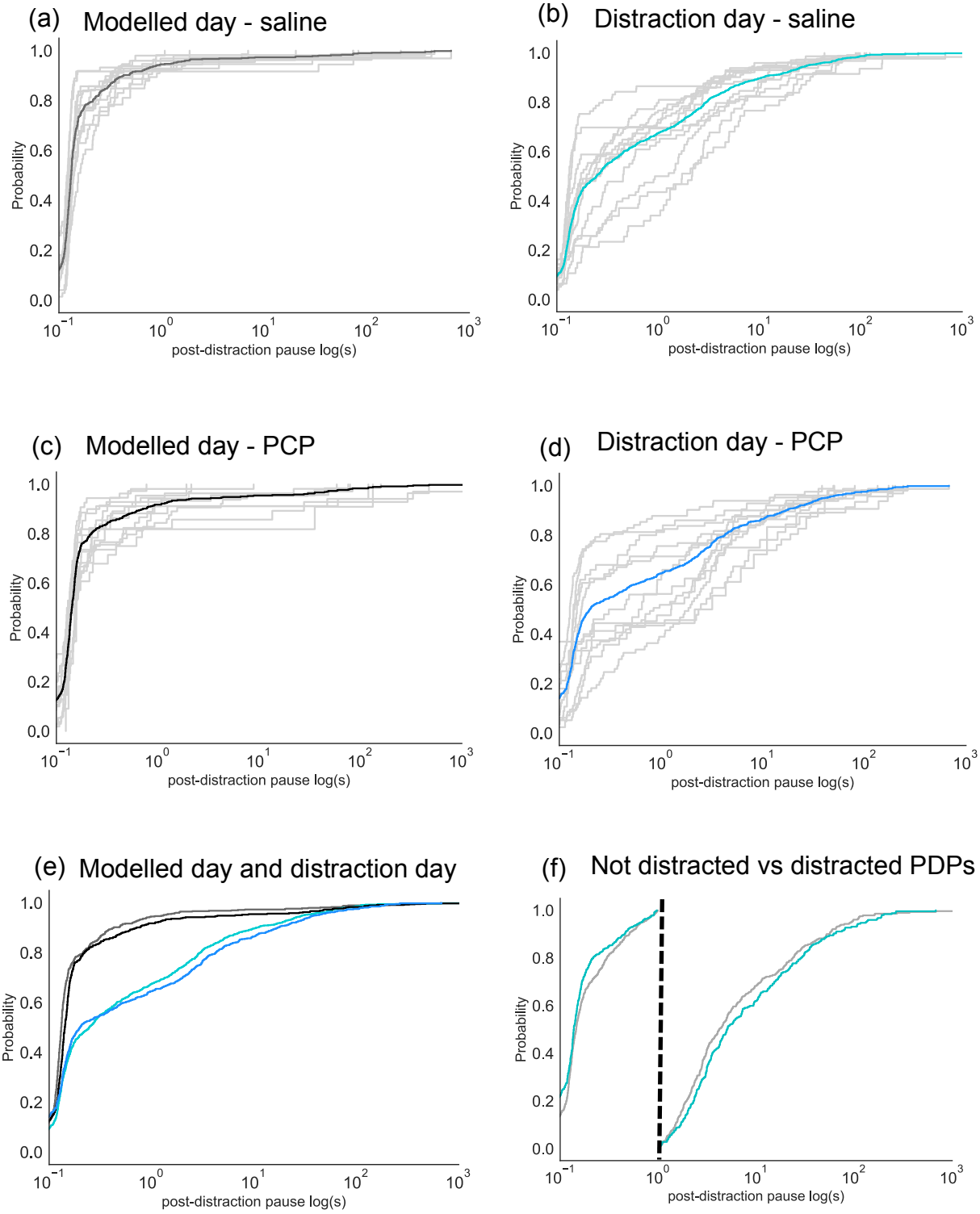

(a) Modelled day - saline
(b) Distraction day - saline
(c) Modelled day - PCP
(d) Distraction day - PCP
(e) Modelled day and distraction day
(f) Not distracted vs distracted PDPs
Probability
post-distraction pause log(s)

Figure 3.16 – *Cumulative plots for post distractor pauses in females*

Cumulative probability (or normalised cumulative frequency) of post-distractor pauses. Light grey lines on all plots show individual rats and darker lines represent the mean of all rats. **(a)** Modelled post distractor pauses on lick training day in saline rats, mean in dark grey. **(b)** Distraction day post distractor pauses in saline treated rats, mean in turquoise. **(c)** Modelled post distractor pauses on lick training day in PCP treated rats, mean in black. **(d)** Distraction day post distractor pauses in PCP treated rats, mean in blue. **(e)** Mean PDPs for modelled day and distraction day for both saline and PCP treated rats shown together, there are more short pauses observed on the modelled day for both saline (black) and PCP (dark grey) treated rats compared to the distraction day, saline in turquoise and PCP in blue. **(f)** Mean PDPs plotted separately for distracted (right) and not distracted trials (left) for both saline (dark grey) and PCP (turquoise) rats. Dashed line shows the boundary of 1 second which classifies a pause as distracted.

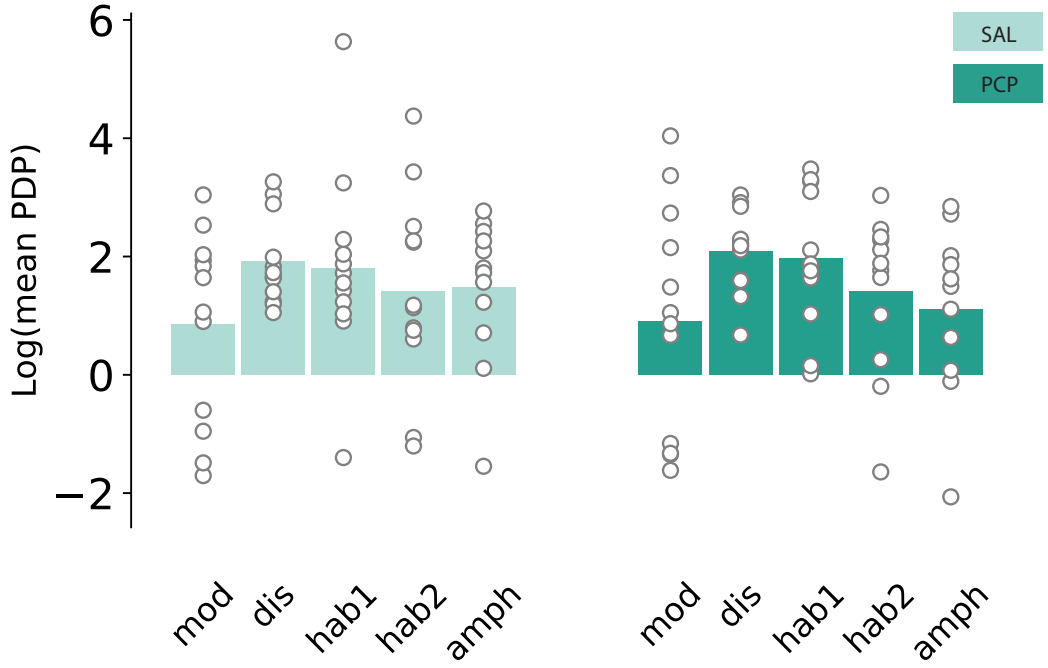


Figure 3.17 – *Post distractor pauses across 5 sessions for saline and PCP pretreated females*

Mean PDPs are shown for the modelled, distraction, habituation1, habituation 2 and amphetamine days. Pale green bars show the group averages across days for saline rats and the teal bars show PCP treated rat averages. Circles show individual rat mean PDPs.

3.4.2.6 Novel object recognition in saline and PCP females

To assess the efficacy of PCP pre-treatment, NOR was performed. A robust deficit is typically observed in PCP treated animals, especially in female rats. To determine that rats do not show a side preference during acquisition a 2 x 2 ANOVA was performed, exploration times were compared for left and right sides in saline and PCP treated animals at acquisition. There was no significant difference in object exploration times between left (M = 19.78 sec) and right (M = 22.57 sec) sides ($F(1, 22) = 2.43$, $p = .13$). There was a significant effect of pretreatment, with PCP animals (M = 18.18 sec) showing a general decrease in exploration globally when compared with saline animals (M = 24.17, $F(1,22) = 4.80$, $p < .05$). There was no interaction between side and drug treatment ($F(1,22) = 1.47$, $p = .24$). (**Figure 3.18a**).

To assess whether rats showed NOR, familiar and novel object exploration times were compared between saline and PCP animals during the retention trial using 2 X 2 ANOVA. Rats explored the novel object (M = 18.52 sec) significantly more than the familiar object (M = 13.79 sec, $F(1,22) = 7.042$, $p < .05$). There was no main effect of drug ($F(1,22) = 0.32$, $p = .58$) and no interaction effect ($F(1,22) = 2.02$, $p = .17$). T-test comparing discrimination index (DI) between treatment groups showed that DI was not significantly different between PCP (M = 0.16) and SAL (M = 0.22) animals ($t(22) = 0.55$, $p = .59$). Thus, as with the NOR experiment in male rats, unexpectedly, PCP pretreatment did not lead to a deficit in novel object exploration (**Figure 3.18b)**.

One sample t-tests were also performed to show that rats had a discrimination significantly different from zero. Saline animals showed significant DI ($t(11) = 4.30$, $p < .001$), however, PCP treated animals did not ($t(11) = 1.63$, $p = .13$). This suggests that there is some evidence of a PCP-induced deficit in NOR, with the discrimination of the objects (DI) not significantly different from zero for PCP treated rats (**Figure 3.18c)**.

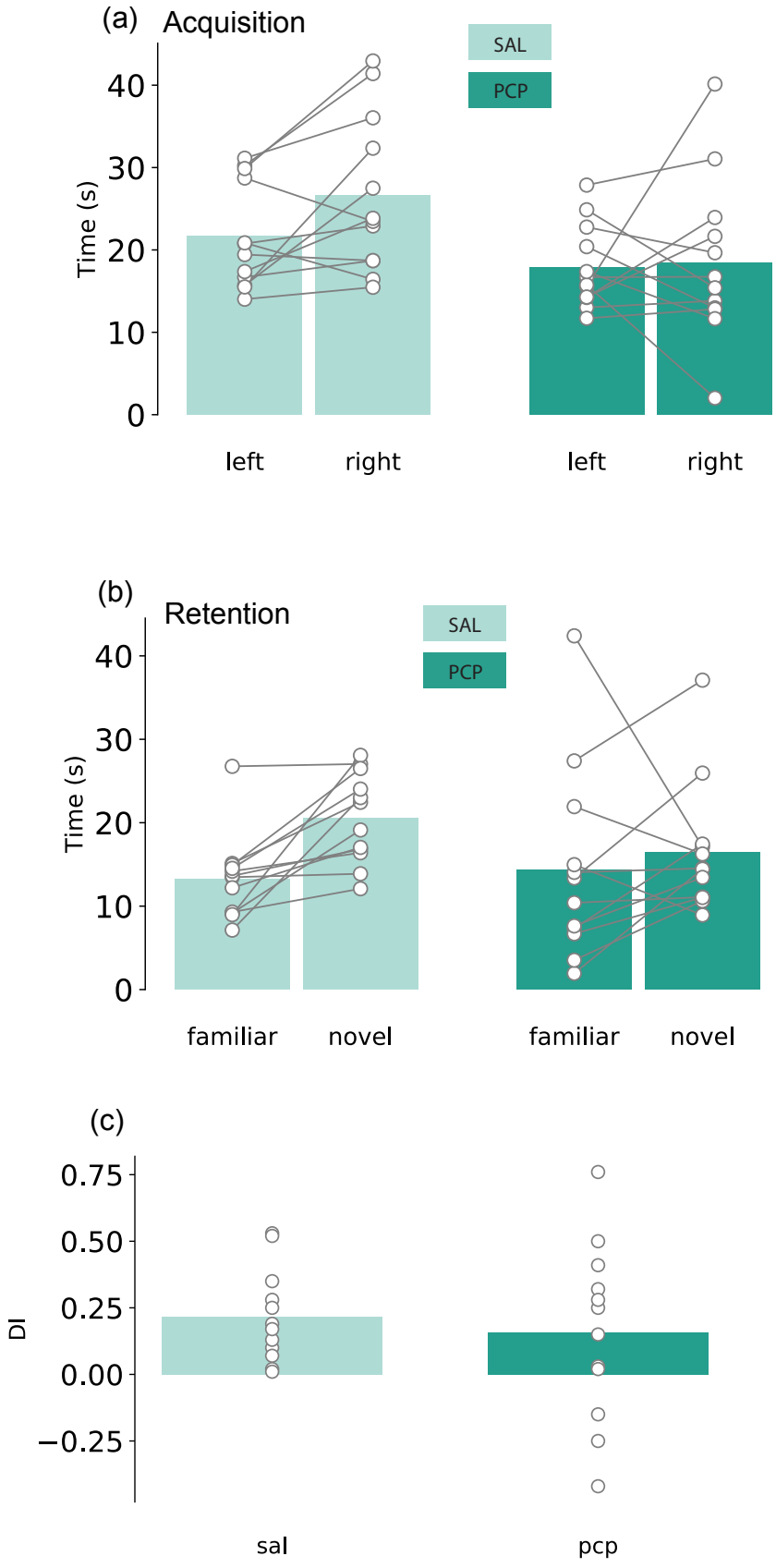

(a)
Acquisition
SAL
PCP
Time (s)
0
10
20
30
40
left
right
left
right
(b)
Retention
SAL
PCP
Time (s)
0
10
20
30
40
familiar
novel
familiar
novel
(c)
DI
−0.25
0.00
0.25
0.50
0.75
sal
pcp

Figure 3.18 *Novel object recognition in saline and PCP pretreated female rats*

Exploration times during acquisition and retention trials of NOR as well as discrimination index in saline and PCP treated rats. **(a)** Acquisition – time spent exploring left and right object, there is no side preference in both groups **(b)** Retention – novel vs familiar object exploration, both saline and PCP treated rats show increased exploration of the novel object and there is no PCP deficit **(c)** Discrimination index – comparison of saline and PCP discrimination indices, both groups show significant discrimination of the novel object.

3.4.2.7 Individual differences in females

Individual differences were assessed to probe relationships between: (1) individual variability in the palatability for saccharin and percentage distracted, and (2) NOR scores and percentage distracted. Linear regressions were performed on saline and PCP data separately to assess this.

To assess a possible contribution of palatability to distraction – i.e. if rats that have an elevated preference for saccharin are less likely to be distracted – linear regression was performed. Linear regression was not significant for saline animals ($r^2 = 0.0001$. $p = .97$) or PCP pretreated animals distracted ($r^2 = 0.02$, $p = .66$) indicating palatability did not significantly predict percentage distracted at test in either case (**Figure 3.19a**)

To assess the relationship between individual NOR discrimination index scores and percent distracted, linear regressions were performed for saline and PCP treated animals separately. Both saline treated animals ($r^2 = 0.03$, $p = .58$) and PCP treated animals ($r^2 = 0.07$, $p = .42$) showed no significant relationship between DI and percent distracted. (**Figure 3.19b**).

To assess the relationship between individual NOR discrimination index scores and licking on the lick training day, linear regressions were performed for saline and PCP treated animals. Both saline treated animals ($r^2 = 0.07$, $p = .43$) and PCP treated animals ($r^2 = 0.09$, $p = .33$) showed no significant relationship between DI and percent distracted. (**Figure 3.20**).

Individual differences in palatability do not seem to influence percent distracted in female rats. Discrimination index scores from NOR are also not predicted by percent distracted. Neither are DI scores predicted by licking on the lick training days. This is the case for both saline and PCP treated female rats.

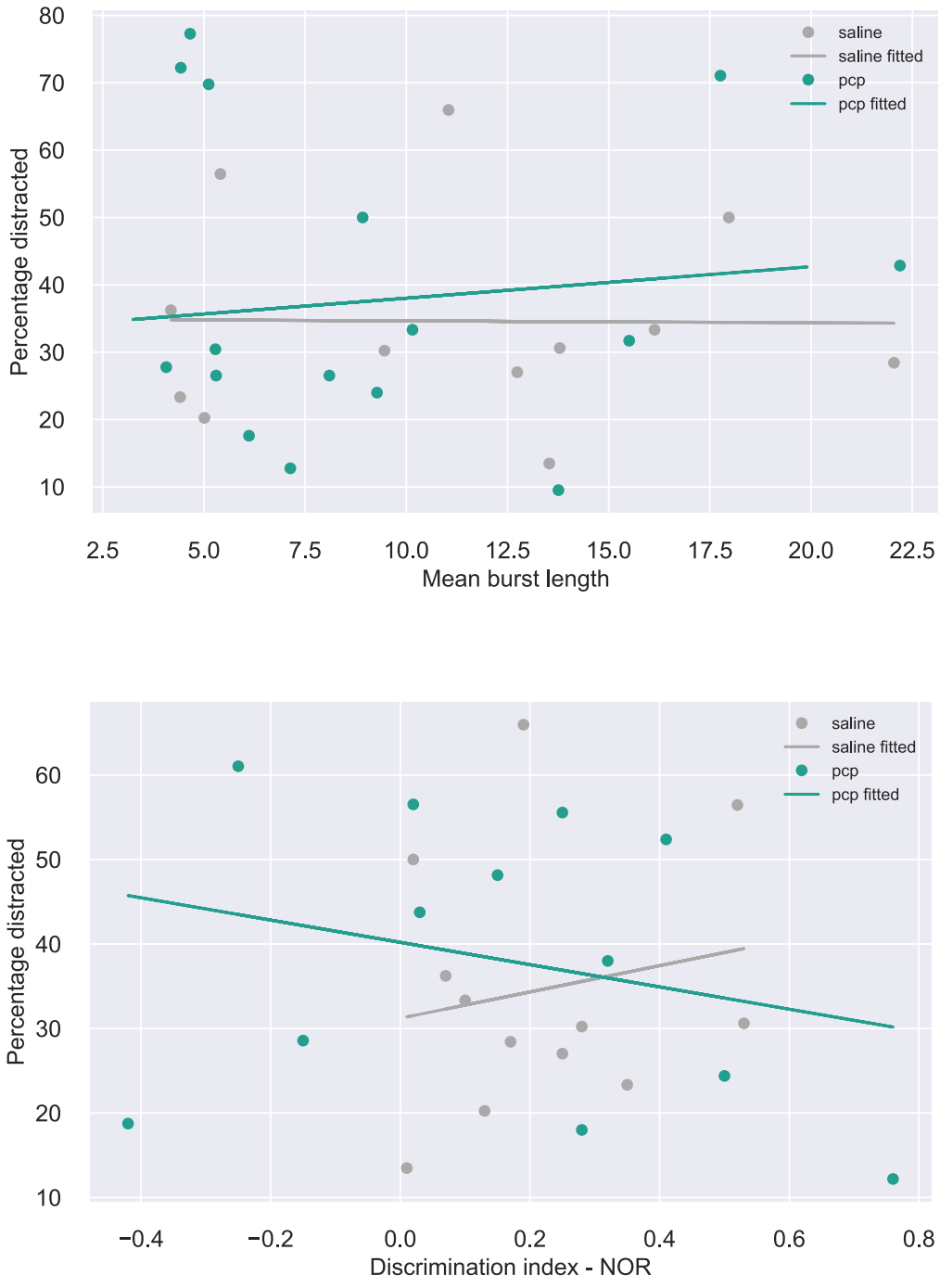


Figure 3.19 – *Linear regressions show no relationship between palatability of saccharin and percent distracted in saline or PCP female rats*

(a) Scatter plot showing percent distracted against burst length (a measure of palatability). Lines of fit for saline (grey) and PCP (teal) show no significant relationship between the two variables in either group. **(b)** Scatter showing NOR discrimination index against percent distracted. Again, there is no relationship in either saline (grey) or PCP treated rats (teal).

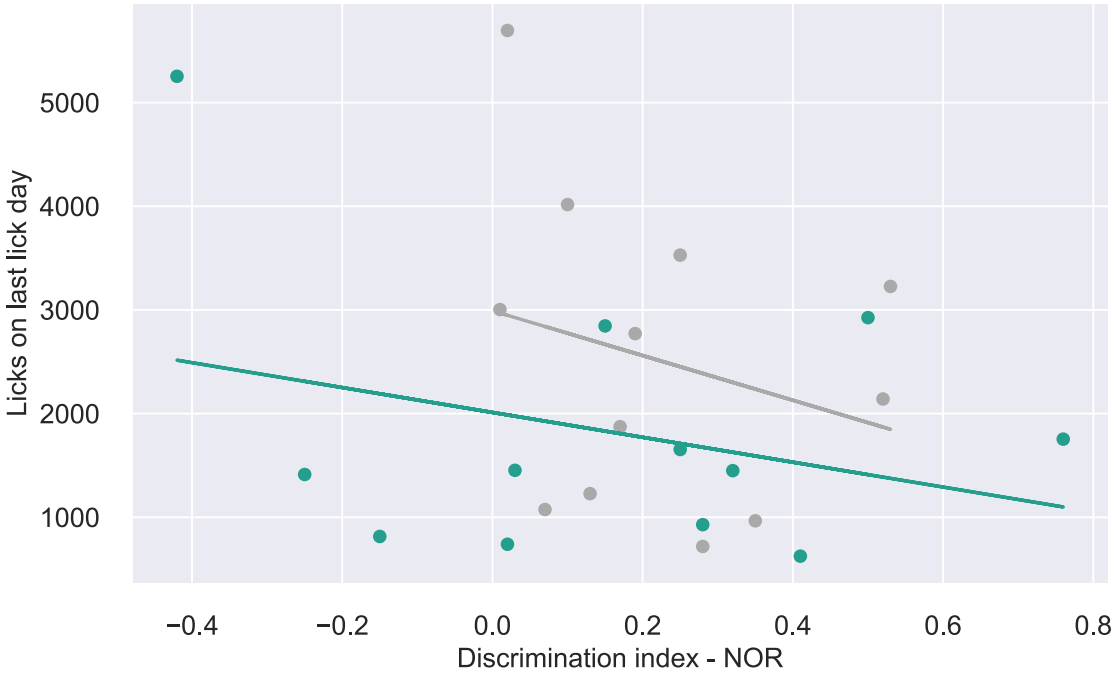


Figure 3.20 – *Linear regression show no relationship between discrimination index and licking in saline or PCP female rats*

Scatter showing NOR discrimination index against total licks on last lick training day. Data points are for individual rats with saline animals in grey and PCP treated in teal. Lines of fit for saline (grey) and PCP (teal) show no significant relationship between the two variables in either group.

3.4.2.8 NOR in males and females combined

Male and female rats tested separately do not show a NOR deficit in response to PCP pretreatment. To assess whether there was an effect of PCP pretreatment when these data are grouped (males and females combined) 2 way ANOVAs were used to compare the exploration times as well as the DI of all rats in the retention trial for familiar versus novel objects and in saline and PCP treatment groups.

Combined analysis matches the results of individual cohort analyses. There was a significant main effect of object familiarity, regardless of drug treatment or sex rats showed longer exploration times for novel (M = 21.59 s, SEM = 1.28 s) versus familiar objects (M = 14.52 s, SEM = 1.23 s, $F(1,50) = 19.23$, $p < .0001$) in the retention phase. There was no main effect of drug pretreatment ($F(1,50) = 0.77$, $p = .386$) or sex ($F(1,50) = 3.93$, $p = .053$) and no interaction effect were observed. For DI there was no main effect of either drug treatment ($F(1,50) = 0.87$, $p = .357$) or sex ($F(1,50) = 0.61$, $p = .44$). These results further support a lack of PCP induced deficit in NOR.

To assess possible differences in locomotor activity between saline and PCP treated animals and between sexes, 2 way ANOVA was used to for total distance travelled during the retention trial and for the average speed of locomotion within the test. There was no effect of PCP treatment on locomotr activity. For total distance travelled there was no main effect of drug ($F(1,49) = 0.496$, $p = .484$). There was also no main effect of drug on average speed ($F(1,49) = 0.622$, $p = .434$). PCP pretreatment did not affect locomotor activity during the NOR test phase.

There were significant differences between male and female rats in locomotor activity. There was a main effect of sex, with female rats showing greater total distance travelled (M = 6.54 m, SEM = 0.3 m) than males (M = 5 m, SEM = 0.30, $F(1,49) = 11.72$, $p < .001$). Females also exhibited faster locomotion with higher average speed measure (M = 0.04 m/s, SEM = 0.002) compared to male rats (M = 0.03 m/s, SEM = 0.002, $F(1,49) = 12.12$, $p < .001$).

3.5 DISCUSSION

3.5.1 Licking microstructure in the PCP model

The aim of this chapter was to assess distraction in the subchronic PCP model using a novel licking based task. The data presented here extend those of the initial pilot experiment introduced in Chapter 2 to apply this assay to a well characterised model of schizophrenia. PCP pretreated rats were compared to saline controls across a number of parameters and these experiments were conducted in both male and female rats.

Firstly, we found that licking microstructure was similar between saline and PCP treated rats (both male and female). There were no obvious motor impairments in licking and they consumed saccharin in a similar way to saline controls, appearing to also show no difference in palatability measures. This is evidenced by their similar overall consumption of saccharin, their licks per cluster (palatability), and their total number of clusters. This is crucial to determine as the distraction assay relies on licking microstructure to deliver distractors and to determine pauses that provide an index of distraction. Any subtle difference in motor function or consumption and licking patterns could confound interpretation of distraction test results. Differences, particularly in palatability, could have effects on distraction such that decreased preference or palatability for saccharin could lead to an increased tendency to distraction, as animals are less engaged in ongoing licking for saccharin. Once we had established this important information, we could assess distraction.

3.5.2 Distraction across sessions, habituation effects

The distraction test produced a number of distracted trials similar to the pilot experiment with rats pausing in response to distractors on around 50% of trials. The three cohorts presented here all show the same pattern of responses with repeated testing and, importantly, the amount of distraction resulting from modelled distractors on lick days without presentation of distractors was

significantly lower than real distractors. This shows that baseline levels of three lick bursts (which are used to trigger distractors and determine whether the animal pauses or not) are not particularly high and indicates that the observed effects of pausing during licking are due to real distraction and not some natural development of licking patterns. With repeated testing, rats habituate to distractors. This occurs after just one session in males and after two sessions in females, which appear slower to habituate

Habituation can be somewhat reversed by amphetamine, this effect was seen in both males and females and PCP pretreatment did not modulate this effect. Amphetamine increased animals' propensity to become distracted by salient stimuli in the environment even after they had experienced multiple test sessions and reached a distracted level which was not significantly different from the modelled day (no distractors). We hypothesis that the most likely neurochemical explanation for this is due to amphetamine's well-established ability to increase dopamine. The mechanisms involved here require further study. In addition, we predicted that, due to the known disruption of the dopaminergic system in schizophrenia and models of schizophrenia, that we would see a modulation of the amphetamine effect in PCP animals. This was not the case.

3.5.3 Distraction and subchronic PCP treatment

There seems to be no obvious effect of PCP pre-treatment in enhancing distractibility as predicted. There are several possible reasons for this lack of effect. The distraction assay we have developed may be assessing a facet of distractibility, namely the interruption of ongoing consummatory behaviour by salient stimuli, which PCP animals do not show a deficit in. That is, they may show increased distraction in other ways but they perform without issue in this particular task. Alternatively, it could be that this assay is not sensitive enough to measure true deficits in distractibility within this model. Or even the disruptions to NOR seen by Neill et al., may not be involved distraction processes at all. However this is unlikely given evidence that altering the

parameters of the NOR task to avoid periods of distraction during the ITIs abolish the deficit suggesting a real role for distraction (Neill et al., 2010).

One possible explanation for the lack of increase in distraction is that there were fundamental issues with the PCP pretreatment. We observed a surprising lack of NOR deficit in all three cohorts of animals, both male and female rats which is very unusual and contradicts previous findings. Errors in the drug administration, dilution or other human error, do not seem likely to explain this lack of effect in three separate cohorts. This can also not be attributed to us using a dose that was too low as several previous studies have validated the doses we used in both male and females (Neil et al., 2010) rats. We did use a 5 day protocol whereas many studies use 7 days of pretreatment. However, previous published work from our lab and others shows this to be effective in producing the NOR deficit (Asif-Malik et al., 2017; McClean et al., 2017). It is possible that there was a strain effect, typically Wistar rats are used and our animals were Sprague Dawley rats. It could be that the dose and 5 day schedule of PCP treatment that we used interacted with some feature of the strain which resulted in a lack of NOR deficit. Perhaps we require higher dosage or longer time course of PCP treatment to show the robust deficits reported in our lab previously with female Wistars.

Interestingly, it is worth noting that the levels of NOR observed in the present experiment for both males and females are not typical for saline control animals from other studies from our and other labs (Yavas & Young 2017). As such, the discrimination indices in particular are low compared to previous studies within the literature and from previous experiments within this lab. For example, a typical discrimination index for saline treated rats is 0.43 (Yavas & Young 2017) whereas here we see between 0.22 and 0.29 suggesting a weaker novel object recognition effect in saline rats. This suggests a problem with the expression of novel object recognition memory itself as well as (or perhaps instead of) a lack of deficit in the PCP animals. Although there is significantly more exploration of the novel object in all observed cohorts this is not as large an effect as expected and there may be a strain or experimental issue with the testing as discussed above. All procedures were identical to previous studies in the

current lab so it is surprising not to observe a robust NOR effect in saline treated animals though. This highlights the importance of validating deficits in any model system by using multiple tests and not relying on a single assay. In fact, the development of the distraction assay was hoped to become one of these tests which could be useful in this model.

These issues mean that it is not yet possible to say there is no difference in PCP treated animals within the current task. The distraction assay may prove to be a useful tool to assess deficits and pharmacological targets in the PCP and other models. The ambiguity introduced by the lack of NOR effect highlights fundamental issues with relying on behavioural measures of deficit in the PCP model. There is a need to find other methods to validate successful PCP treatment and assess the deficits in the PCP model. An approach using convergent behavioural and neurochemical approaches may provide more reliable results.

3.5.4 Order of testing

One possible confound and important variable to consider when determining why we did not observe a NOR deficit, and in discussing the distraction results, is the order in which we tested the rats on these different assays. These animals underwent lick training and the distraction assay, then several injections including amphetamine before the NOR procedure. These rats had become habituated to distracting stimuli throughout the distraction task and were less responsive (did not pause) to external distractors. It is possible that the task itself induced some form of resistance to distraction through repeated testing. Maybe rats are habituated not only to salient stimuli but also to novelty as these tasks involve the repeated presentations of novel stimuli. However, the NOR and distraction contexts are vastly different and we would not expect the distraction task to interfere with such a different, and innate behaviour. Furthermore, this explanation cannot account for the small effect we observed in saline animals where we seem to see a poorer recognition memory or

encoding than previous experiments, even in controls. Further experiments could run these same tests in the opposite order to assess this possibility.

3.5.5 Conclusions and next steps

The described experiments have shown the distraction assay to be a replicable test with 3 cohorts of both male and female animals showing the same interruptions of licking in response to external distractors. Although it is difficult to make any reliable conclusions about the effects of PCP on distraction using this task, we have shown that there is a clear habituation effect in this task with rats becoming used to distractors and ignoring them with repeated testing. In addition we further characterise the effects of amphetamine in reversing this habituation.

As it is unclear whether PCP treatment affects distraction and because there is no obvious and clear explanation for the lack of NOR deficit, all further experiments will focus on non-treated animals. It is crucial to understand the basic mechanisms involved in the distraction from ongoing consumption and this can be applied to disease models in the future once we have characterised neural responses in control animals. The following chapters will use calcium based measurements to investigate the neural activity during licking for saccharin and during distraction from that ongoing consummatory behaviour. We record from VTA cell bodies (**Chapter 4**) and from terminals in the NAc originating from the VTA (**Chapter 5**) before assessing the contribution of dopamine to these neural responses (**Chapter 6**).

CHAPTER 4

Fibre photometry recordings from ventral tegmental area (VTA) cell bodies during distraction

4.1 INTRODUCTION

4.1.1 Recording neural activity

A major goal of neuroscience is to understand how neural activity encodes and orchestrates behaviour. The ability to record neural activity in the brain of awake and behaving animals and time lock this activity to important events is fundamental in striving towards this goal. Many methods exist for assessing real-time neural activity, several of which have already been discussed including electrophysiology, microdialysis and fast scan cyclic voltammetry. However, recent advances in calcium-based methods have provided new insights into the neural circuits underlying motivated behaviours. Key technological advancements in genetically encoded calcium indicators and viral strategies to target their expression have been instrumental in these developments. The ideal properties of calcium sensors used in such methods are fast temporal resolution, cellular specificity, good signal to noise ratio and the ability to remain stable for long periods of time for repeated, sustained measurements of neural activity (Jennings & Stuber, 2014, Chen et al., 2013).

4.1.2 Calcium signalling

Calcium is fundamental in neuronal signalling, the relationship between calcium and neuronal activity is complex and multifaceted. However, it is known that increases in intracellular calcium and subsequent calcium release from intracellular stores is crucial for neuronal activity and transmitter release (Katz & Miledi, 1967). Calcium influx at presynaptic terminals is required for exocytosis via synaptic vesicles (vesicle release (Katz and Miledi, 1967). Calcium imaging methods take advantage of the rapid calcium influx occurring across the cytoplasmic membrane during action potential generation (Baker et al., 1971; Tank et al., 1988). Using these changes in intracellular calcium, modern calcium imaging tools can measure dynamic changes in calcium concentrations as a proxy for neuronal activity and action potential generation (Kerr et al., 2000; Regehr et al., 1989). These methods allow for measurement of temporal

differences in calcium concentration of populations of neurons and permit long term (days to week) recordings of deep brain structures.

4.1.3 Calcium indicators

The development of calcium indicators has facilitated the recordings of calcium fluctuations in live, behaving animals. The most important advancement in calcium imaging and the reason we are able to make real-time measurements using methods such as fibre photometry, have been the development of genetically encoded calcium indicators (GECIs), in particular the GCaMP family. Before the development of modern GECIs, calcium sensors consisted mainly of calcium sensitive dyes such as Fura-2 (a combined calcium chelator and fluorescent chromophore, Tsien, 1980; Grynkiewicz, Poenie & Tsien, 1985) which required complex cell-loading procedures and had poor signal to noise ratios. In addition, the fact that GECIs are genetically-encoded means that their expression can be precisely targeted to subpopulations of neurons using sophisticated genetic tools (Jennings & Stuber, 2014).

Developed by Nakai, Ohkura and Imoto (2001), GCaMP has been the most important calcium indicator and its development has been fundamental in the application of recording methods such as fibre photometry. GCaMP is an engineered fluorescent protein, it consists of circularly permuted enhanced green fluorescent protein (cpGFP). The N-teminus of this calcium sensor is linked to M13 (a fragment of myosin light chain kinase) and the C-terminus is linked to calmodulin (CaM) (Akerboom et al., 2009). M13 acts as a CaM binding peptide so that when calcium is present and binds to CaM, the M13 and CaM domains interact (Zhao et al., 2011). This conformational change around the chromophore of the cpGFP leads to water-mediated changes (Wang et al., 2008), which result in the emission of green fluorescence at approximately 515 nm (Chen et al., 2013; Gore et al., 2014). **Figure 4.1** shows the structure and function of GCaMP genetically encoded calcium indicator.

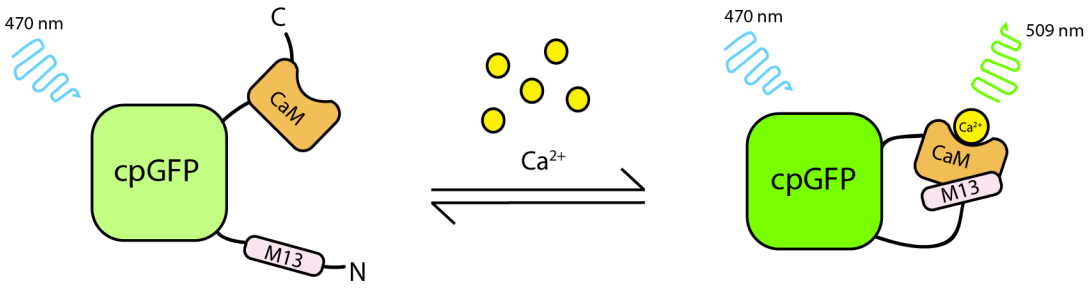


Figure 4.1 – *Structure and function of GCaMP*

Circularly permuted GFP containing a calmodulin binding site and an M13 myosin light chain kinase element. In the presence of calcium, which binds to the CaM site, the CaM binding site and M13 and CaM interact causing a change in the properties of the chromophore. The result is a rapid increase in fluorescence emission of 515 nm.

Since its original design (Nakai et a., 2001) mutagenesis experiments have optimised these indicators, such engineering has led to increased sensitivity with faster responses, greater affinity for calcium, improved fluorescence emission and better signal to noise ratios. Several variants have been engineered and published including a GCaMP3 (Tian et al., 2009) GCaMP5 (Akerboom et al, 2009) and the GCaMP6 (Chen et al., 2013) versions. The indicator used in this thesis is GCaMP6s and GCaMP6 is the most widely used variant in fibre photometry experiments. The GCaMP6 indicators are ultrasensitive and exist in three varients, GCaMP6s, 6m or 6f (slow, medium or fast referring to their kinetics) (Chen et al., 2013). In our experiments we utilise the s variant, which is the most sensitive albeit slower than others. In fact, it is sensitive enough to produce large fluorescence transients in response to single action potentials (Chen et al., 2013)

4.1.4 Fibre photometry

Fibre photometry is a method used to measure the calcium fluctuations and changes in fluorescence induced by GECIs. Typically, a virus such as an adeno-associated virus (AAV; in our case we used AAV9.Syn.GCaMP6s.WPRE.SV40) is used as a vector for the GECI, usually GCaMP. Such viral constructs can infect neurons specifically and, in the case of more complex strategies, they are able to infect neurons in a cell specific manner. Chapter 6 discusses such strategies in relation to targeting dopamine neurons within the VTA in further detail. The basic premise of this method, which uses relatively simple optical equipment, is to record the emitted fluorescence of the GECI, GCaMP, during behaviour and to investigate real time neural activity in response to behavioural events.

In fibre photometry, an optical fibre is implanted chronically and LEDs are used to cause excitation of GCaMP. The same optic fibre used to deliver the excitation wavelength also collects emitted fluorescence from the target area. A system of dichroic mirrors (use and details in the Methods section) is used to separate the input and output signals by wavelength. The output signal is then filtered and collected by a photoreceiver. In this way, the emitted fluorescence from a GECI during behaviour can be recorded in real time (Adelsberger et al., 2005; Cui et al., 2014; Gunaydin et al., 2014).

In fibre photometry, the collected signal is a ‘bulk’ fluorescence signal (Siciliano & Tye, 2018). This refers to the fact that measured fluorescence arises from a population of neurons expressing GCaMP around the fibre tip rather than spatially precise, individual neuronal responses. In other words, this method reports summed neural activity. The majority of fibre photometry experiments use two light sources, one to excite the GCaMP at its calcium-modulated wavelength (~470 nm) and another to control for autofluorescence and motion artefacts, by exciting GCaMP at its isosbestic wavelength (Barnett et al., 2017; Tian et al., 2009). In the case of GCaMP, this isosbestic wavelength is approximately 400 nm (violet light). Signals elicited by this wavelength are

independent of calcium as the way in which GCaMP absorbs this wavelength is not modulated by calcium and this reports non-specific autofluorescence (Barnett et al., 2017; Tian et al., 2009; Jercog, Rogerson & Schnitzer, 2016). The two different wavelengths modulated at different, non-overlapping carrier frequencies, which allows the resulting signal recorded at the photoreceiver to be demodulated

There have been several landmark studies using fibre photometry which have examined deep brain structures in the awake behaving animal. The first photometry studies from the Deisseroth lab (Gunaydin et al., 2014) examined neurons projecting from the VTA to the NAc and implicated this pathway in social interaction. These experiments were amongst the first to measure transmitter release indirectly using fibre photometry within terminal regions and demonstrated the utility of fibre photometry.

This chapter will use fibre photometry to record from neurons within the VTA during saccharin consumption and in response to distractor stimuli. Although there is considerable evidence for a primary role of the prefrontal cortex in attention there are many related areas, which can influence how we perceive stimuli. The ability to selectively attend to important, relevant behavioural stimuli within the environment is essential. Attentional functions involve the constant monitoring of salient stimuli and there is an important contribution of VTA dopaminergic inputs to cortical areas which subserve attention (Totah, Kim & Moghaddam, 2013). VTA projections provide real-time information during learning about the state of environmental cues and the VTA is an important site for the integration of information about internal need states and external stimuli.

4.2 AIMS, OBJECTIVES & HYPOTHESES

4.2.1 Aims

1) Investigate VTA neural activity during licking for saccharin
2) Characterise VTA neural activity in response to distractor stimuli
3) Compare VTA activity when an animal is distracted versus when they are not distracted
4) Determine how VTA responses to distractors develop over repeated testing

4.2.2 Objectives

1) Inject AAV virus containing GCaMP6s into VTA cell bodies and implant photometry fibres
2) Record calcium fluctuations using fibre photometry in the VTA during licking and in response to distracting stimuli using the behaviour described in chapters 2 and 3
3) Compare VTA responses when the animal is distracted and not distracted by stimuli
4) Perform histology to identify TH positive and GFP positive neurons and determine co-localisation of these markers

4.2.3 Hypotheses

1) Licking for saccharin will produce a change in VTA neural activity (e.g. calcium transients will be observed at the start of periods of licking)
2) During distraction testing, distractor stimuli (lights, auditory cues) will evoke transient activity increases in VTA cell bodies (due to the sensory and salient nature of these stimuli)
3) There will be greater neural activity during distracted versus non-distracted trials
4) There will be significant overlap (co-localisation) between TH-positive and GFP-positive cells in the VTA showing that the calcium signals recorded during photometry are predominantly dopaminergic

4.3 METHOD

4.3.1 Animals

Fourteen male Sprague Dawley rats (300g - 350g at time of surgery) were used in fibre photometry experiments (Charles River, UK). All animals were housed in pairs in individually ventilated cages under temperature controlled conditions (21°C ± 2°C; 40-50% humidity) and kept under 12 h light/dark cycle, with lights on at 7am. Larger groups were avoided to protect head implants from damage and paired animals were observed frequently for signs of aggression. Rats had access to food and water ad libitum, except for a period of food restriction before testing and during experimental sessions, when only saccharin was available. All procedures were carried out under the appropriate license authority in accordance with the Animals (Scientific Procedures) act (1986). Project licence 70/8069 personal licence I9202C6A3.

4.3.2 Viral injection and implant surgery (VTA)

Rats were placed in an anaesthetic induction chamber and anesthetised with isoflurane (5% isoflurane at 2 litres per minute). Once anaesthetised and the pedal reflex absent, the rat was transferred to a rat anaesthetic mask and maintained at 2% isoflurane whilst the scalp was shaved and cleaned with chlorhexidine. Local anaesthetic (bupivocaine 150 µl) was injected subcutaneously along the incision site on the scalp. In addition, a non-steroidal anti-inflammatory (meloxicam 1 mg/kg) and saline (2 ml) were given via intraperitoneal injection. The rat was transferred to the aseptic surgical area. Teeth were positioned over the incisor bar of a stereotaxic frame with fitted anaesthetic mask (David Kopf Instruments: CA). The rat was mounted in the frame using blunt ear bars (David Kopf Instruments) coated in topical anaesthetic cream (EMLA cream). A rectal temperature probe was inserted and a pulse oximeter was used to monitor oxygen saturation and heart rate throughout the surgery (typical ranges 96-100% O_2, 280 – 400 BPM). A thermostatic blanket was used to maintain a stable body temperature throughout surgery (37-38 °C).

For viral injection, an incision in the scalp was made and the skull was cleaned with sterile cotton buds. Bregma was measured using a hypodermic needle attached to one arm of the frame and the AP and ML co-ordinates of the ventral tegmental area (VTA) were marked on the skull (AP – 5.8 mm, ML + 0.7 mm). A 0.7 mm hole was drilled at the VTA using an electric dental drill for the virus injection/fibre implantation. A further 4 holes were drilled for later positioning of anchor screws – these were typically drilled 2 anterior and 2 posterior to the VTA, with one in each of the cranial bones. A 10 µl Hamilton syringe loaded with the GCaMP6s virus (AAV9.Syn.GCaMP6s.WPRE.SV40, with an undiluted titre of 7.528 x 10^{13} GC/ml) diluted 1:4 with aCSF was then lowered into the VTA (AP – 5.8 mm, ML + 0.7 mm, DV – 8.1 mm), **Figure 4.2** shows this viral construct and **Figure 4.3** the injection and implantation site. 1 µl of diluted virus was injected over 10 minutes (rate 100 nl / min) into the VTA using a pump (Harvard Apparatus, CA). The needle was left for 5 minutes following injection before being removed. Once the virus injection needle was removed, through the same VTA hole an optic fibre cannula was implanted to DV – 8.0 mm (0.1 mm above the virus injection site) (ThorLabs CFM14L10, 400 µm, 0.39 NA, 10 mm length). Four anchor screws were inserted and a headcap was formed using two types of dental cement (C&B Supabond and regular dental acrylic, Prestige Dental, UK). The headcap was built such that approximately 5 mm of the fibre ferrule was left protruding to allow the fibre to be coupled to recording apparatus during behavioural testing. Rats were housed in pairs immediately for recovery and 4 weeks was left before recording to allow ample time for virus expression.

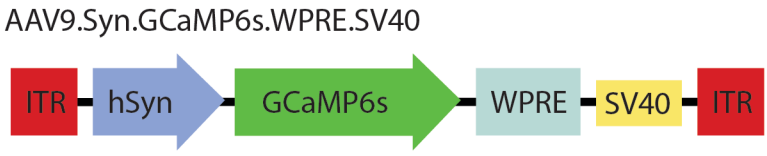


Figure 4.2 – *Adeno-associated viral construct*

Adeno-associated virus (AAV) construct for delivering GCaMP6s calcium indicator to neurons in the VTA.

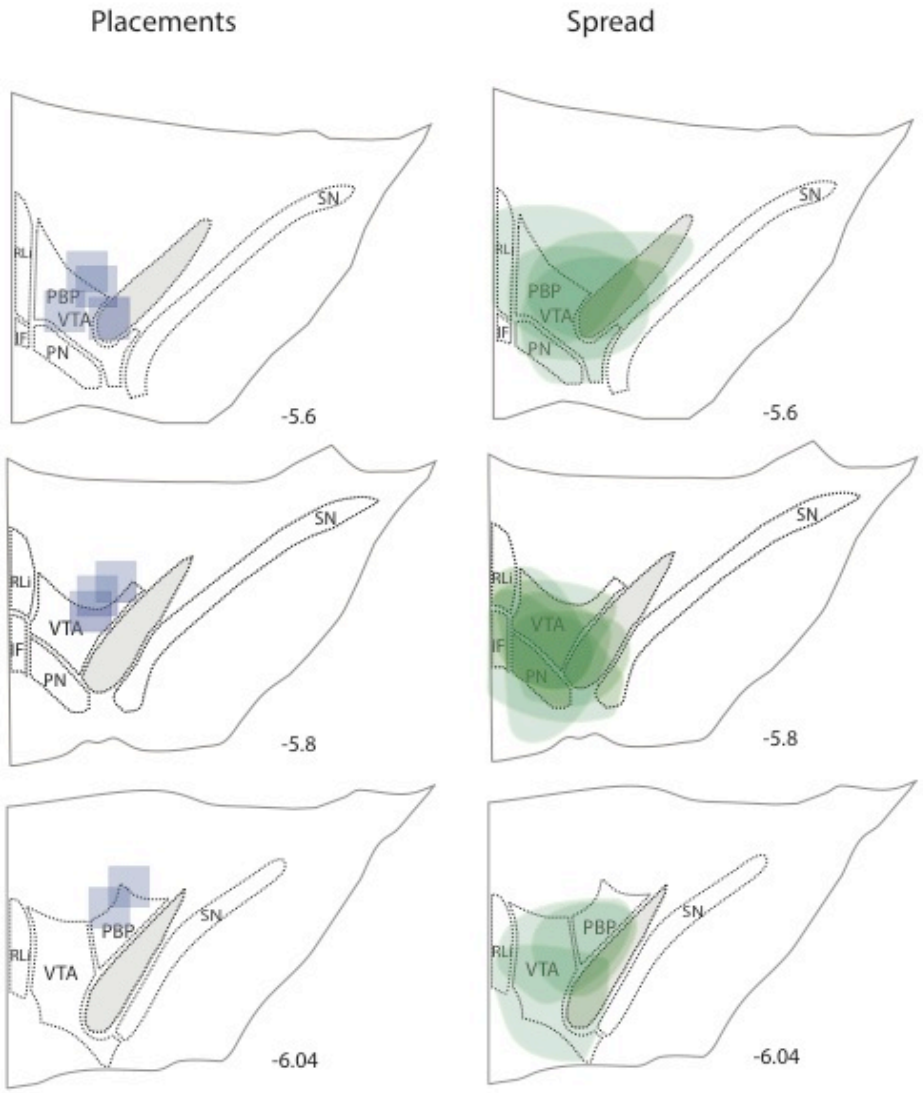


Figure 4.3 – *Fibre placement and viral spread in the ventral tegmental area*

Histological verification of fibre placements was conducted. Blue squares show electrode placements in the VTA and green densities represent viral spread (GFP positive neurons) in all animals (n=12). Anterior-posterior spread is shown with atlas images spanning -5.60 to -6.04 from Bregma (atlas images are modified from Paxinos and Watson (2005)). VTA subnuclei are labelled as follows: VTA (ventral tegmental area), PBP (parabrachial pigmented area), PN (paranigral nucleus), RII (rostral linear nucleus), IF (interfascicular nucleus)

4.3.3 Photometry set up

The fibre photometry recording set up consisted of two fibre-coupled light sources powered by LED drivers (Thorlabs, LEDD1B T-Cube LED driver). To deliver the excitation wavelength, a 470 nm blue LED was used (Thorlabs, M470F3) and to control for non-specific non-calcium modulated fluorescence changes, a 405 nm violet LED was used (Thorlabs, M405F1). Both light sources were attached to kinematic slip plates to allow for precise alignment and adjustment. Individual bandpass filters were used for the blue light source (filter 470 nm, Thorlabs, MF469-35) and the UV light source (405 nm, Thorlabs, FB405-10). The fibre-coupled LED light sources were passed through separate collimators, narrowing the light into a focused beam, before being directed into the first of two filter cubes both fitted with dichroic mirrors. The first dichroic mirror (Thorlabs, DMLP425) allowed blue light to pass through and reflected the violet wavelength, this directed both light sources to a second filter cube. Here, both 405 and 470 wavelengths are reflected from a dichroic mirror (Thorlabs, MD498), through a 400 μm core patch cord cable and into the implanted optic fibre cannula. Green light emitted from GCaMP fluorescence was received through the same fibre cable and fluorescence from calcium dependent and non-calcium dependent activity was relayed, via a 469 nm filter (Thorlabs, MF525-39), to a femtowatt silicon photoreceiver (Newport #2151) which was used to count individual photons of light output from the brain. Tucker Davis software (Synapse) was used to analyse the fluorescence changes during behaviour. **Figure 4.4** shows a detailed diagram of the fibre photometry set up.

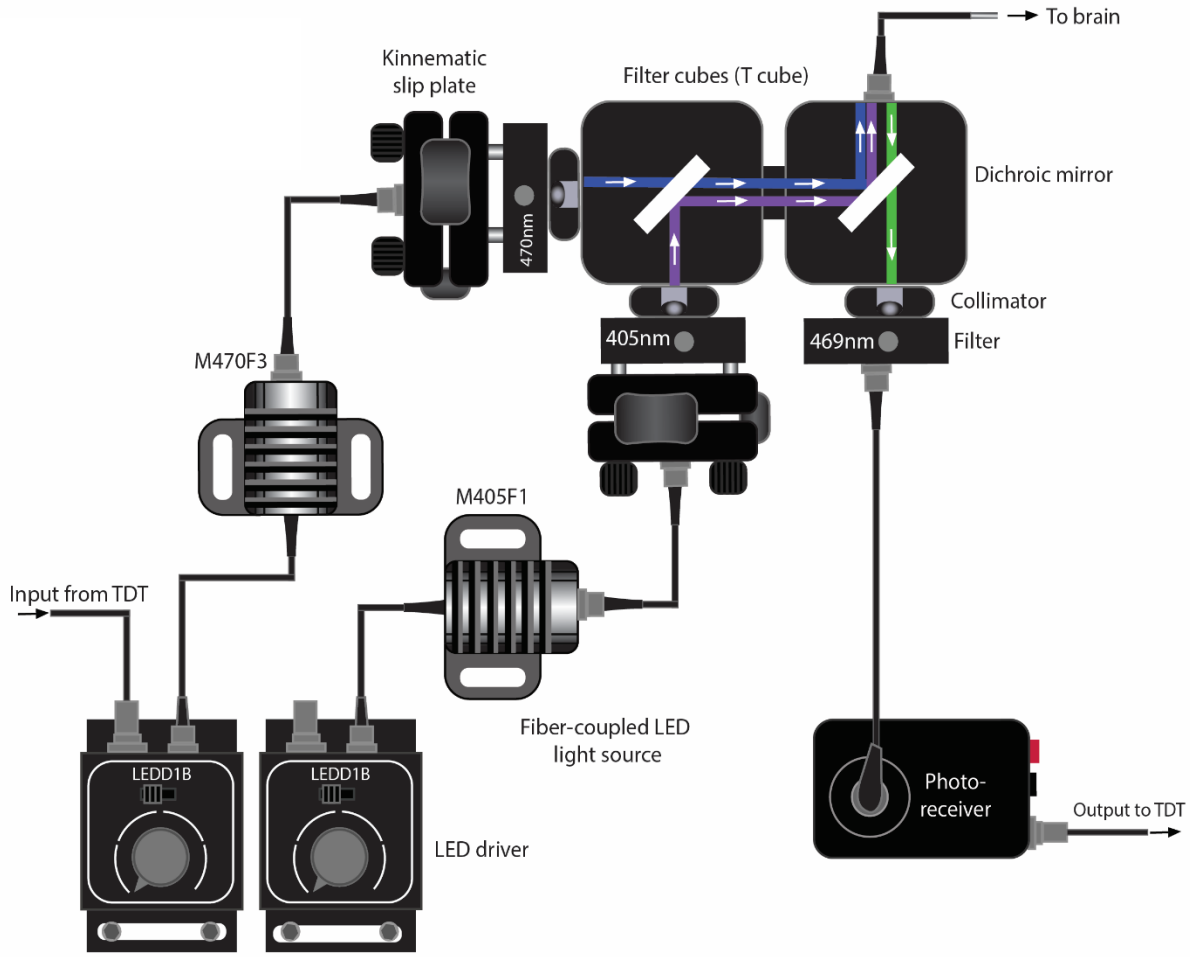


Figure 4.4 – *Fibre photometry set up*

Detailed schematic of the fibre photometry set up. Two fibre coupled LEDs are powered by drivers, 405 nm violet and 470 nm blue light sources. The light path passes through a series of dichroic mirrors directing these excitation wavelengths to the recording fibre and rat. Emission (515 nm) from GCaMP6s within the rat brain is collected via the same fibre optic and patch cord and a photoreceiver collects this before it is processed by the Tucker Davis software, described in the following section.

4.3.4 TDT Software

Tucker Davis Technologies (TDT) Synapse software was used to control all photometry settings and to record light output from the brain. A key function of the Synapse programme is to demodulate the mixed light signals from the brain. The received green light signal is a mixture of signals evoked by both blue and violet light. Synapse allows the two light sources to be delivered at different sinusoidally modulated frequencies (blue light 470 nm at 211 Hz and violet light 405 nm at 539 Hz). These carrier frequencies are sufficiently different (with non-overlapping Fourier spectra) to be separable by mathematical demodulation, which Synapse performs on-line during recordings. The raw signal, before demodulation, was sampled at 6.1 kHz, and the demodulated signals were sampled and recorded 1017 Hz. Synapse also recorded behavioural signals by registering TTLs from the MEDPC system, which recorded licks, distractor stimuli and whether the animal was distracted or not on a given trial, as well as video files captured via connected webcams (10 Hz).

4.3.5 Distraction testing

All behavioural experiments were carried out in operant behaviour chambers similar to those used in behavioural experiments Chapters 2 and 3. (MedAssociates, UK). Chambers were 25 cm X 32 cm X 25.5 cm and housed inside aluminium sound attenuating chambers (built in-house). Operant chambers are described previously, in the case of photometry experiments there was a hole in the ceiling of the chamber to allow the tether to be connected from the fibre photometry light sources to the animals' head. An interconnecting ceramic sleeve was used to couple the ferrule on the rat's head to the fibre optic cable from the photometry system.

The lick training and distraction programmes were written in MED-PC. For lick training days, rats were placed in the operant chamber with access to a spout. Rats could freely lick for saccharin (0.2%) during a 60 minute session. A contact

lickometer recorded individual licks and these were registered using MED-PC software. In addition, TTLs were generated for each individual lick and sent to Synapse, to be synchronised with photometry recordings. Before the first licking session rats were food restricted overnight (given 10 g per rat 24 h before testing). Rats were trained to lick for saccharin for 3-6 days until they reached a set criterion of 1000 licks within 60 minutes. No rats were excluded according to this criterion. TTLs were also used to record the timings of distractors and whether the animal was distracted or not on the Tucker Davis Synapse programme.

4.3.6 Immunohistochemistry

To verify that photometry fibres were implanted in the correct brain area, electrode placements were histologically verified and compared to images from a rat brain atlas (Paxinos et al., 1985). Immunohistochemistry was performed to assess viral expression of the GCaMP6s AAV within the VTA and to determine what proportion of these cells were also positive for tyrosine hydroxylase (TH), therefore dopaminergic. Following behavioural experiments all rats were anaesthetised using isoflurane (5%) before being terminally anesthetised using pentobarbital (50 mg/ml; 1.5 ml per 300g body weight). Once breathing had subsided and pedal reflex was absent, trans-cardiac perfusion was carried out. The sternum was cut using surgical scissors and either side of the ribs were cut and the chest wall retracted. The diaphragm was severed and the heart exposed. A reusable feeding perfusion needle (18061-22, Fine Science Tools) attached to a variable flow peristaltic pump (Fisher Scientific) was inserted into the left ventricle and the right atrium was promptly cut with small dissection scissors. Phosphate buffered saline (Sigma Aldridge, UK) was pumped through the heart at 10 ml/min flow rate. After 5 minutes the pump system was switched to 4% paraformaldehyde (PFA), the fixative was perfused via the pump for ten minutes following the first signs of fixation (paw twitching, liver clearing). Following fixation the head was removed and the brain harvested and placed in fresh ice cold 4% PFA. Brains were kept in PFA at 4°C for 24 hrs before being transferred to 30% sucrose solution (with 0.02% sodium azide to prevent

bacterial and fungal growth). Once brains had sunk in the sucrose solution, approximately 48 hours, they were sliced using a freezing sledge microtome. A coronal cut was made to remove the anterior most surface of the brain leaving a flat surface for mounting and the cerebellum was removed using a razor blade. The stage was cooled using dry ice and maintained at this temperature throughout. Each brain was affixed to the freezing stage with optimal cutting temperature medium (OCT) anterior side down so that the brain was sliced from posterior to anterior. Once fixed to the stage, crushed dry ice was applied to cover the brain and left for 5 minutes to freeze the specimen. The dry ice was removed from the brain and slicing began. Sequential 40 μm slices were taken and removed from the blade using a paintbrush dipped in PBS. All sections were taken and placed in 24 well plates for later staining and electrode verification.

The two primary antibodies used were: rabbit anti-tyrosine hydroxylase (AB152, Millipore, UK) and chicken anti-GFP (A10262, Fisher Scientific, UK), the secondary antibodies were: donkey anti-Rabbit IgG (H+L) highly cross-adsorbed, Alexa Fluor® 594 (Fischer Scientific, UK) and goat anti-chicken IgY (H+L) secondary antibody, Alexa Fluor® 488 conjugate (Life Science Technologies). Preliminary experiments determined an appropriate concentration of both primary antibodies (1:1000) and established that the protocol was successful to run both antibodies in parallel. Secondary antibodies were applied at 1:250 dilution.

Slices were selected for staining, VTA slices containing the fibre track as well as NAc terminal regions were chosen for staining. Slices were transferred to 6 well plates (one well per brain with no more than 10 slices per well) and washed with PBS three times for 5 minutes. Slices were blocked in 3% normal goat serum, 3% normal donkey serum, and 3% Triton X detergent for 1 hour at room temperature. Primary antibodies were diluted in blocking solution. The primary antibody solution was left on the slices for 18 hours at room temperatures, well plates were constantly agitated by an automated plate shaker. Following incubation with primary antibodies slices were washed again with PBS three

times for 5 minutes. Secondary antibodies were diluted in PBS and applied for 90 minutes at room temperature before three final washes in PBS.

Slices were mounted onto slides using VectorShield Hard Set mountant (Vector, UK) and coverslipped. Images were taken using an epifluorescent microscope (Leica, UK) and cells were counted using images from the red and green channels and Image J software. Regions of interest were manually selected and fluorescence co-localisation quantified.

4.3.7 Data analysis

Behavioural data were analysed as previously described in Chapter 3. ANOVA was used to compare percent distracted across test days and Bonferroni corrected t-tests were used to follow up significant main effects.

Fibre photometry data, consisting of blue and violet signals as well as licking and event-related TTL pulses, were extracted from Synapse data files using custom MATLAB scripts which converted these files into .mat files that Python could access (https://github.com/mccutcheonlab/tdt-convert). Once data were extracted, all further analyses were completed using Python 3.6 or SPSS. Blue and violet signals were processed and aligned to events of interest. Custom written functions were used to average individual trials and to calculate epochs aligned to various events, such as distractor presentations. These epochs were then background subtracted and scaled to give change in fluorescence (ΔF) relative to the background signal.

Four parameters were calculated from averaged traces aligned to specific events that were calculated as follows and are detailed in **Figure 4.5**:

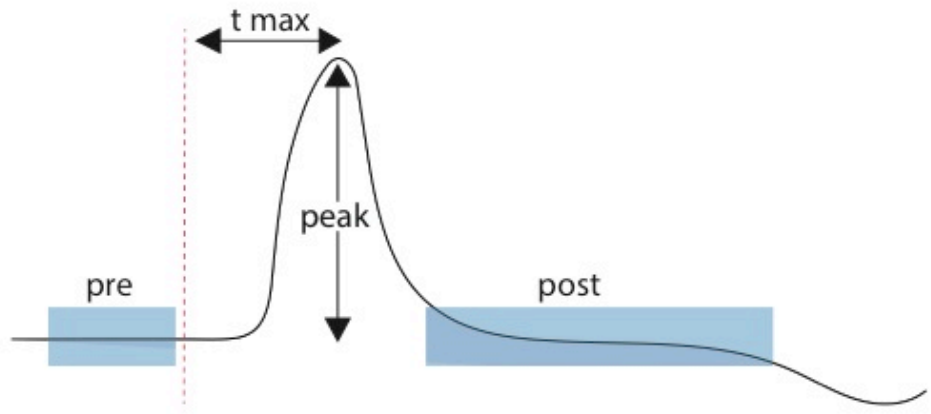


Figure 4.5 – *Schematic of calculated photometry peak parameters*

Peak – the maximum value following a given event, expressed as percentage change from baseline (5 seconds before the event)
T-Max – the time to peak, the time in seconds from the event to the maximum value (within a 3 second window from the beginning of the event)
Pre-event period (pre) – mean activity change across the 5 seconds before the event
Post-event period (post) – mean activity change across 20 seconds following an event

All Python 3.6 code used to extract, subset and plot these data can be found on the Github repository of Kate Peters (*https://github.com/KatePeters/Thesis*). The MATLAB conversion script and other functions can be found on the github repository of Dr James McCutcheon or the McCutcheon lab page (*https://github.com/mccutcheonlab*). All files are open access and all data are freely available upon request.

To assess the parameters of the photometry signals in different event conditions, MANOVA, ANOVA and planned comparison t-tests were used and are described in detail in each results subsection. For all hypothesis tests, SPSS was used, alpha was set at .05 unless otherwise specified, and all tests were two-tailed. Assumptions of normality, sphericity and homogeneity of variance were met unless otherwise specified.

4.4 RESULTS

4.4.1 Licking behaviour

Runs were defined as periods of sustained licking activity separated by pauses of greater than 10 seconds and were calculated for the final lick training day. For all rats combined there was a total of 301 runs of licking. The mean number of licks per run was 143 and the median was 80 licks. The shortest runs were 1 lick long and the longest was 1301 licks. **Figure 4.6** shows a histogram of all run lengths for all rats.

For later analysis, runs were divided into quartiles so the upper and lower 25% could be assessed separately. All run lengths were ordered from shortest to longest and the boundaries were calculated for the upper and lower quartiles. These cut-offs were <20 licks for the shortest runs and >169 licks for the longest.

For SHORT runs: Mean was 7 licks, median was 6 licks and the range was 1 – 19 licks.

For LONG runs: Mean was 400 licks, median was 358 licks and the range was 170 – 1301 licks.

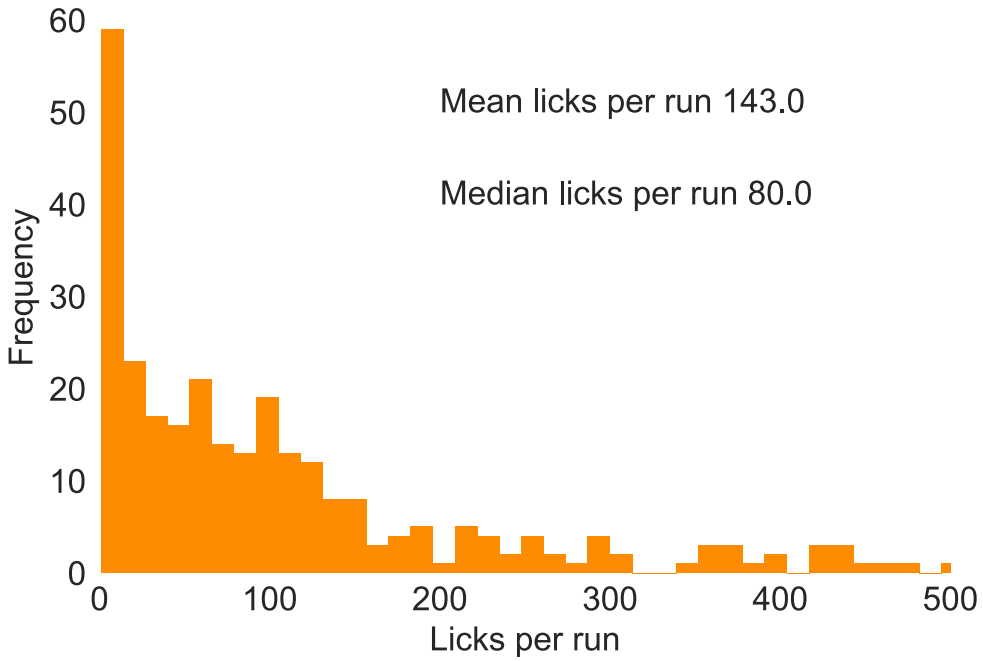


Figure 4.6 – *Histogram of all run lengths*

Histogram of all licking run lengths, this distribution is skewed, showing a long tail to the right. There is a large proportion of short runs and many unique values in the tail representing very long runs of licks.

4.4.2 Percent distracted across days

Rats underwent the same distraction test as previously described (**Chapter 2 and Chapter 3**). **Figure 4.7** shows representative raster plots of licking responses during distractor presentations. There are clear pauses in licking during the distraction day, which are not observed on the modelled day when these licks are aligned to modelled distractors.

To assess distraction behaviour and to verify that this was not affected by fibre photometry implants or during tethered behavioural testing, percentage distracted was compared across days as with Chapters 2 and 3. Percent distracted across three test days is shown in **Figure 4.8**. One way ANOVA comparing percent distracted on the MODELLED day, DISTRACTION day and HABITUATION day showed a significant main effect of day ($F(2,22) = 26.67$, $p < .001$). Bonferroni corrected post hoc comparisons revealed that compared to the modelled day ($M = 3.56\%$) there was significantly higher percent distracted on both distraction ($M = 51.62\%$, $p < .001$) and habituation ($M = 26.60\%$, $p < .05$) days. There was significantly lower percent distracted on habituation day when compared to distraction day also ($p < .01$) – showing the same habituation effect seen in previous behavioural experiments using this task.

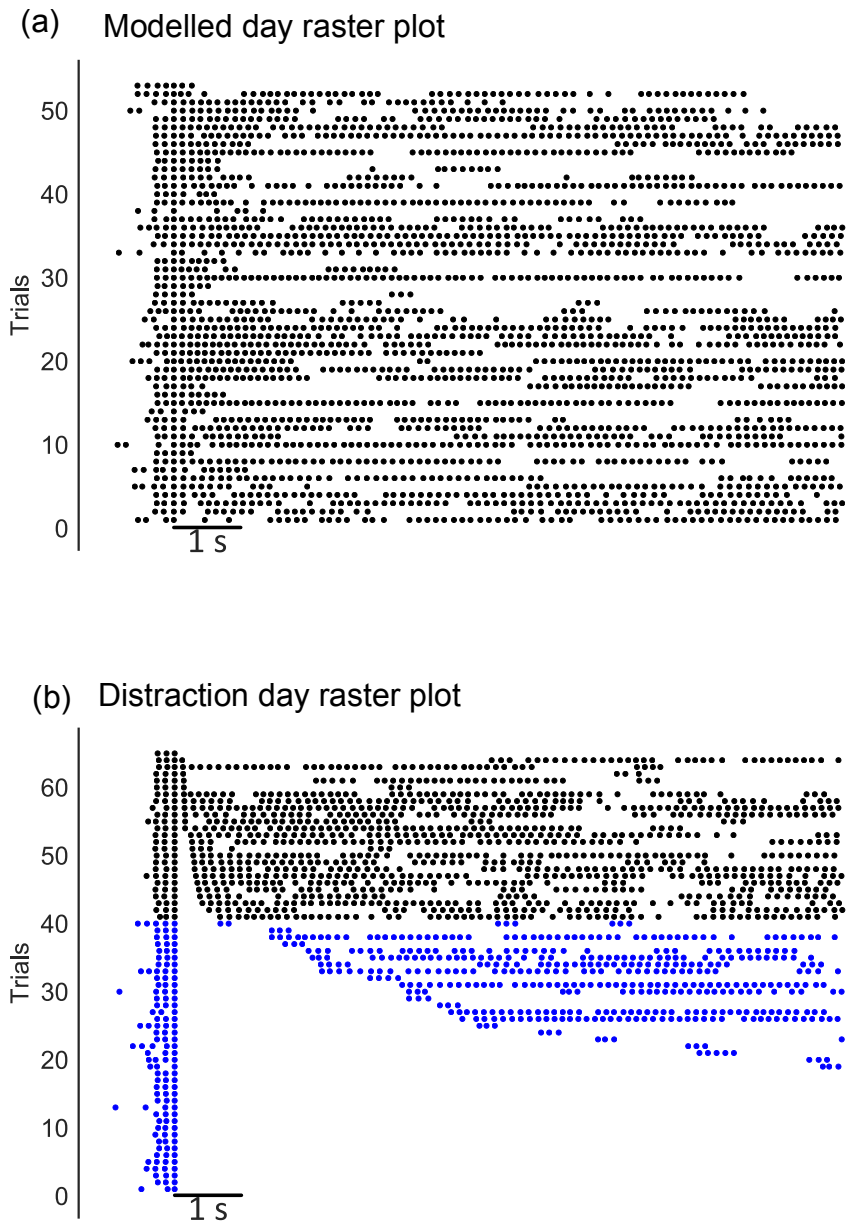


Figure 4.7 – *Raster plots of licks aligned to distractor presentations*

Raster plots show individual licks in rows (black dots), aligned to the onset of distractor stimulus, each row is a distraction trial. These are plots from one representative rat on **(a)** the modelled day with licks aligned to modelled distractors and **(b)** the main distraction test. Blue dots show where pauses following the distractor are >1 second, and thus that trial is classified as distracted. This rat has a percent distracted value of over 50%.

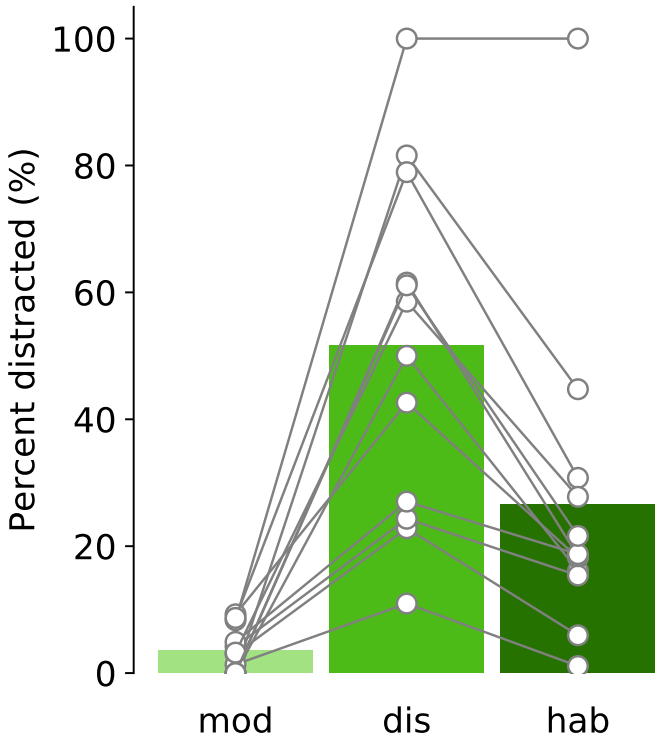


Figure 4.8 – *Percent distracted across three sessions*

Percent distracted on the modelled, distraction and habituation days are shown. Bars represent means and individual white circles are individual rat data. As with previous experiments rats show a clear increase in percent distracted on the distraction day compared to the modelled day, and in addition, rats show a habituation effect on the following day.

4.4.3 Photometry signals in the VTA in response to behavioural events

For each event all trials from all rats were averaged and binned into 30 second epochs aligned to the event of interest before the four parameters were calculated (peak, t max, pre and post event periods).

To assess the important parameters of the photometry signal which vary in response to different events, an initial MANOVA was performed. This was used to compare the four different parameters of the photometry signal (PRE-EVENT PERIOD, PEAK, TMAX and POST-EVENT PERIOD) for all events. MANOVA was used to determine which of these four parameters would be investigated in further analysis to compare specific pairs of events for licking and distraction events separately. The independent variable in MANOVA was "event' and had 8 levels as follows:

ALL RUNS – photometry signal aligned to the first lick in each run
SHORT RUNS – aligned to the first lick in the short runs only (lower quartile of run lengths)
LONG RUNS – aligned to the first lick in the long runs only (upper quartile of run lengths)
DISTRACTORS – aligned to the presentation of a distractor (all distractors)
DISTRACTED – aligned to distracted trials, where a distractor is presented and the rat is distracted (i.e. pauses licking)
NOT DISTRACTED – aligned to not distracted trials, where a distractor is presented and the rat is not distracted (i.e. continues to lick)
MODELLED DISTRACTOR – aligned to 'modelled' distractors, using data from a lick training day, the 'modelled' distractor events are where distractors would be presented if this was a distraction day (i.e. following three lick bursts)
HABITUATION – aligned to distractor presentations (all distractors) on the habituation day following initial distraction

Multivariate testing showed there was a significant effect of event on the calculated photometry parameters ($F(28,304) = 2.36$, $p < .001$; Wilks' $\Lambda = 0.493$). Following this significant result, univariate tests (ANOVAs) were

performed for each measure individually to assess which measured parameters where driving this difference. There were significant main effects of event for the peak ($F(7,87) = 2.33$, $p < .05$), tmax ($F(7,87) = 2.14$, $p < .05$) and post-event period ($F(7,87) = 4.89$, $p < .001$) but no main effect for the pre-event period ($F(7,87) = 1.33$, $p = .246$).

A priori planned comparisons for pairs of events which are of interest were performed only for those measures with significant ANOVA results (peak, t and post but not pre-event period). The planned comparisons were as follows and are presented in the following sections:

(1) **Short runs vs Long runs** – to assess differences between short and long runs of licks on photometry measures (section 4.4.4)
(2) **All runs vs All distractors** – to assess differences between photometry responses to runs and distractors (section 4.4.5)
(3) **Distracted vs Not distracted** – to assess photometry responses in distracted and not distracted trials on the distraction test day (section 4.4.6)
(4) **Modelled distractors vs Distractors** – to assess responses to modelled distractors (lick day) and to real distractors (distraction test day) (section 4.4.5)
(5) **Distractors vs Habituation** – to assess habituation effects, comparing distractor responses on distraction day and habituation day (section 4.4.7)

4.4.4 Photometry signals in the VTA increase during licking

To assess whether licking produced consistent changes in the fibre photometry signals measured within the VTA, the calculated 30 second epochs were aligned to the first lick in each run and then averaged.

Figure 4.9 shows individual trials, the blue and violet signals are shown with the licks plotted above. Although it is often difficult to see reliable signal changes on a trial by trial basis before group averaging, there are clear transient increases leading up to licking runs.

Figures 4.10 shows all trials for two representative rats, each light blue trace is a single trial and the darker blue lines are the mean response for that rat.

Figure 4.11 shows the whole group average of all trials aligned to the first lick in the run for all rats (n = 12 rats).

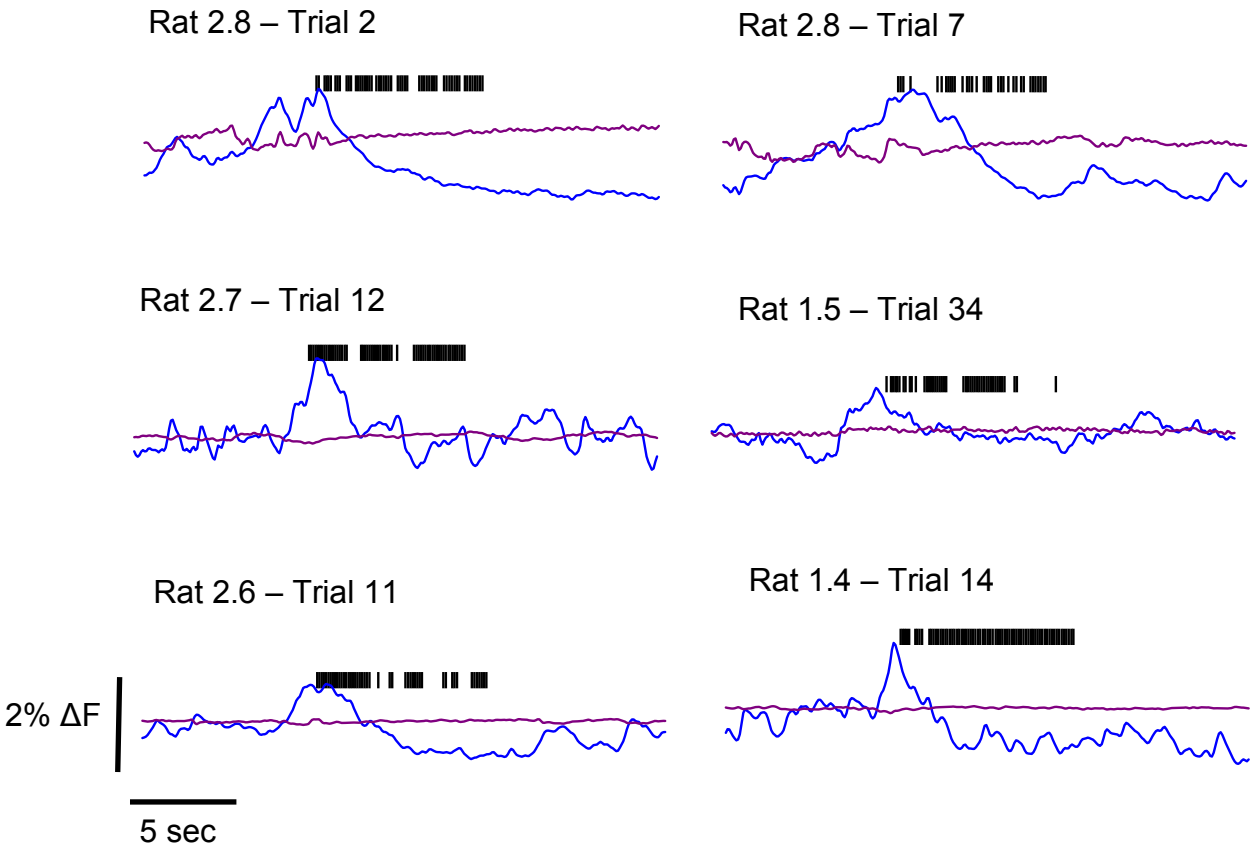


Figure 4.9 – *Individual trials aligned to the first lick in a run*
Photometry traces for individual trials with lick runs shown in five representative rats. Blue shows the 470 nm calcium modulated signal and purple shows the violet 405 nm control. All epochs are 30 seconds with the first lick occurring after 5 seconds. Black lines show individual licks. There are consistent changes in the photometry signal recorded during licking, namely a peak is observed at the onset of a run with some evidence of suppression in activity with sustained licking.

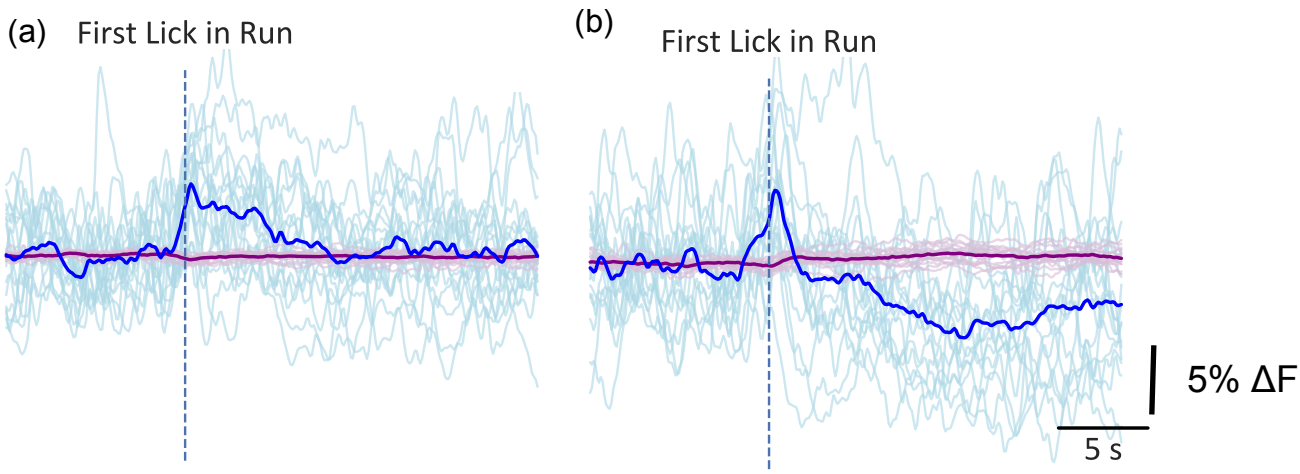


Figure 4.10 – *All licking trials aligned to first lick in a run for two representative rats*

Figures show all lick run trials for two representative rats. An epoch of 30 seconds is shown with photometry trials aligned to the first lick in the run. **(a)** Rat 2.7, calcium modulated signals for individual trials (pale blue) are shown for a total of 18 runs. Dark blue trace shows the mean of all trials for this rat and dark purple the mean of the control violet signal. **(b)** Rat 2.2, individual trials for 17 runs are shown (pale blue) with the mean trace in dark blue. Vertical dashed lines show the timing of the first lick within each run.

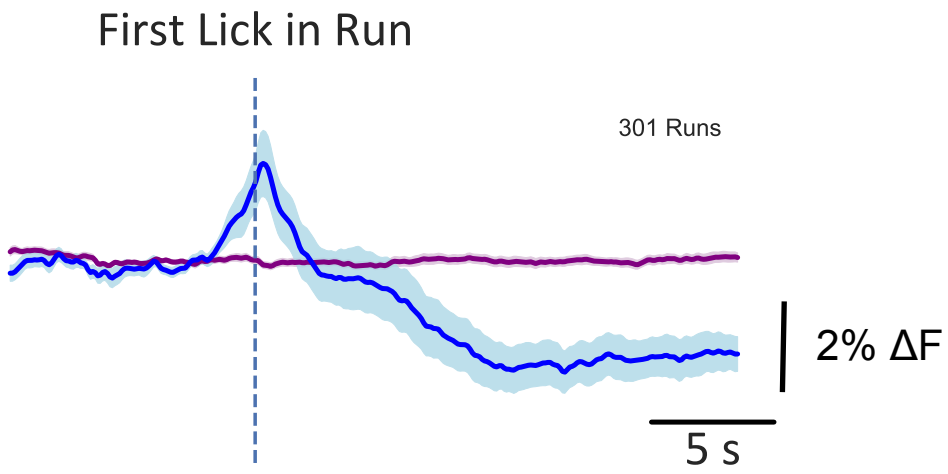


Figure 4.11 – *Averaged photometry trace for all runs in all rats*

VTA neural activity is increased in response to the initiation of licking runs. Photometry trace aligned to the first lick in the run for all runs from all rats. Dark blue trace shows the mean calcium modulated (470 nm) signal in response to the start of a run, with shaded light blue error (SEM), the purple trace shows the 405 nm control signal and associated error in light purple (SEM). Dashed vertical line represents trial onset (in this case the first lick in each run). Scales are shown as percentage change in fluorescent signal.

4.4.5 VTA activity is different for long versus short runs

To assess possible differences in photometry responses in the VTA between short and long runs of licks, t-tests were performed. Photometry signals were compared for signals aligned to the first lick in short runs (lower quartile of run lengths) and long runs (upper quartile of run lengths).

There was no difference in peak height between short and long runs ($t(11) = 0.88$, $p = .40$). There was also no significant difference in tmax (time to reach peak) $t(11) = 0.39$, $p = .70$, **Figure 4.13a, 4.13b**. There was a significant difference in the photometry signal during the post-event period (**Figure 4.13d)**. In response to long runs activity was significantly lower in the post-event period (M = - 2.7% ΔF, SEM = 1.2% ΔF) when compared to short runs (M = 1.2% ΔF, SEM = 0.6% ΔF, $p < .05$). There was a significant depression in activity following long runs which was not seen in short runs where there was a slight and sustained elevation (**Figure 4.12**).

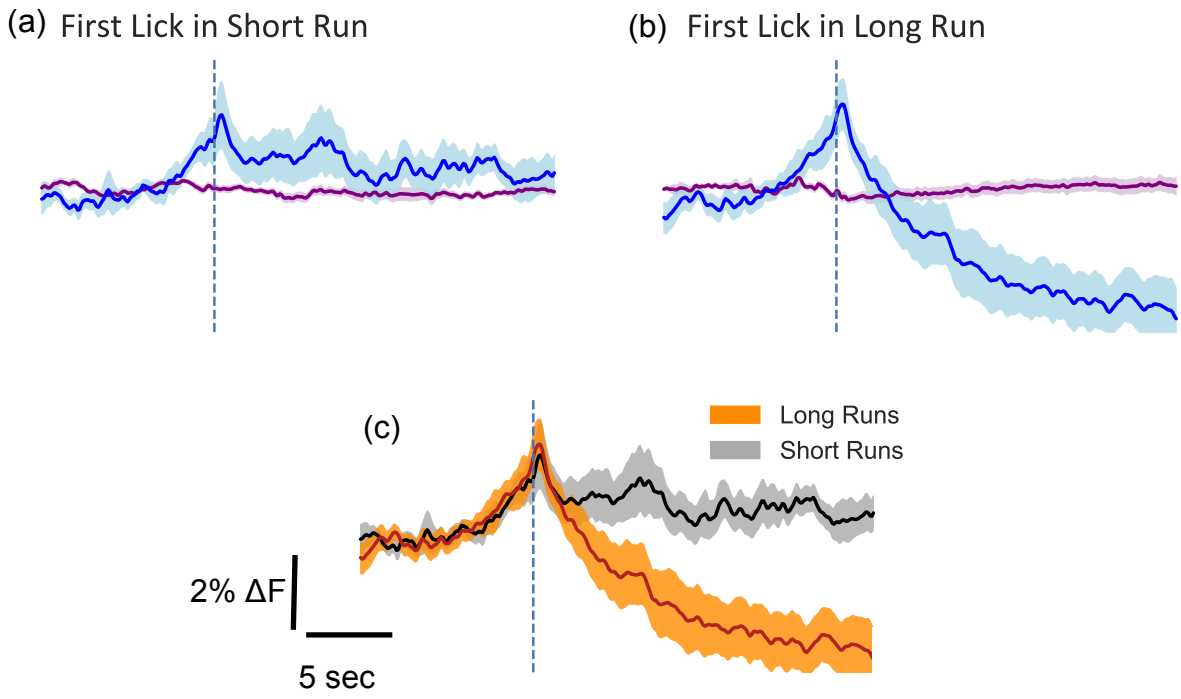


Figure 4.12 – *Averaged photometry traces for short and long runs*

VTA neural activity during licking initiation (the start of a run of licking) differs depending on the length of the run. **(a)** Averaged photometry trace for all rats and for all short runs, n = 75 runs, (defined by lower quartile of run lengths) aligned to first lick in the run. **(b)** Averaged photometry trace for all rats and for all long runs, n = 75 runs (defined by upper quartile of run lengths). In a and b, blue traces show the mean calcium modulated (470 nm) signal with SEM in shaded area and purple trace shows the 405 nm control signal. (c) VTA responses to short and long runs shown on the same plot, average responses for long runs (orange) show a significant suppression of activity following an initial peak, this is not observed with short runs (grey).

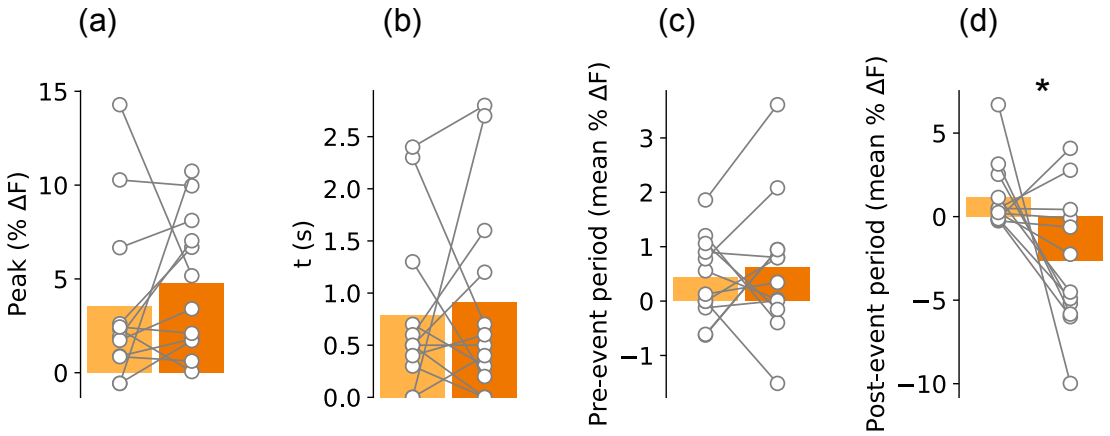


Figure 4.13 – *Calculated photometry parameters for short vs long runs*

Parameters calculated from averaged photometry signals for short (light orange) and long runs (dark orange). **(a)** Peak height is similar in short and long runs. **(b)** tmax, the time to reach peak height in short vs long runs **(c)** Pre-event period, the mean change in fluorescence in the five seconds preceding the start of the run is similar in short and long runs. **(d)** Post-event period, there is a significant elevation in the 20 seconds following the onset of short runs compared with a substantial suppression of activity seen with the onset of long licking runs. * $p < .05$

4.4.6 Photometry signals in the VTA following distractors

Figure 4.14 shows individual trials from representative rats with the 30 second epochs aligned to the presentation of a distractor. Filled circles represent distracted trials and open circles show not distracted trials. There are observable transient increases in photometry signals within these individual trials time locked to the distractor presentation and these are further analysed in the following section.

Figure 4.15 shows all trials for two representative rats, each light blue trace is a single trial and the darker blue lines are the mean response for that rat. **Figure 4.16a** shows the whole group average of all trials aligned to distractor presentation for all rats (n = 12 rats).

To assess whether photometry responses in the VTA were in response to distractors and not a general feature of baseline activity, photometry measures were compared between the modelled day and the distraction day. There was significantly higher peak response to real distractors (M = 3.4% ΔF, SEM = 0.5% ΔF) versus modelled distractors on the licking day (M = 1.8% ΔF, SEM = 0.4% ΔF, $t(11) = 2.74$, $p < .05$) (**Figure 4.17a)**. The tmax of the peak observed in response to real distractors (M = 1.1 sec, SEM = 0.14) was significantly longer than that of modelled distractors (M = 0.4 sec, SEM = 0.18, $t(11) = 3.503$, $p < .01$), **Figure 4.17b**. There were no differences in the post-event period measures ($t(11) = 0.97$, $p = .35$).

Despite the occurrence of transient peaks on the modelled day (where no distractors are present, **Figure 4.16c**), transients observed following real distractors are significantly higher and often there is a double peak with responses to the initiation seen before a second peak that follows the distractor itself.

To investigate and distinguish licking related peaks from responses to distractors, the signals evoked by distractors were compared to those evoked for all lick runs. There was no difference in the peak ($t(11) = 0.56$, $p = .96$) or tmax ($t(11) = 2.065$, $p = .063$) measures (**Figure 4.18a, b**). However, in the post-event period, responses to lick runs (M = - 1.7% ΔF, SEM = 0.6% ΔF) were significantly depressed when compared to those following distractors (-0.7% ΔF, SEM = 0.2% ΔF, $t(11) = 3.3$, $p < .01$), (**Figure 4.18d**).

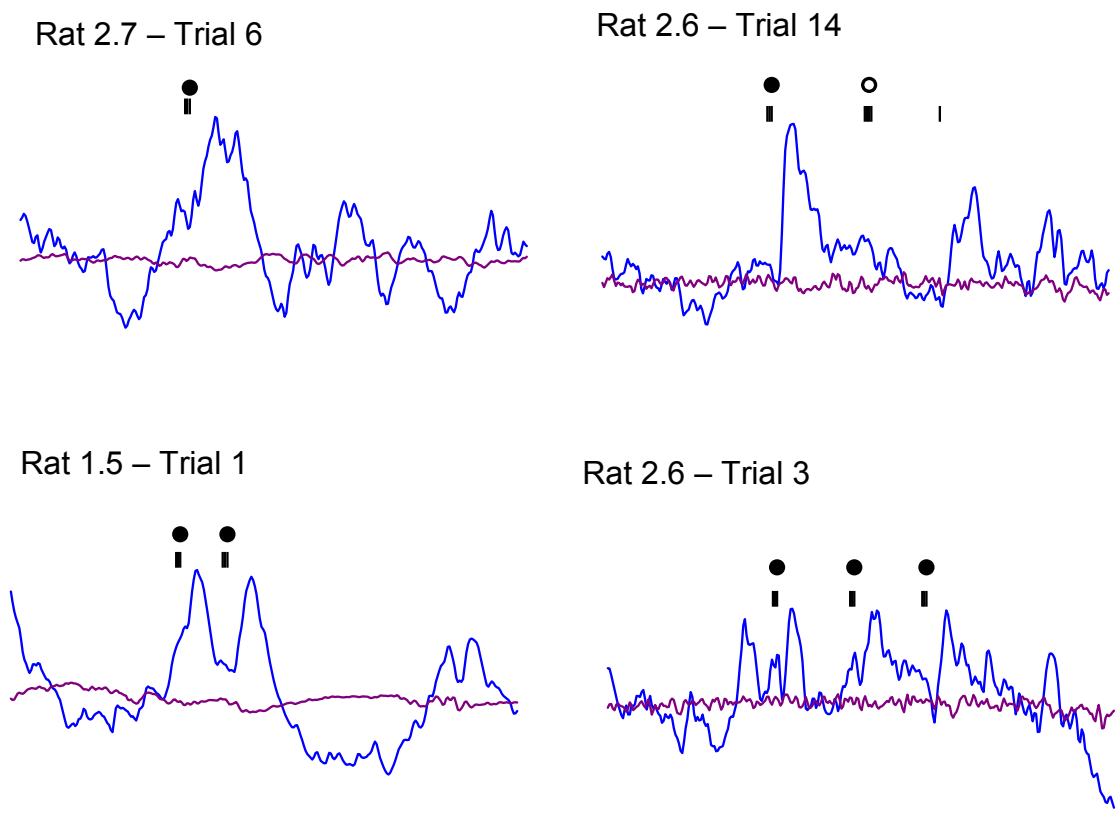


Figure 4.14 – *Individual trials aligned to distractor presentations*

Photometry traces for distractor presentations on the first distraction test day from three representative rats. Blue shows the 470 nm calcium modulated signal and purple shows the violet 405 nm control. All epochs are 30 seconds with the distractor occurring after 5 seconds. Black lines show individual licks. Black filled circles show trials in which the rat was distracted and open circles show non distracted trials. There are peaks observed following distractors and these are larger than those seen on the lick day.

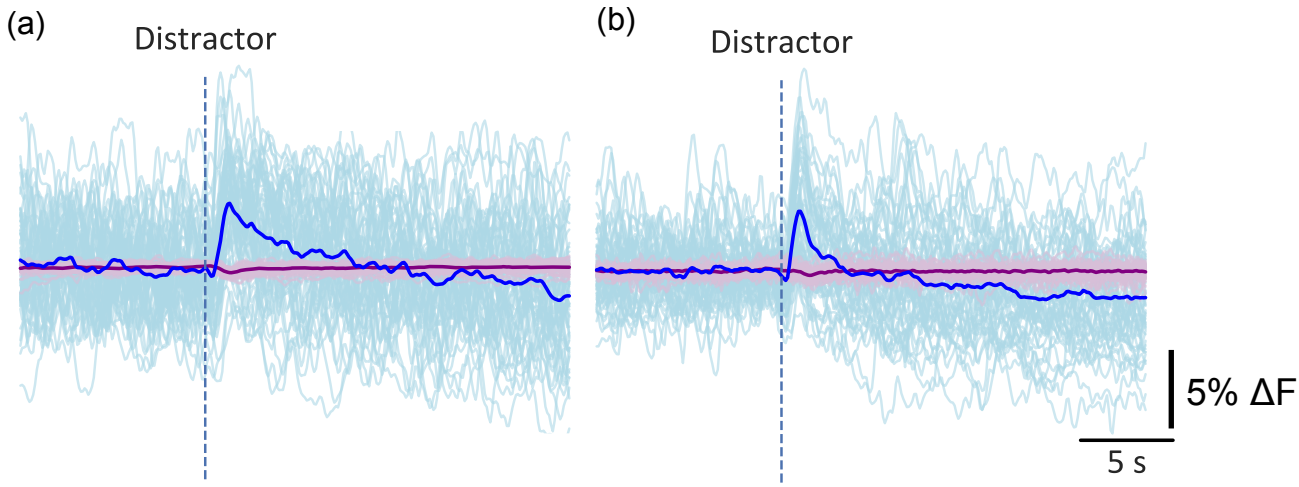


Figure 4.15 – *VTA responses to distractor presentations in two representative rats*

Traces show all distractor presentations for two representative rats. An epoch of 30 seconds is shown with photometry trials aligned to distractor. **(a)** Rat 2.1, calcium modulated signals for individual trials (pale blue) are shown for a total of 57 distractor presentations. Dark blue trace shows the mean of all trials for this rat and dark purple the mean of the control violet signal. **(b)** Rat 2.6, individual trials for 54 distractor presentations are shown (pale blue) with the mean trace in dark blue. Vertical dashed lines show the timing of the distractor presentation.

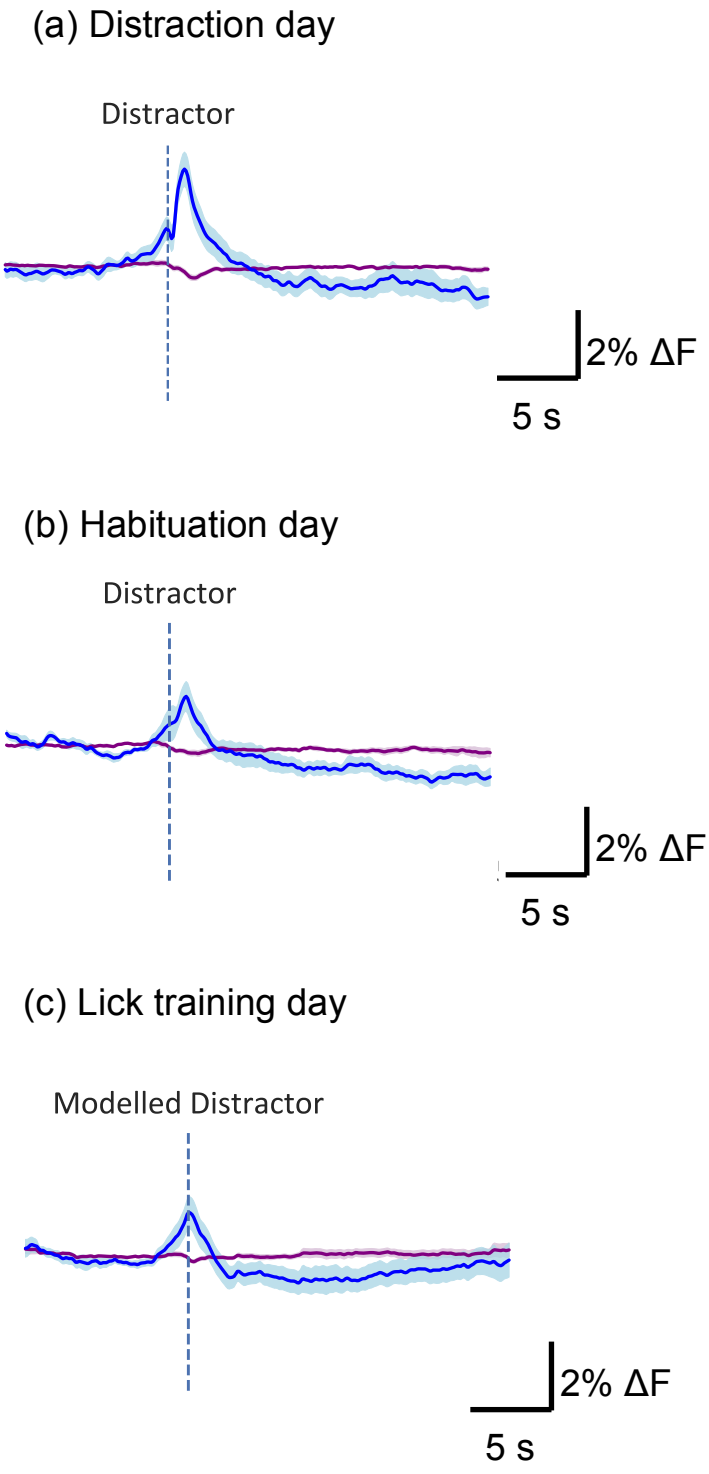


Figure 4.16 – *Averaged photometry traces for distractor presentations*

VTA neural activity during distractor presentation differs across days. **(a)** Averaged photometry trace for all rats and all distractors aligned to distractor presentation on the main test day. **(b)** Averaged photometry trace to distractor presentations on the habituation day, a decreased peak is observed. **(c)** Averaged trace for modelled distractors (calculated on the last lick day). As seen earlier, the lick related peak is seen as distinct, relative to the peak evoked by real distractors.

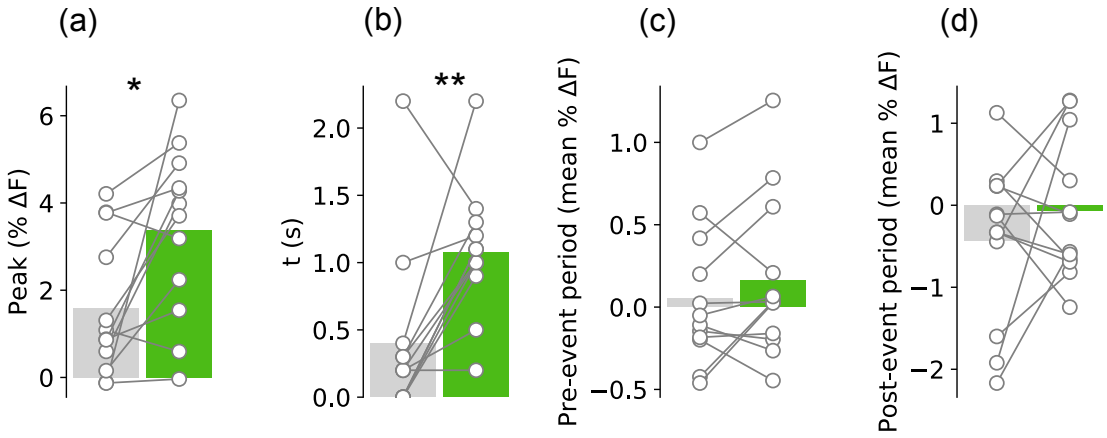


Figure 4.17 – *Calculated photometry parameters for modelled vs real distractor presentations*

Parameters calculated from averaged photometry signals for modelled distractors (grey) and distractor presentations on the distraction test day (green). **(a)** Peak height is larger in the case of real distractors. **(b)** tmax, the time to reach peak height is similar on the modelled and distraction days. **(c)** Pre-event period, the mean change in fluorescence in the five seconds preceding the distractor is similar on the modelled and distraction day. **(d)** Post-event period, there is a significant decrease following modelled distractors compared with real distractors on the distraction day. This is likely activity related to licking (as no real distractors were presented). * $p < .05$, ** $p < .01$

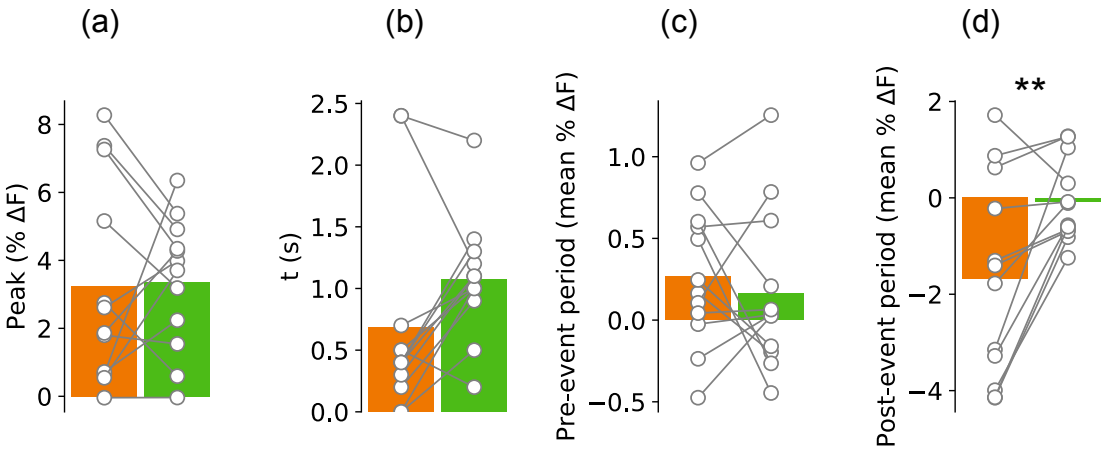


Figure 4.18 – *Calculated photometry parameters for licking runs vs distractor presentations*

Orange bars represent means for lick-related activity and green for distractor related. **(a)** Peak height for runs and distractor. **(b)** tmax for runs and distractors. **(c)** Pre-event period, the mean change in fluorescence in the five seconds preceding the start of the run is similar to that preceding distractor presentations. **(d)** Post-event period, there is a significant suppression of activity following licking runs, which is not observed in response to distractor presentations. ** $p < .01$

4.4.7 VTA responses are larger for distracted versus not distracted trials

Figure 4.19 shows photometry traces from representative rats for distracted and non distracted trials and **Figure 4.20** shows averaged photometry traces for all rats comparing distracted and non distracted trials.

To compare the VTA responses to distracted versus not distracted trials, t-tests were performed to examine the following parameters: peak, tmax and post-event period measures, **Figure 4.21.** Peak responses on distracted trials were significantly higher (M = 4.5% ΔF, SEM = 0.7% ΔF) than responses on non-distracted trials (M = 3.3% ΔF, SEM = 0.6% ΔF, $t(11) = 2.27$, $p < .05$). There was also a significant difference in the post event period, with a significantly greater decrease in activity during non-distracted trials (M = -1% ΔF, SEM = 0.4% ΔF), compared with distracted trials, which showed a small increase in activity (M = 0.9% ΔF, SEM = 0.4% ΔF, $t(11) = 2.77$, $p < .05$). There was no effect on the timing of peaks as measured by the tmax value ($t(11) = 1.23$, $p = .24$).

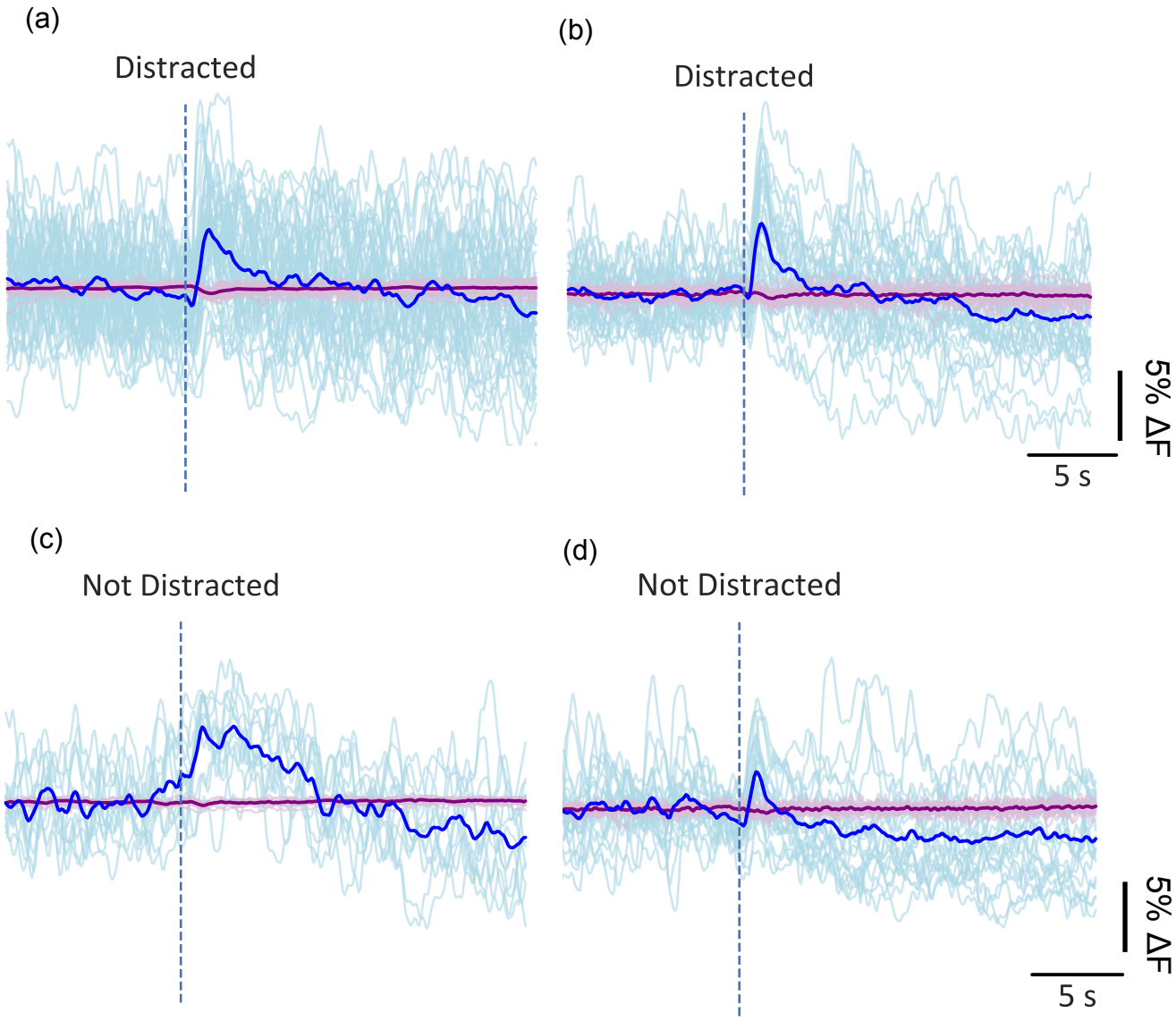


Figure 4.19 – *VTA responses on distracted and not distracted trials in two representative rats*

Distracted trials, **(a-b)** Calcium modulated signals for distracted trials in two rats, (a) Rat 2.1 (n=45), (b) Rat 2.6 (n=33). **(c-d)** Calcium modulated signals for not distracted trials in the same two representative rats, (c) Rat 2.1 (n=12), (d) Rat 2.6 (n=21). Individual trials are shown in light blue traces with means in dark blue for the blue 470 nm calcium modulated signals. Vertical dashed lines show the timing of the distractor presentation.

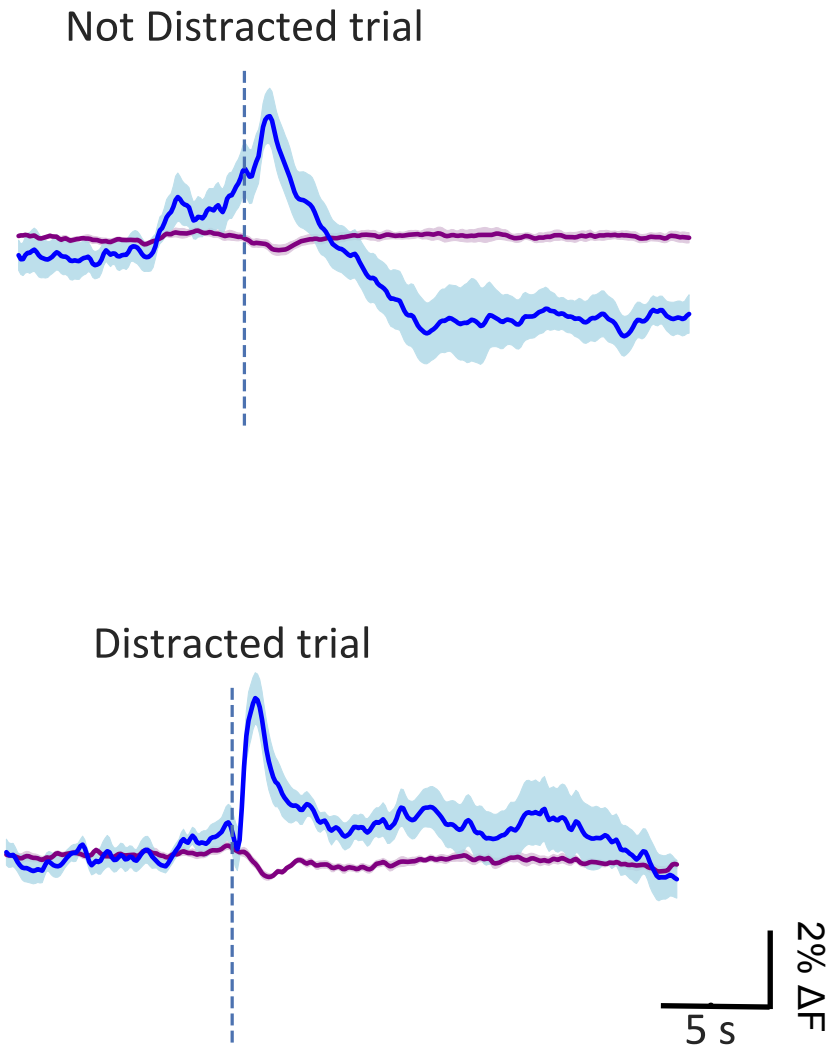


Figure 4.20 – *Averaged photometry traces for distracted and not distracted trials following distractor presentation*

VTA neural activity following distractor presentation in **(a)** Distracted trials, where rats pause following a distractor, and **(b)** Not distracted trials, where rats continue to lick in the face of distractors. Averaged photometry trace for all rats and all trials aligned to distractor presentation. There is a significantly larger and sharper peak for distracted versus not distracted responses.

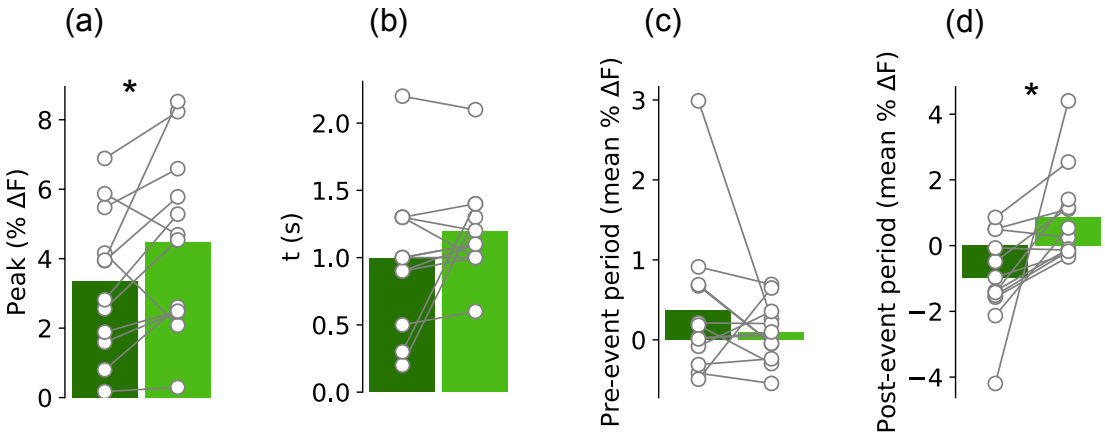


Figure 4.21 – *Calculated photometry parameters for not distracted and distracted trials*

Parameters calculated from averaged photometry signals for not distracted trials (dark green) and distracted trials (light green), **(a)** Peak height appears larger for distracted trials. **(b)** tmax is similar in distracted and non distracted traces. **(c)** Pre-event period, the mean change in fluorescence in the five seconds preceding the distractor is similar whether the rats are distracted or not. **(d)** Post-event period, there is a significant suppression of activity following not distracted trials, which is opposite to the increased activity observed following distracted trials; this could be related to the responses to continued licking in non distracted trials where rats do not pause. * $p < .05$.

4.4.8 VTA responses to distractors habituate across multiple sessions

Figure 4.16b shows the photometry responses in the VTA when distractors are presented on the habituation day. There is a decrease in the photometry peak seen on the habituation day compared with distraction day in response to distractors.

To assess whether the VTA response to distractors decreases over time with repeated distractor exposure, responses to distractor presentations were compared between the first distraction day and the habituation day. Peak responses to distractors were significantly lower on the habituation day (M = 2.3% ΔF, SEM = 0.5% ΔF), compared to the distraction day (M = 3.2% ΔF, SEM = 0.6% ΔF , $t(10) = 2.85$, $p < .05$), **Figure 4.22a**.

There was no effect of habituation on tmax ($t(10) = 1.198$, $p = .076$) (**Figure 4.22b)** or on activity during the post-event period ($t(10) = 0.55$, $p = .60$) (**Figure 4.22d**).

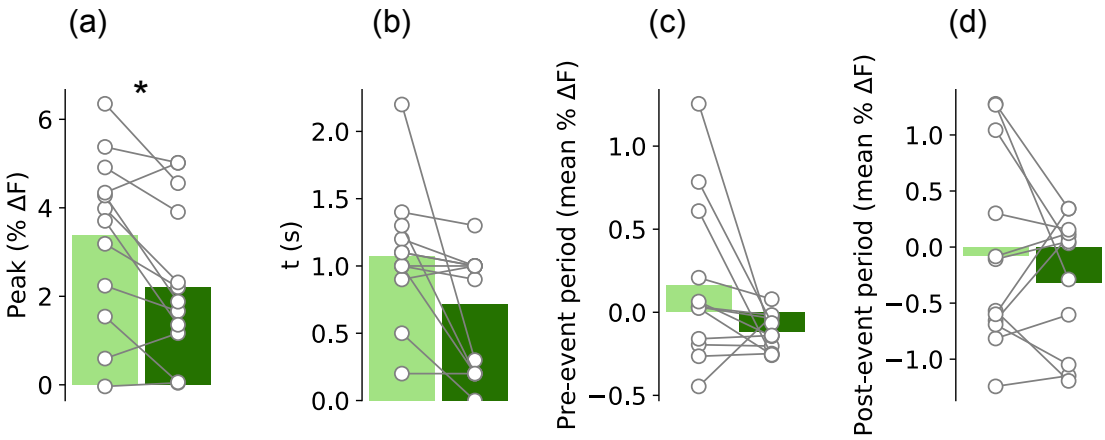


Figure 4.22 – *Calculated photometry parameters for distractor presentations on the distraction day vs the habituation day*

Parameters calculated from averaged photometry signals for responses to distractors on the distraction test day (light green) and the habituation day (dark green). **(a)** Peak height is decreased on the habituation day. **(b)** tmax is similar across days. **(c)** Pre-event period, the mean change in fluorescence in the five seconds preceding the distractor is similar across days. **(d)** Post-event period, there is no difference in activity in this period across days. * $p < .05$.

4.4.9 No effect of cue identity on distracted peaks

Figure 4.23 shows averaged photometry traces for all rats comparing distracted trials for white noise containing distractors and for non white noise containing distractors.

To compare the VTA responses to white noise versus non white noise distactors, t-tests were performed to examine the following parameters: peak, tmax and post-event period measures, **Figure 4.24.**

There was no effect of cue identity on peak height $t(11) = 1.72$, $p = .113$, nor on tmax $t(11) = 0.157$, $p = .878$, nor on the post event period measure $t(11) = 0.545$, $p = .596$, **Figure 4.24**. Cue identity did not influence the magnitude or timing of the VTA photometry response.

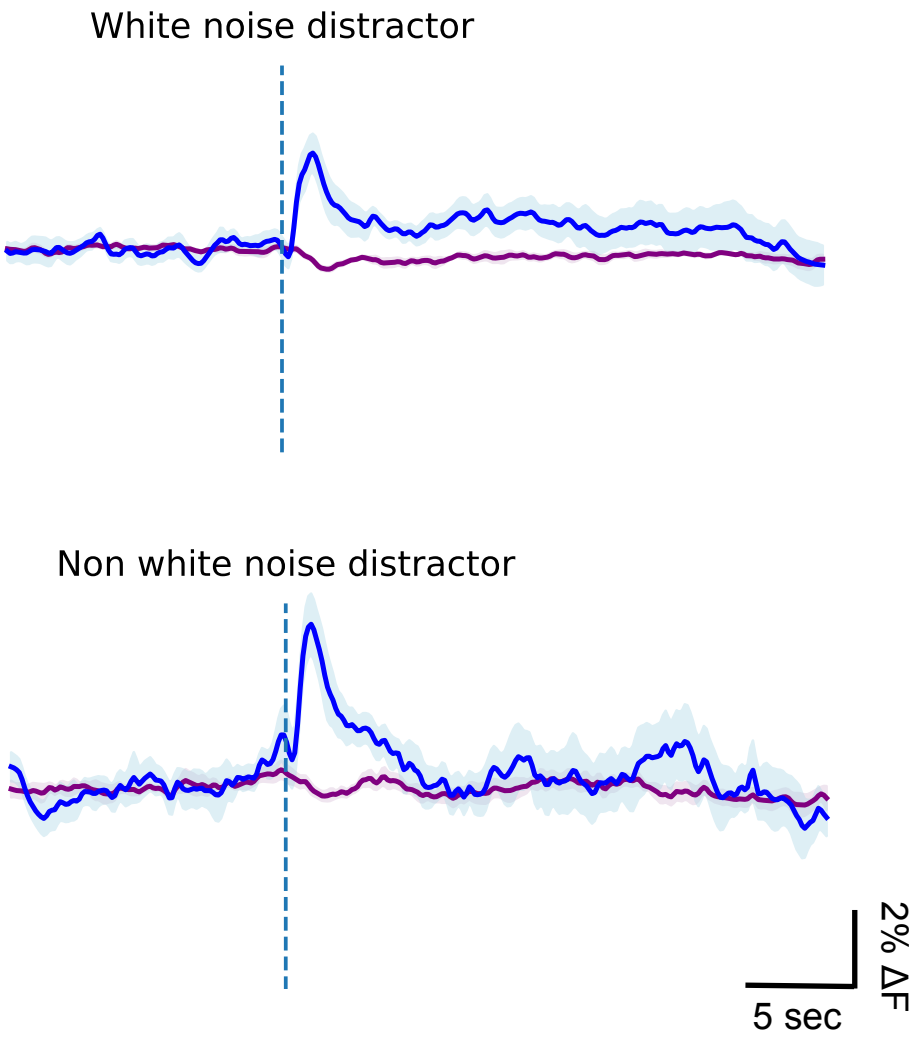


Figure 4.23 – *Averaged photometry traces for distracted trials for white noise and non white noise containing distractors*

VTA neural activity following distractor presentation in **(a)** Distracted trials, where rats received white noise containing distractors **(b)** Distracted trials where rats received non white noise containing distractors. Averaged photometry trace for all rats and all trials aligned to distractor presentation.

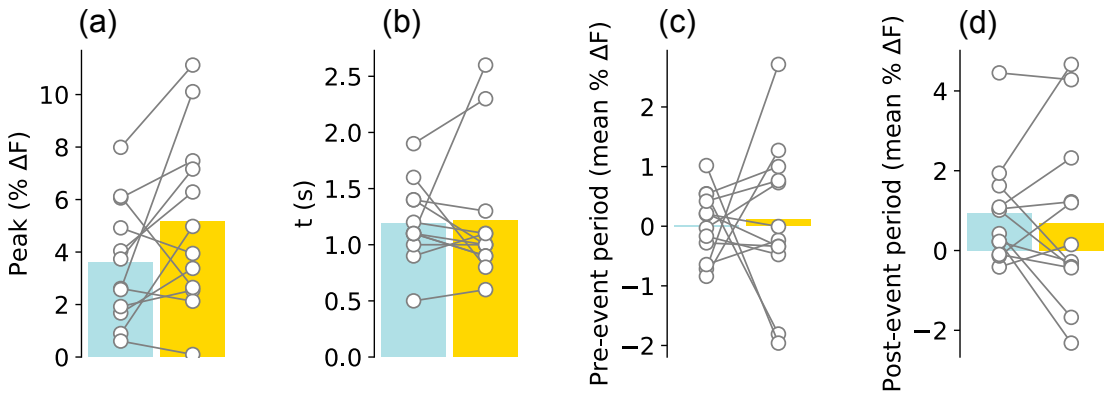


Figure 4.24 – *Calculated photometry parameters for distractor presentations white noise versus non white noise distractors*

Parameters calculated from averaged photometry signals for responses to distractors, those containing white noise (light blue) and those not containing white noise (gold). **(a)** Peak height **(b)** tmax **(c)** Pre-event period **(d)** Post-event period.

4.4.10 Correlations of photometry peaks with percent distracted

VTA neural activity may be involved in the pausing response to distractors. It is possible that individual differences in neural activity when presented with distractors may predict the percent distracted for that rat.

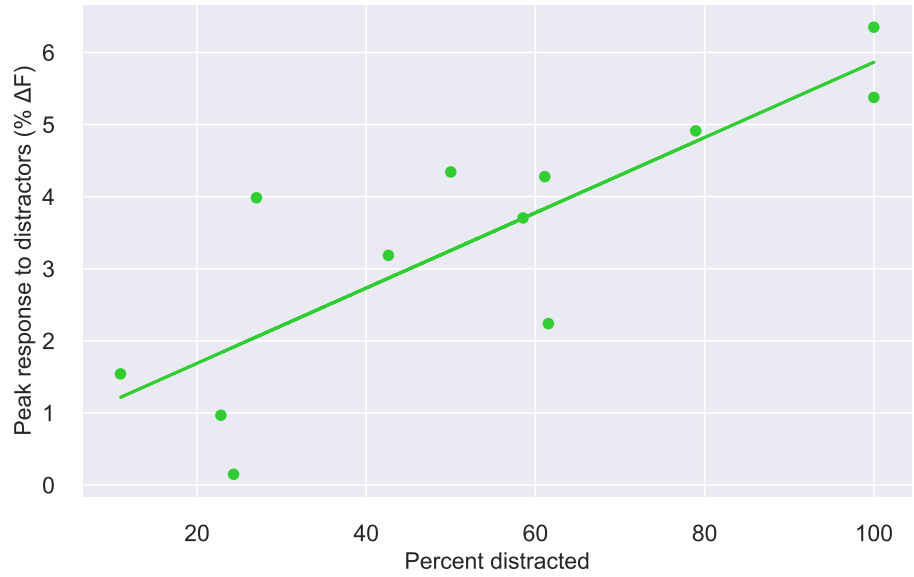
Linear regression was performed to investigate whether, for individual rats, photometry peak signals could predict the percent distracted observed behaviourally. These analyses showed a significant correlation between the peak response to distractor presentations and the percent distracted within session ($r2 = 0.68$, $p < .001$). Thus, the average increase in activity evoked by each distracting stimulus significantly predicts the distractibility of each rat. **Figure 4.25a** shows percent distracted plotted against peak response to distractors with a line of best fit demonstrating the positive relationship.

As peaks in the VTA photometry signal to distracted trials are higher than those accompanying not distracted trials, it is possible that the above correlation is due to a bias of distracted trials contributing to a higher mean response. To attempt to overcome this potential bias the liner regression was repeated using only responses to distracted trials.

In comparing only distracted trials with percent distracted in individual rats the linear regression was still significant ($r2 = 0.38$, $p < .05$), **Figure 4.25b**. This suggests that there is a relationship between the magnitude of VTA responses to distracted trials and the percent distracted that is observed behaviourally. Those rats with larger peak responses to both distractors and distracted trials show a greater overall propensity to distraction. VTA activity appears to drive percent distracted.

When percent distracted is correlated with the relative peak (peak in response to distracted trials – peak in response to non distracted trials) there is no significant relationship ($r2 = 0.0008$, $p = .930$, **Figure 4.26)**

(a)



(b)

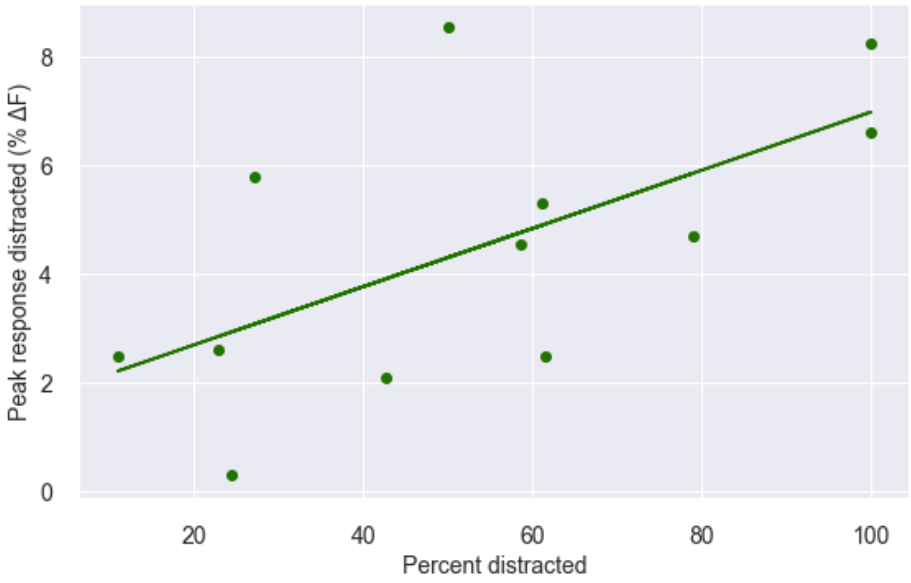


Figure 4.25 – *Relationship between VTA responses to distractors and percent distracted*

(a) Scatter plot showing percent distracted against peak fluorescent change in VTA following distractors. Lines of fit (light green) shows a positive relationship with increased distraction linked to greater peak responses. **(b)** Scatter shows linear regression results for percent distracted against VTA responses to distractors in distracted trials only, a significant positive relationship was also found.

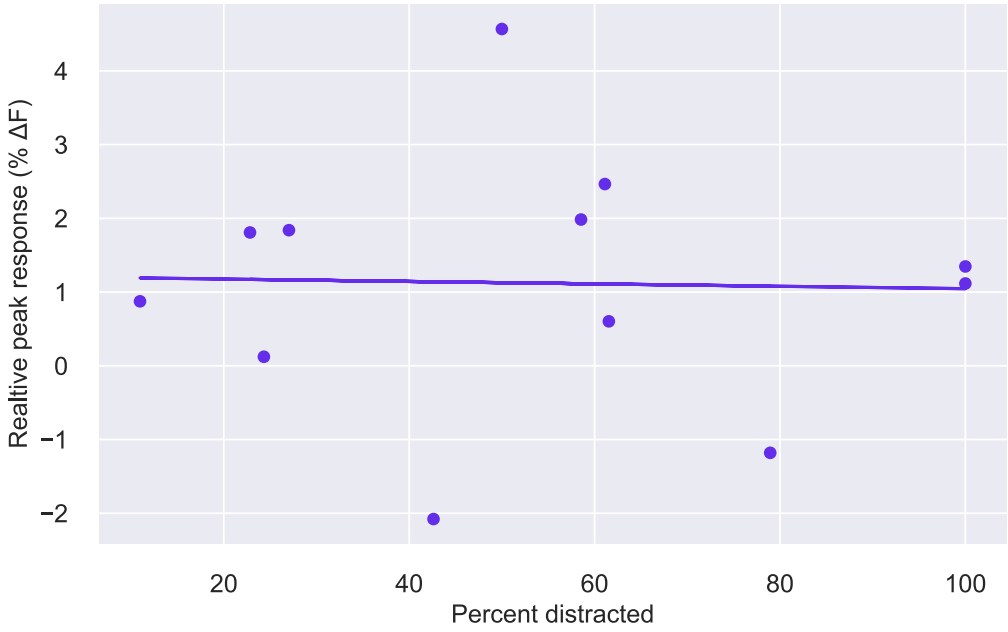


Figure 4.26 – *Relationship between relative VTA responses to distracted versus non distracted trials and percent distracted*

Scatter plot showing percent distracted against the relative peak during distraction, relative peak is calculated as the mean peak for each rat on distracted trials minus the mean peak for non distracted trials. Scatters show individual rats (purple) and a line of best fit is shown.

4.4.11 No relationship between TH or GFP expression and percent distracted

To rule out the possibility that percent distracted was related to expression of GFP or TH – for example a lesion effect could account for why there are larger peaks in the most distracted rats – linear regressions were performed to investigate the relationship between percent distracted and GPF and TH expression respectively.

There was no correlation between percent distracted and total number of GFP positive cells in the VTA (r2 = 0.066, p = .448, **Figure 4.27**, green). Neither was there a significant relationship between percent distracted and the number of TH positive cells (r2 = 0.077, p = .408, **Figure 4.27**, red). There is no evidence of a relationship between a dopamine / or non cell specific lesion contributing to the percent distracted effect.

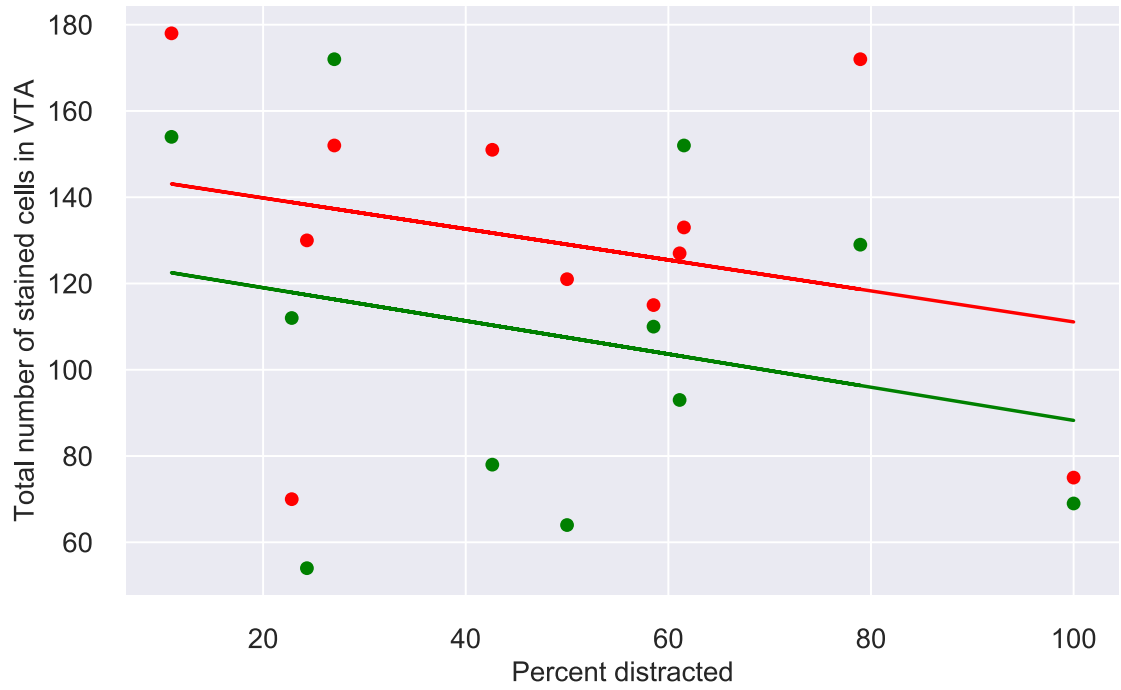


Figure 4.27 – *No relationship between percent distracted and GFP or TH staining*
Scatter plot showing total number of stained cells against percent distracted for GFP (green points are individual rats and green line is best fit) and TH (red points are individual rats, red line of best fit). There is no relationship between the number of stained cells in either GFP or TH and percent distracted.

4.4.12 Immunohistochemistry – extent of TH and GFP overlap

All brains were assessed for fibre placements and viral expression. Two animals were excluded for poor viral expression and/or incorrect fibre placement. **Figure 4.28** shows a representative image of the fibre placement in the VTA, green staining shows GFP positive neurons where GCaMP6s is expressed at the fibre tip.

For each rat, a representative sample of VTA slices were taken and used to quantify viral expression. Images of the VTA were taken at 10X magnification and, within the field of view, the total number of GFP positive cells (green) were counted as well as the total number of TH positive cells (red).

Cells were manually counted using Image J by selecting regions of interest (ROIs) where GFP positive neurons were observed. Within these ROIs, TH positive neurons were counted and the extent of co-localisation was assessed. Specificity was calculated as the total number of GFP positive neurons divided by the number of co-localised (GFP+TH positive) neurons.

The mean number of GFP positive cells within the field of view was 107 (SEM = 10). The mean number of TH positive cells was 119 (SEM = 12). The mean specificity across all rats tested was 7.46% (SEM = 1.37%). This specificity measure suggests a very low level of co-localisation around the fibre tip (**Figure 4.29)**. This suggests that a substantial proportion of those recorded cells were not dopaminergic, or for some other reason were not stained for TH as well as GFP.

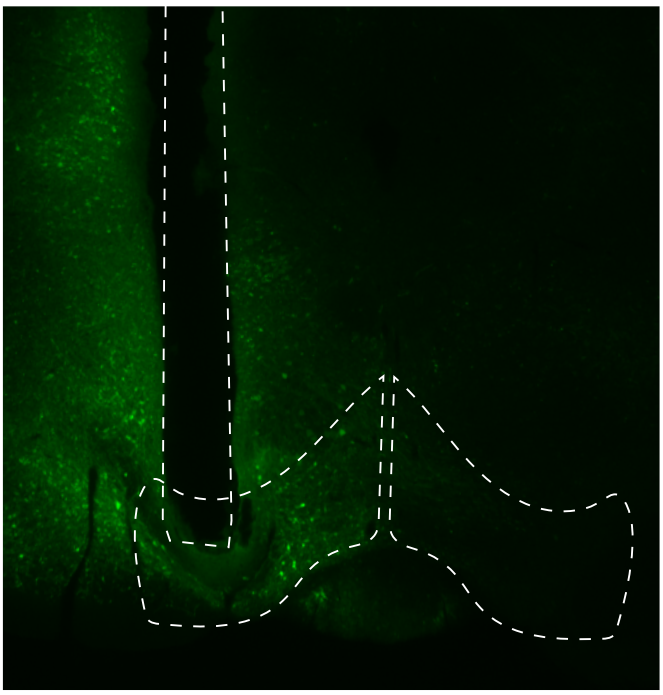


Figure 4.28 – *Fibre tip placement and viral expression within the VTA*

Representative VTA rat brain slice, image taken using an epifluorescent microscope using a 2.5X objective, shows the expression of GCaMP6s (green bright areas) around the tip of a photometry fibre implant. Dotted white lines indicate the path of the fibre track and highlight the VTA.

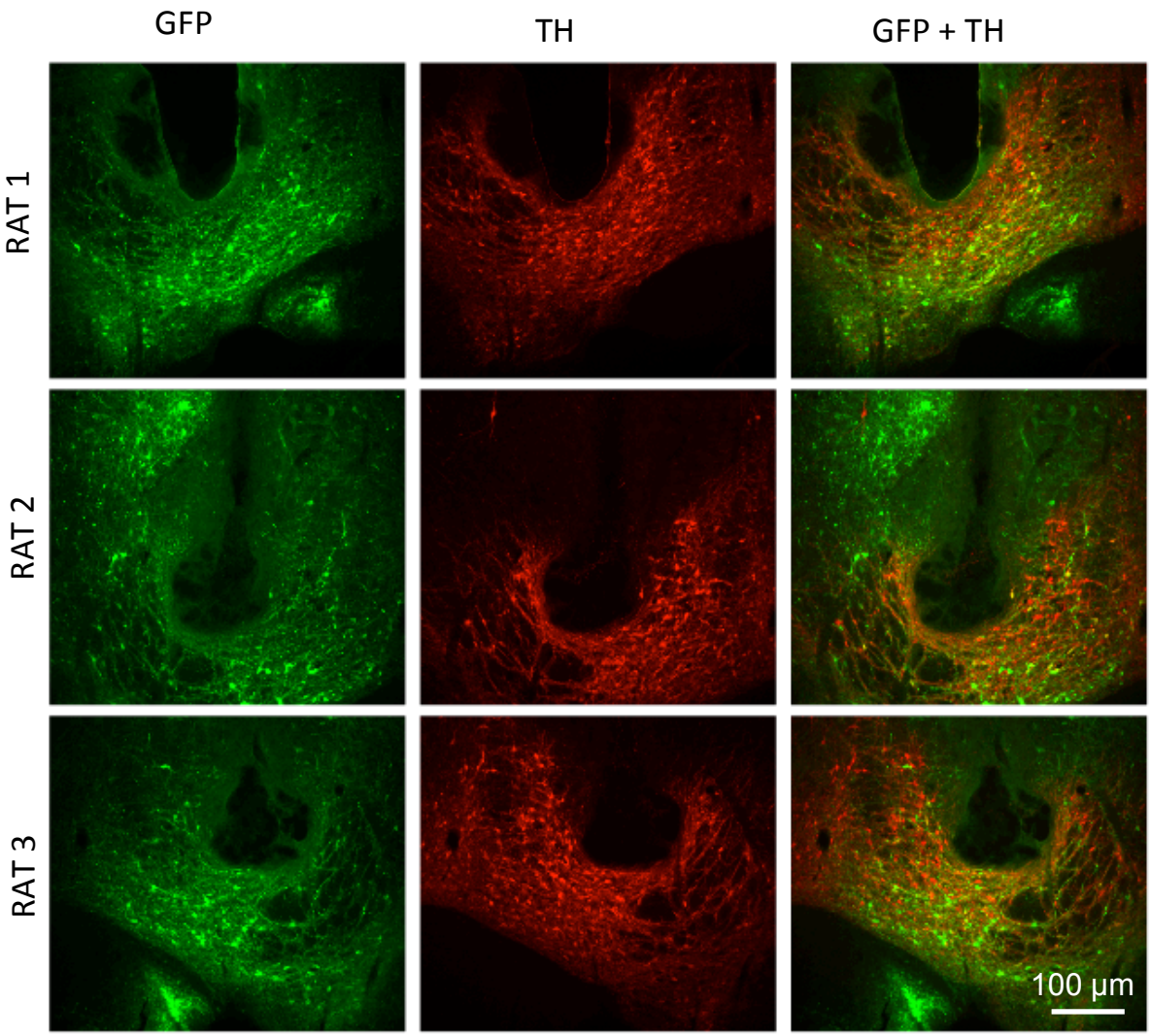


Figure 4.29 – *GFP and TH expression at the fibre tip in VTA slices*

Examples of GFP positive (green), TH positive (red) and overlay images (merged) showing expression of the GCaMP6s virus in the VTA around the fibre tip in three rats. Fibre placements were verified using a brain atlas as reference and all fibres were within the VTA territory. Despite expression of the virus and positive TH staining for dopamine, there is little overlap in localisation of these markers showing a lack of dopamine neuron specificity.

4.5 DISCUSSION

Here we recorded from VTA cell bodies during the distraction task developed in the preceding chapters. We characterise several responses within the VTA during distraction from ongoing saccharin consumption. Firstly, we replicated the behavioural results in the distraction assay in tethered animals with head implants. Percent distracted and the habituation effect previously shown in Chapters 2 and 3 were observed again verifying no obvious detrimental effect of tethering on performance of this task. Our results show increased VTA activity during licking, when rats initiate runs of licks there are transient peaks in the calcium response (neural activity). When separated into short and long licking periods we found similar peak responses however there was a substantial suppression of activity in response to long runs which was not seen with short runs. Sustained licking produces decreased activity in the VTA for a prolonged period (> 10 seconds).

We also report VTA activity increases in response to the presentation of salient distractors. Not only does increased VTA activity accompany distractor presentations but these peaks are also larger when the animal is distracted compared to when they are not distracted and continue licking. We compared the VTA responses seen during distraction to those of modelled distractors and verified that these responses are not characteristic of non specific activity changes but are in fact due to salient distractors. Our data show a habituation of the neural signal with decreased peak height on the second test day mirroring the behavioural habituation we see (decreased percent distracted and resistance to the interrupting effect of distractors with repeated testing).

These data support the role of the VTA in both driving consumption and in monitoring external stimuli in the environment. However, there are several important considerations when interpreting these data. Firstly, the analysis method used involves binning data into epochs centred around events. In the case of licking runs it is important to note that within a 30 second epoch several short runs could occur and each epoch may not represent a single event (with longer runs this is less common as these typically last longer than 30 seconds).

We observed a suppression in activity following an initial peak with long runs, this was not seen in short runs where there was an opposite sustained elevation. It may be the case that this activity results from the summed peaks of multiple events (ie. The starts of several short runs within a single 30 second epoch) this accumulation of peak activity could explain why we observe what appears to be a prolonged elevation.

With this in mind the distraction task could be modified in several ways to attempt to tease apart these responses as well as further differentiate responses to licking and distractor stimuli. We could gather enough licking data, or combine data across multiple lick training days, to exclude all of the short runs that are closely preceded and/or followed by other runs. That is, isolate just short bursts which occur in isolation. This would allow us to assess the photometry signals of just short runs without the possible confound of multiple events in a single epoch.

As licking and distractor presentations are closely linked in time (the three lick bursts trigger distractors) it is sometimes difficult to tease apart responses from these different events. We could further modify our distraction paradigm to counteract this. For example, instead of consistently triggering distractors after a set number of licks (3 lick bursts, O'Connor et al., 2015), we could use a variable number of 10, 20, 30 licks within sessions to allow for the measurement of licking before the distractor is presented. In this way the very beginning of the runs will not always coincide with the distractor presentations, the two responses can be separated temporally. This could also have the added benefit of reducing habituation, perhaps prolonging the number of days which animals show high levels of distraction by making distractors less predictable. We could also introduce periods within the session where distractors are not delivered at all, to separate out licking responses further.

The responses we have described to distractors (and the increased responses on distracted trials compared to non distracted trials) are consistent with a role for the VTA in monitoring environmental stimuli and directing attention. These responses may be mediated by fast, primitive inputs to the VTA from the superior coliculus. Further experiments could combine photometry recordings with optogenetic inhibition or activation of this circuitry and assess the consequences for distraction behaviour. These VTA responses may have an adaptive role in orienting behaviour towards important and salient stimuli and may be modulated by the motivation to engage in consumption.

It was predicted that a large proportion of the VTA neurons recorded in these experiments would be dopaminergic. However, our immunohistochemistry data suggest that this is not the case. A substantial proportion of the neurons stained for GFP – the marker of GCaMP expression – were not positive for TH and the overall co-localisation was less than 10%. This was surprising given the large dopaminergic population in the VTA (Morales & Margolis, 2017), despite using a non-specific virus we expected greater co-localisation. It is possible the neurons expressing GCaMP6 were somehow perturbed and this the expression of TH was reduced. In fact, there is evidence that TH, which is under dynamic control, is sensitive to oxidative stress and perhaps high levels of GCaMP expression drive down that of TH (Stuber, Stamatakis & Kantak, 2015) However this is speculative and requires further investigation.

If these neurons are however truly not dopaminergic then this raises the question of their molecular identity. We assumed based on the literature that the candidate neurons might be GABAergic given the relative abundance of such neurons within the VTA (Beier et al., 2015). We attempted to stain neurons using GABA antibodies however we had methodological problems and were not successful in identifying and quantifying GABAergic neurons. Future work should use immunohistochemical techniques to determine the identity of neurons close to the fibre implant in these experiments.

The following chapter will attempt to gain increased dopamine specificity by recording in terminal regions within the NAc. We will use the same non-specific GCaMP virus injected into the VTA, but will take advantage of the dense dopaminergic projections to the NAc to achieve increased specificity.

There are also a number of viral strategies, some of which we have evaluated in Chapter 6, which may produce dopamine specific expression of GCaMP and enable the determination of the contribution of dopamine to the VTA signals we have observed here.

CHAPTER 5

Fibre photometry recordings from nucleus accumbens (NAc) terminals during distraction

5.1 INTRODUCTION

5.1.1 Dopamine specificity

In the previous chapter, we recorded neural responses to distractor stimuli during ongoing saccharin consumption. We observed real time calcium fluctuations in cell bodies in the VTA, which we were able to time lock to specific behavioural events. However, the virus we used to express the genetically encoded calcium indicator, GCaMP6s, was non-specific. This virus in theory targets all neurons with equal probability as expression is driven by a *synapsin* promoter. However, it was hoped that, as a significant portion of cell bodies within the VTA are dopaminergic, that the majority of GCaMP6s expressing neurons would be dopaminergic. According to our immunohistochemistry, this was not the case. Despite a large dopamine neuron population within the VTA, our findings suggest a specificity of targeting to dopamine neurons of less than 10%.

There are various strategies used in fibre photometry experiments to produce cell specific targeting of GCaMP expression. Several of these are discussed in detail in the next chapter (**Chapter 6),** including combinatorial viral approaches, which use Cre recombinase systems and transgene mediated cell specific expression approaches using transgenic animals. However, fibre photometry can also be used to record calcium fluctuations within terminal regions and not just cell bodies (Barker et al., 2017; Li et al., 2017). In fact, fibre photometry has been shown to track neurotransmitter release in terminal regions quite well (Parker et al., 2016)

The VTA has a vast array of projection targets (as discussed in detail in **Chapter 1**) and, in particular, there are dense dopaminergic projections from the VTA to the NAc shell and core. Between 65 and 85% of the projections from the VTA to the NAc are dopaminergic (Morales and Margolis, 2017). By taking advantage of this reasonably high level of dopamine specificity in projection targets we can use the same non-specific virus injected into the VTA but instead record the signal in terminal regions, such as the NAc. Assuming the

virus is completely non-specific with no preference for any neuronal type, then we would expect at least 65% of neurons recorded in the NAc (after viral injection in the VTA) to be dopaminergic. This would be a level of specificity far higher than that which we report earlier.

5.1.2 Properties of terminal release

In addition to advantages of greater cell specificity with terminal recordings, it is also important to consider possible differences between calcium responses seen in cell bodies and the relationship to consequent dopamine release at terminals. Several studies have shown that firing in cell bodies does not always translate into increased transmitter release in terminals in a one to one fashion. Within the mesolimbic dopamine system in particular, there are intricate local modulatory influences on dopamine transmission which can produce unexpected and contradictory results when comparing cell body firing and terminal dopamine release (Rice & Patel, 2015). For example, there is a key influence of somatodendritic release in modulating cell firing within the mesolimbic pathway. The activation of autoinhibitory dopamine D2 receptors is one mechanism by which firing and release may not be so simply related (Rice & Patel, 2015).

In addition, terminal regions such as the NAc receive many other modulatory influences. Not only are there glutamatergic inputs to the striatum and NAc , but dopamine terminals themselves can also release glutamate (Hnasko et al., 2010; Stuber et al., 2010). There are complex neuromodulatory mechanisms involved in the local control of NAc dopamine release (Catchope and Cheer, 2014) Dopamine release may be triggered independently of cell body firing via several mechanisms including glutamatergic and cholinergic influences in terminal regions (Catchope and Cheer, 2014). Therefore, as well as utilising the well-characterised VTA projection anatomy to record dopamine neurons more specifically in terminals, we will also investigate possible differences in the signals obtained from cell body and terminal recordings.

5.2 AIMS, OBJECTIVES & HYPOTHESES

5.2.1 Aims

5) Investigate VTA neural activity in neurons projecting to the NAc during licking for saccharin
6) Characterise VTA → NAc neural activity in response to distractor stimuli
7) Compare VTA → NAc activity when an animal is distracted versus when they are not distracted

5.2.2 Objectives

5) Record calcium fluctuations using fibre photometry in the NAc from neurons originating in the VTA during licking and in response to distracting stimuli using the behaviour described in chapters 2 and 3
6) Determine whether there are differences in neural activity during licking and distraction within NAc subregions (core and shell)

5.2.3 Hypotheses

5) Licking for saccharin will produce a change in neural activity in the NAc terminals of VTA neurons (e.g. calcium transients will be observed at the start of periods of licking)
6) During distraction testing, distractor stimuli will evoke transient activity increases in VTA → NAc terminals
7) There will be greater neural activity during distracted versus non-distracted trials

5.3 METHOD

5.3.1 Animals

Ten male Sprague Dawley rats (300g – 350g at the time of surgery) were used in these fibre photometry experiments (Charles River, UK). All animals were housed in pairs in standard conditions as previously described (**Chapter 4, Section 4.3.1**). One rat died during surgery due to a fault with the oxygen system so the number of animals reported is nine.

5.3.2 Viral injections and fibre implants

Identical surgical procedures were used as in **Chapter 4** with the only exception being the placement of fibre photometry probes in the NAc and not the VTA. AAV9.Syn.GCaMP6s.WPRE.SV40 was diluted to 1:4 with aCSF and 1 µl of diluted virus was injected over 10 minutes (rate 100 nl / min) into the VTA (AP – 5.8 mm, ML + 0.7 mm, DV – 8 mm). Photometry fibres were implanted in either the NAc core (AP + 1.3 mm, ML + 1.3 mm, DV – 6.5 mm) or NAc shell (AP + 1.7 mm, ML + 0.9 mm, DV – 6.5 mm). For these experiments, rats were given 5 weeks before behavioural testing, to allow for the virus to be expressed in VTA cell bodies and to travel and express in NAc terminals.

5.3.3 Behavioural testing and photometry recordings

Rats were trained to lick for saccharin and the distraction assay was performed. Photometry recordings were undertaken during the task as described in **Chapter 4, Section 4.3.5.** There were successful recorded signals in only 4 of the 10 rats, with 3 of these recorded from the core and one from the shell co-ordiantes.

5.3.4 Data analysis

Data analyses were as described in **Chapter 4, Section 4.4.7.** The four parameters (peak, tmax, pre-event period and post-event period) were calculated from averaged traces aligned to specific events as in **Chapter 4, Figure 4.5.** To assess the parameters of the photometry signals in different event conditions, MANOVA, ANOVA and planned comparison t-tests were used and are described in detail in each results subsection.

5.4 RESULTS

5.4.1 Photometry signals in the VTA in response to behavioural events

In **Chapter 4, Section 4.4.3,** MANOVA was used to assess which photometry parameter would be investigated further. However, when testing the current animals, less than half showed a discernible photometry signal (changes in fluorescence during behavioural events above background noise). Due to this, four rats were excluded from analyses and the final number of rats with signals was n=5. MANOVA is sensitive to small numbers of cases and when estimating covariance matrices this type of tests is less accurate, given the low number of animals here MANOVA is not appropriate. Individual univariate tests (ANOVAs) were performed for each measure (peak, tmax, pre-event period and post-event period) to assess which measured parameters were relevant. The independent variable in each of these four ANOVAs was 'event' with 8 levels detailed below:

ALL RUNS – photometry signal aligned to the first lick in each run
SHORT RUNS – aligned to the first lick in the short runs only (lower quartile of run lengths)
LONG RUNS – aligned to the first lick in the long runs only (upper quartile of run lengths)
DISTRACTORS – aligned to the presentation of a distractor (all distractors)
DISTRACTED – aligned to distracted trials, where a distractor is presented and the rat is distracted (i.e. pauses licking)
NOT DISTRACTED – aligned to not distracted trials, where a distractor is presented and the rat is not distracted (i.e. continues to lick)
MODELLED DISTRACTOR – aligned to 'modelled' distractors, using data from a lick training day, the 'modelled' distractor events are where distractors would be presented if this was a distraction day (i.e. following three lick bursts)
HABITUATION – aligned to distractor presentations (all distractors) on the habituation day following initial distraction

There were significant main effects of event for the peak ($F(7,21) = 2.55$, $p < .05$), and post-event period ($F(7,21) = 3.71$, $p < .01$) but no main effect for the pre-event period ($F(7,21) = 0.79$, $p = .60$) or tmax ($F(7,21) = 1.41$, $p = .25$). Therefore, only peak and post-event period measures were compared between groups further.

A priori planned comparisons for pairs of events were performed only for those measures with significant ANOVA results (peak, and post-event period). The planned comparisons were as follows:

(1) **Short runs vs Long runs** – to assess differences between short and long runs of licks on photometry measures
(2) **All runs vs All distractors** – to assess differences between photometry responses to runs and distractors
(3) **Distracted vs Not distracted** – to assess photometry responses in distracted and not distracted trials on the distraction test day
(4) **Modelled distractors vs Distractors** – to assess responses to modelled distractors (lick day) and to real distractors (distraction test day)
(5) **Distractors vs Habituation** – to assess habituation effects, comparing distractor responses on distraction day and habituation day

5.4.2 NAc activity is different for long versus short runs

Runs of licks were calculated and photometry signals were binned into 30 second epochs aligned to the first lick in a run. For all runs, **Figure 5.1** shows the group average of all runs in all rats (n = 5) aligned to the first lick in the run.

To assess possible differences in photometry responses in the NAc between short and long runs of licks, runs were divided into short and long runs (based on lower and upper quartiles). **Figure 5.2** shows averaged NAc photometry responses for all rats for both short (**Figure 5.2a**) and long runs (**Figure 5.2b**). T-tests assessed difference in the photometry signals aligned to short and long runs

There was a significant difference in peak height between short (0.6% ΔF, SEM = 0.4) and long runs (1% ΔF, SEM = 0.5, $t(4) = 2.80$, $p < .05$). **Figure 5.3a** shows that long runs resulted in a greater increase in calcium modulated fluorescence recorded in NAc terminals originating from the VTA.

In addition, there was a significant difference in the post-event period between long and short runs, **Figure 5.3b**. Both responses to short and long runs showed an elevation of activity in the post event period, however, this was higher during long (0.5% ΔF, SEM = 0.2) versus short runs (0.01% ΔF, SEM = 0.05, $t(4) = 2.95$, $p < .05$)

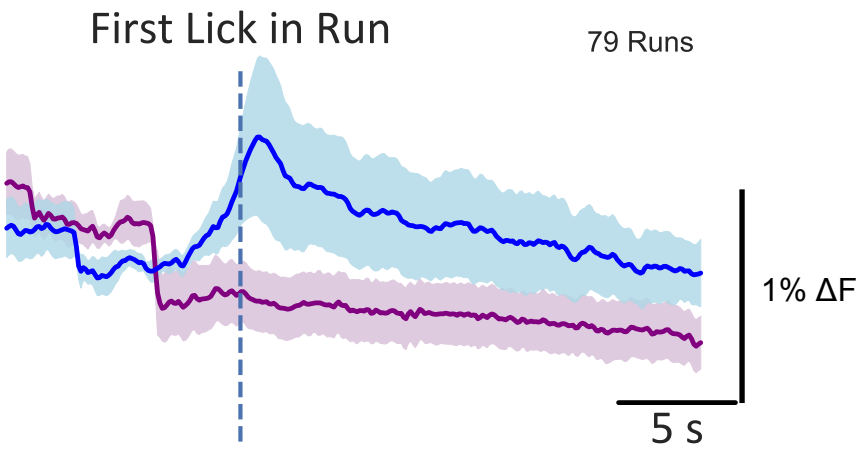


Figure 5.1 – *Averaged photometry trace for all runs in all rats (NAc)*

NAc neural activity is increased in response to the initiation of licking runs. Photometry trace aligned to the first lick in the run for all runs from all rats. Dark blue trace shows the mean calcium modulated (470 nm) signal in response to the start of a run, with shaded light blue error (SEM), the purple trace shows the 405 nm control signal and associated error in light purple (SEM). Dashed vertical line represents trial onset, in this case the start of a run, scales are shown as percentage change in fluorescent signal.

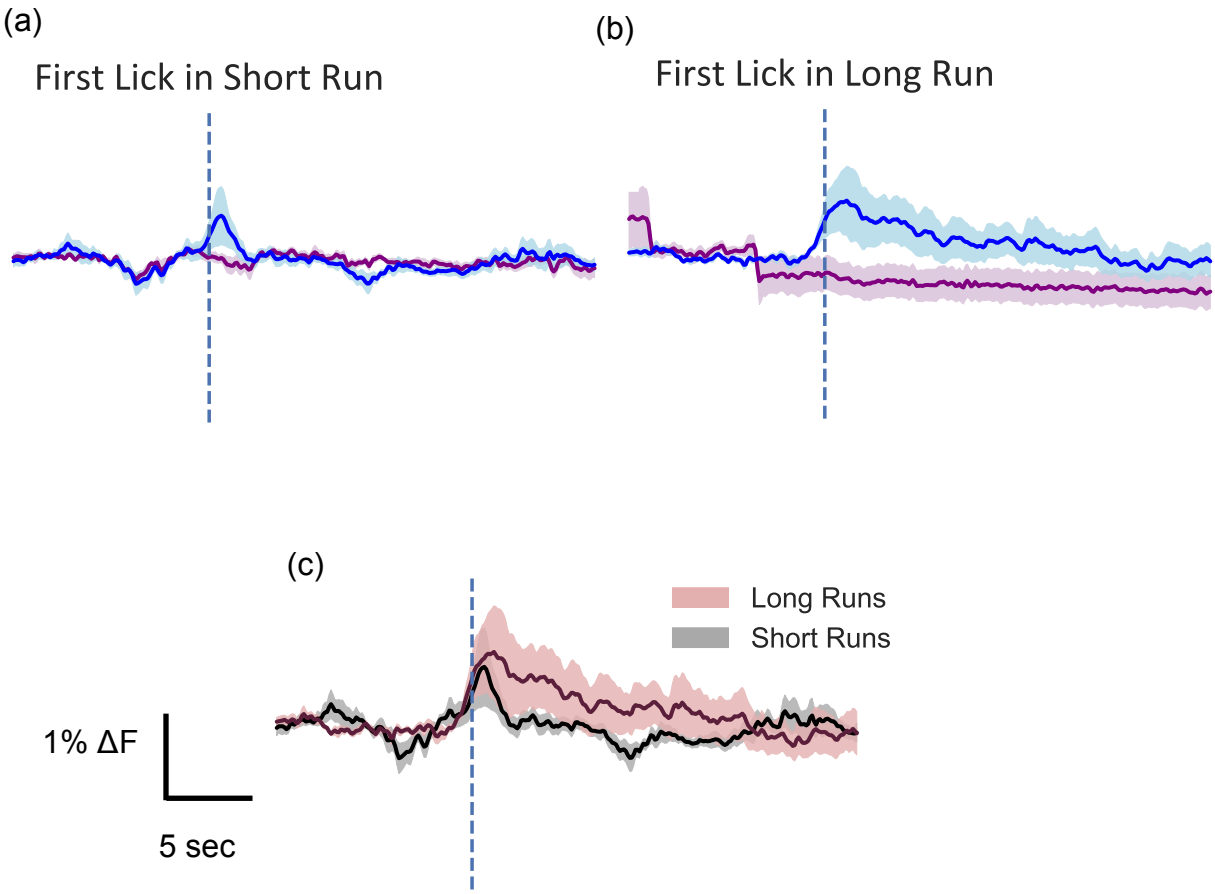


Figure 5.2 – *Averaged photometry traces for short and long runs (NAc)*

NAc neural activity during licking initiation (the start of a run of licking) differs depending on the length of the run. **(a)** Averaged photometry trace for all rats and for all short runs, n = 39 runs, (defined by lower quartile of run lengths) aligned to first lick in the run. **(b)** Averaged photometry trace for all rats and for all long runs, n = 39 runs (defined by upper quartile of run lengths). In a and b, blue traces show the mean calcium modulated (470 nm) signal with SEM in shaded area and purple trace shows the 405 nm control signal. (c) NAc responses to short and long runs shown on the same plot, average responses for long runs (pink) show a significant elevation of activity following an initial peak, this is not observed with short runs (grey).

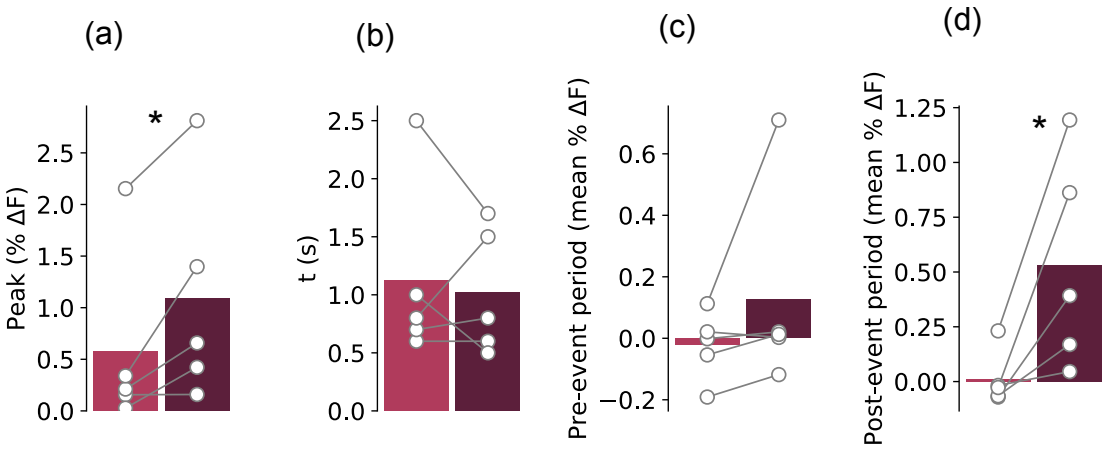


Figure 5.3 – *Calculated photometry parameters for short vs long runs (NAc)*

Parameters calculated from averaged photometry signals for short (pink) and long runs (purple). **(a)** Peak height is greater for long runs. **(b)** tmax, the time to reach peak height is similar for short and long runs. **(c)** Pre-event period for short and long runs. **(d)** Post-event period, there is a significant elevation in the 20 seconds following the onset of long runs compared with short runs. This is in contrast to the VTA data in Chapter 4, where a substantial suppression of activity is seen with the onset of long licking runs. * $p < .05$.

5.4.3 Photometry signals in the NAc following distractors

Figure 5.4a shows the group average of all trials aligned to distractor presentation for all rats (n = 5 rats). To assess whether photometry responses in the NAc were in response to distractors and not a general feature of baseline activity, photometry measures were compared between the modelled day and the distraction day.

There was no difference in peak response between real distractors and modelled distractors t(4) = 0.48, p .66, **Figure 5.5a**). There was also no difference in the post-event period between real distractors and modelled distractors t(4) = 0.15, p = .89, **Figure 5.5d**).

To investigate and distinguish licking related peaks from responses to distractors the signals evoked by distractors were compared to those evoked for all lick runs. There was no difference between licking and distractor related activity for peak, t(4) = 1.54, p = .12, **Figure 5.6a** or post-event period measures, t(4) = 1.76, p = .15, **Figure 5.6d**).

Taken together, these results would suggest that peaks seen in the NAc during distractor presentations are no different from non-specific or lick related changes.

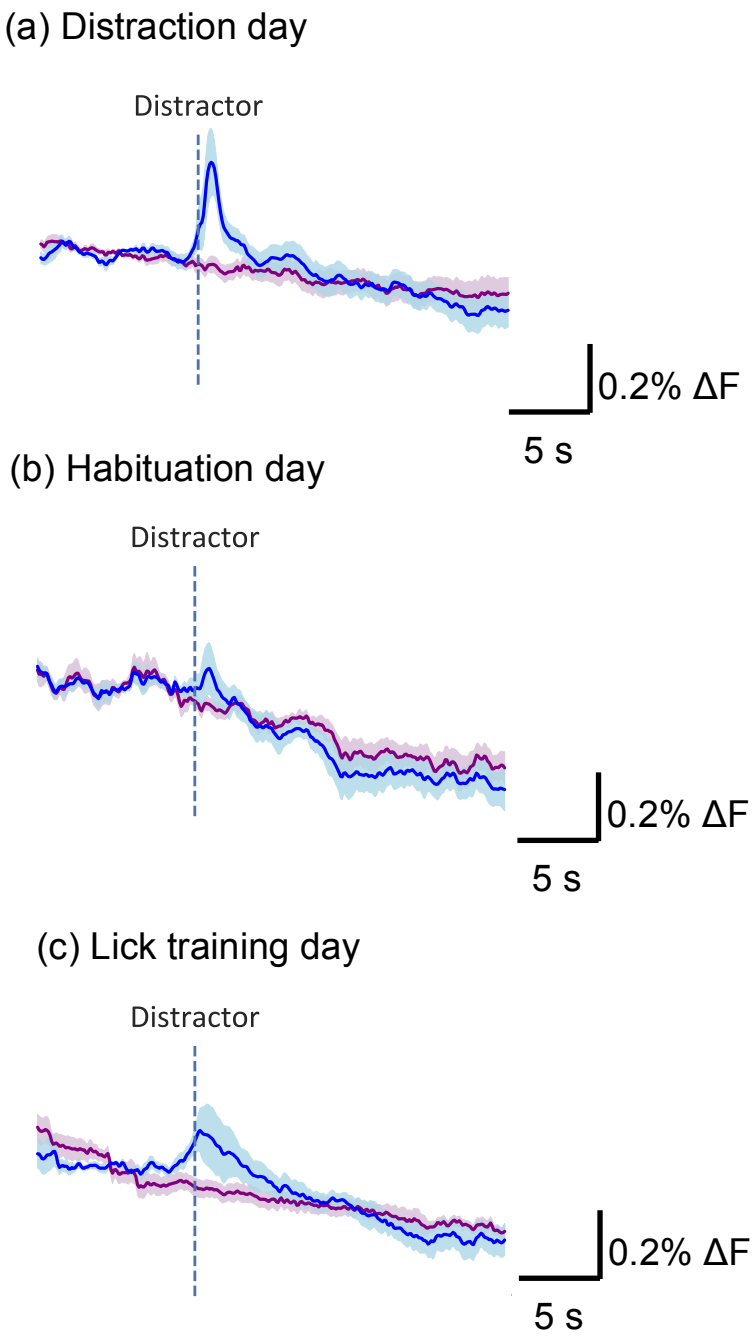


Figure 5.4 – *Averaged photometry traces for distractor presentations (NAc)*

NAc neural activity during distractor presentation differs across days. **(a)** Averaged photometry trace for all rats and all distractors aligned to distractor presentation on the main test day. **(b)** Averaged photometry trace to distractor presentations on the habituation day. **(c)** Averaged photometry trace for modelled distractors (calculated on the last lick day).

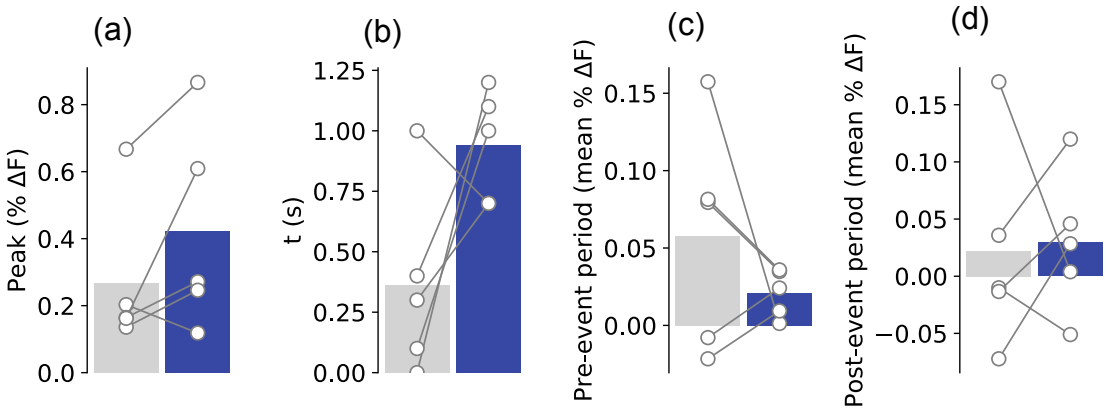


Figure 5.5 – *Calculated photometry parameters for modelled vs real distractor presentations (NAc)*

Parameters calculated from averaged photometry signals for modelled distractors (grey) and distractor presentations on the distraction test day (blue). **(a)** Peak height **(b)** tmax, **(c)** Pre-event period **(d)** Post-event period. There are no statistical differences in these parameters, which is likely due to low numbers of animals used and variability in the data.

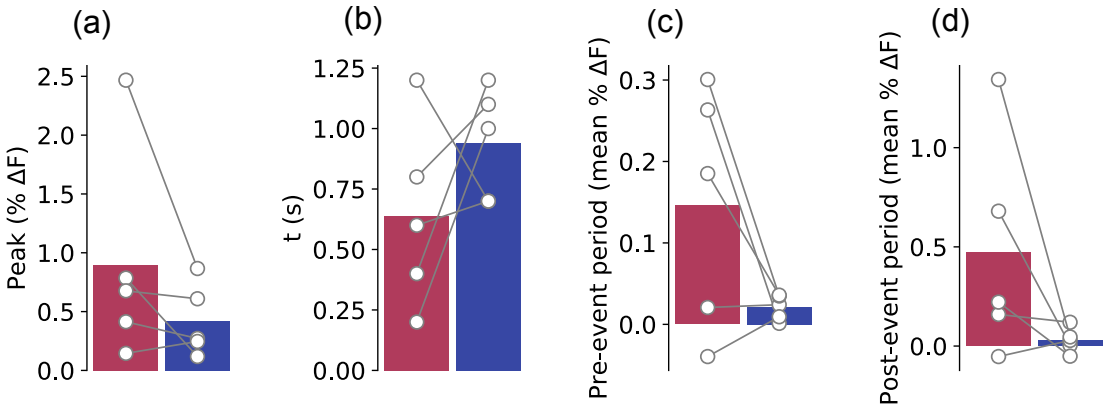


Figure 5.6 – *Calculated photometry parameters for licking runs vs distractor presentations (NAc)*

Parameters calculated from averaged photometry signals for lick runs (shiraz) and distractor presentations on the distraction test day (blue). **(a)** Peak height, **(b)** tmax, **(c)** Pre-event period, and **(d)** Post-event period. There are no statistical differences in these parameters

5.4.4 NAc responses for distracted versus not distracted trials

Figure 5.7 shows averaged photometry traces for all rats comparing distracted and non distracted trials (on the main distraction test day).To compare the NAc responses to distracted versus not distracted trials, t-tests were performed to examine peak and post-event period measures, **Figure 5.8.**

Peak responses on distracted trials were not significantly different from non-distracted trials, $t(4) = 2.17$, $p = .096$, **Figure 5.8a**. There was also no significant difference in the post event period, between distracted and non distracted trials, $t(4) = 0.36$, $p = .74$, **Figure 5.8d.**

Visual inspection of averaged photometry traces suggests that there is increased activity in response to distractors, which is elevated when rats are distracted, however this is not supported statistically because of low numbers of rats used and variability.

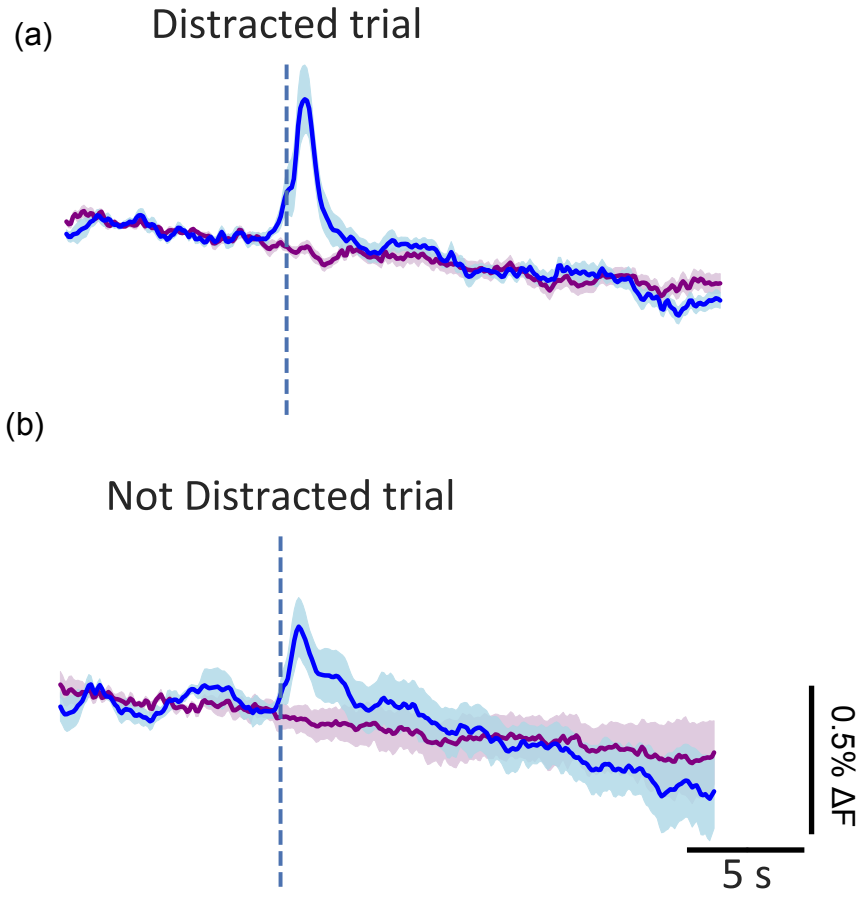


Figure 5.7 – *Averaged photometry traces for distracted and not distracted trials following distractor presentation (NAc)*

NAc neural activity following distractor presentation in **(a)** Distracted trials, where rats pause following a distractor, and **(b)** Not distracted trials, where rats continue to lick in the face of distractors. Averaged photometry trace for all rats and all trials aligned to distractor presentation. There is a larger and sharper peak for distracted versus not distracted responses although this is not statistically significant.

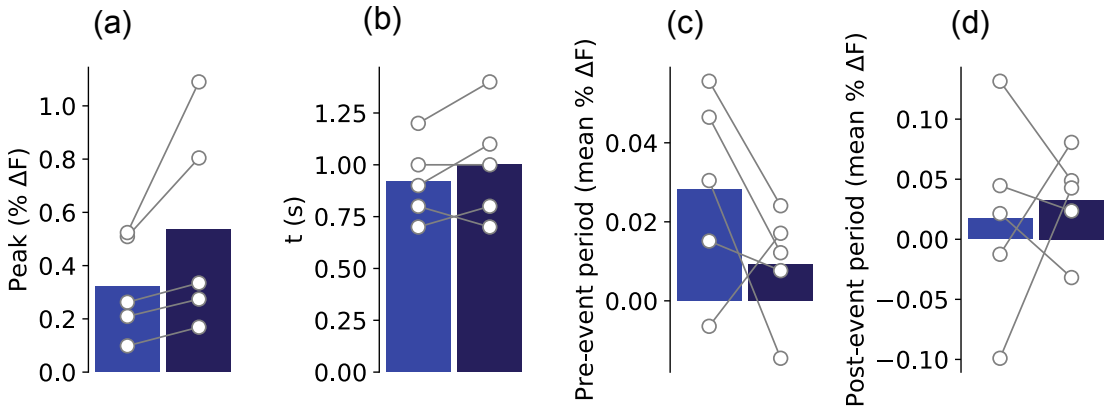


Figure 5.8 – *Calculated photometry parameters for not distracted and distracted trials (NAc)*

Parameters calculated from averaged photometry signals for not distracted trials (purple/blue) and distracted trials (navy/dark blue), **(a)** Peak, **(b)** tmax, **(c)** Pre-event period, and **(d)** Post-event period.

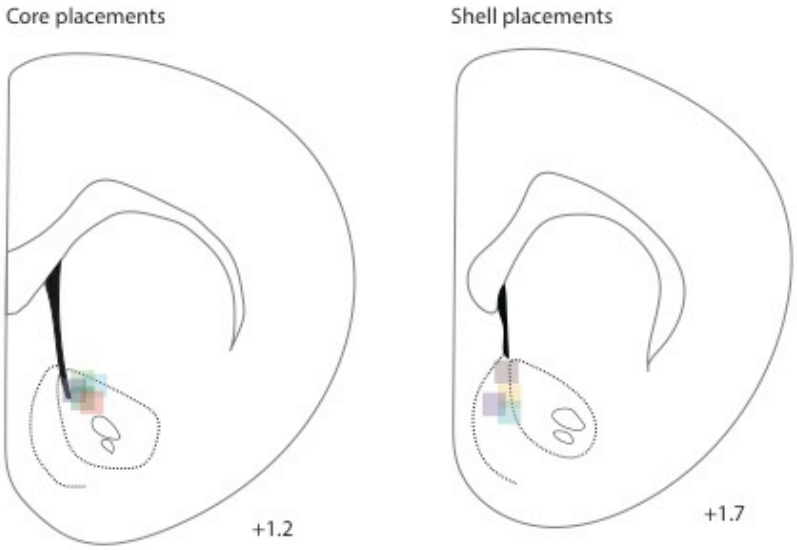


Figure 5.9 – *Fibre placements in the nucleus accumbens core and shell*

Schematic of coronal sections showing electrode placements in NAc core and NAc shell (coloured squares show fibre tip placements for individual rats).

5.5 DISCUSSION

The NAc signals presented in this chapter are similar to those observed when recording from cell bodies in the VTA (Chapter 4). We see transient peaks in NAc activity at the beginning of runs of licks and in response to distractors. Visual inspection of photometry plots also suggests there may be the same differences between peaks for distracted and not distracted trials seen in the VTA (namely larger peaks when rats are distracted). The low number of rats with signals meant that statistical analysis did not find statistically significant effects in most cases, however with more data these responses will likely be similar to those obtained from the VTA.

The similarities between the signals suggests that, despite low levels of specificity reported in out immunohistochemistry results the similarity there is overlap in the population recorded within the VTA and in VTA neurons projecting to the NAc and we assume (based on the high proportion of dopamine neurons within the projections from VTA to NAc) that the majority of this response is mediated by dopamine.

The most significant difference we observe when comparing VTA and NAc responses is when parsing the signals based on different run lengths. In Chapter 4 we found considerable suppression of activity following long runs and an elevation of the GCaMP signal following short runs. Here, in the NAc, we report the opposite effect with long runs showing an unexpected elevation in NAc activity. One possibility here is that multiple populations are involved in generating the VTA cell body signal and that the suppression of activity in VTA during long runs is the result of local neurons, for example inhibitory feedback from VTA GABA interneurons. Although speculative, it is possible that this inhibition allows dopamine neurons to fire in a sustained way during coinsumption, as is seen in our recordings of terminals.

Dopamine release within the terminal regions of the NAc has varied effects on D1 and D2 expressing medium spiny neurons (MSNs) and this may facilitate the selection of appropriate actions (such as continuing consumption or

attending to distractors) in the face of multiple diverse informational inputs. Such dopamine release may be involved in the rapid switching of behaviours and this highlights the importance of the putative dopamine response we have observed to distractors.

These data are consistent with a role of the NAc in responses to both consumption and sensory stimuli in the environment. In line with the role of the LH in authorising feeding proposed by O'Connor et al (2004), Kelley (2004) has also discussed evidence for the modulation of ingestive behaviour by activation of LH cells. Kelley (2004) refers to the NAcSh as a 'sensory sentinel', allowing for adaptive switching between feeding and other stimuli. She argues that medium spiny neurons within the NAcSh release GABA during feeding and this disinhibits the LH. Inputs to the NAcSh from cortex and other limbic structures may use glutamatergic signalling to inhibit downstream feeding in the face of potentially important stimuli in the environment. The shell of the NAc may be especially important in shutting off metabolically driven feeding behaviour and diverting attention to novel, behaviourally relevant stimuli in the environment. However, the signals we report here are mainly from neurons recorded in the accumbens core, further studies are needed to assess potential core and shell differences in this behavioural task and further assess the role of mesolimbic structures in integrating sensory information with internal need states such as hunger.

In Chapter 6 I will investigate several viral strategies for dopamine cell specific expression of GCaMP6 and record from the VTA using cell-specific method.

CHAPTER 6

Fibre photometry in dopaminergic neurons: viral and transgenic strategies

6.1 INTRODUCTION

There are now several viral strategies used in fibre photometry experiments that allow for cell specific targeting. These often make use of Cre/Lox recombinase systems to deliver the genetically encoded calcium indicator to defined neuron cell types. In our case, we are primarily interested in dopamine neurons and in this chapter we evaluate two different strategies using a Cre-dependent system in different ways and assess the ability of each method to selectively infect dopamine neurons in the VTA.

The Cre-lox system has two elements, the enzyme Cre recombinase and the short target sequences that it is able to recombine, these are called Lox sites and contain specific binding sites for Cre protein. Developed in bacteriophage P1 the Cre-Lox system allows for the cell specific expression of GCaMP and or other indicators. A viral construct can be created containing an inverted version of the gene of interest (which cannot be read in this direction). The gene of interest, in our case that which codes for GCaMP,, can be flanked with loxP sites. When this virus is combined with Cre-recombinase the Cre-Lox system works to flip the inverted sequence into a readable direction where it can be expressed.

In the following chapter we present two methods for dopamine specific expression of GCaMP, both utilising the Cre-Lox system, and evaluate their effectiveness before undertaking fibre photometry recordings in dopamine neurons. The first method involves a combinatorial approach with two viruses, one containing the Cre element that is expressed under a TH promoter which drives expression in dopamine neurons specifically. The other is a Cre-inducible GCaMP with loxP site allowing for recombination in the presence of Cre (Gompf et al., 2015).

The second approach is to use the same Cre-inducible GCaMP but instead of adding a second virus this is injected into transgenic animals expressing Cre in a specific neuronal type, TH::Cre rats. Witten et al (2011) developed this TH::Cre rat and this has been used by Steinberg et al., (2014) to demonstrate the importance of dopamine neurons in the NAc in the positive reinforcing effects of intracranial self stimulation and by McCutcheon et al. (2014) in combination with optogenetic inhibition and voltammetry to investigate drug-evoked dopamine transients in the NAc.

6.2 AIMS, OBJECTIVES & HYPOTHESES

6.2.1 Aims

In the following set of preliminary experiments we aim to assess the specificity of two methods for inducing cell type specific expression of GCaMP6s

6.2.2 Objectives

(1) The first objective was to assess the specificity of combinatory viral approach using a TH-specific Cre-expressing virus combined with a Cre-inducible (FLEX) GCaMP virus
(2) The second objective was to assess the specificity of a different, transgenic strategy using a Cre-inducible (FLEX) virus in TH::Cre transgenic rats in targeting dopamine neurons.

6.2.3 Hypotheses

It was predicted that both methods (the combinatorial approach and the transgenic approach) would be more dopamine specific leading to greater expression of GCaMP6s in TH positive neurons compared to the non-specific GCaMP6s virus that targets neurons of any type.

6.3 METHOD

6.3.1 Animals

Two separate experiments were performed in two cohorts of rats. For the first experiment a cohort of 4 male Sprague Dawley rats, weighing 250 – 300 g were used. These animals were only used for immunohistochemistry. For the second experiment a cohort of 10 rats was used. These were TH::Cre rats on a Long-Evans background and weighed 300 – 350 g at the time of surgery. They expressed Cre recombinase under the control of the tyrosine hydroxylase promoter so that they specifically expressed Cre in dopaminergic neurons (Witten et al., 2011). These rats were used for a fibre photometry experiment.

6.3.2 Experiment 1 viral injection

Experiment one aimed to assess the specificity of a combinatorial viral approach. Two viruses were injected into the same brain region at the same time. These where (1) AAV9.rTH.PI.Cre.SV40 (ddTitre = 6.980 x 10^{13} GC/ml), a TH Cre-expressing virus (Addgene plasmid #107788), **Figure 6.1c** shows the structure of this construct (Gompf et al., 2015) (2) AAV9.Syn.FLEX.GCaMP6s.WPRE.SV40 (ddTitre = 6.664 x 10^{13} GC/ml, Addgene plasmid #100843), a Cre-inducible GCaMP virus which will express in the presence of Cre recombinase (provided by the first virus) (**Figure 6.1b**).

The TH-Cre expressing virus was injected into the VTA in four rats at different concentrations (undiluted, 1:1, 1:3 and control aCSF). The Cre-inducible GCaMP was injected at 1:3 in all cases. Both viruses were loaded into the same Hamilton syringe and a total of 1 µl was injected into the VTA (AP – 5.8 mm, ML + 0.8 ML, DV – 8.1 mm) at a speed of 100 nl / min.

6.3.3 Experiment 2 viral injection and fibre implantation

In this experiment, the Cre-inducible, FLEX GCaMP virus (AAV9.Syn.FLEX.GCaMP6s.WPRE.SV40) was injected into the VTA of transgenic, TH::Cre rat (Witten et al., 2011). In these rats, photometry fibres were implanted and rats were allowed 4 weeks for the virus to express before being tested behaviourally using the distraction assay and recorded with the photometry system (as described in **Chapter 4**).

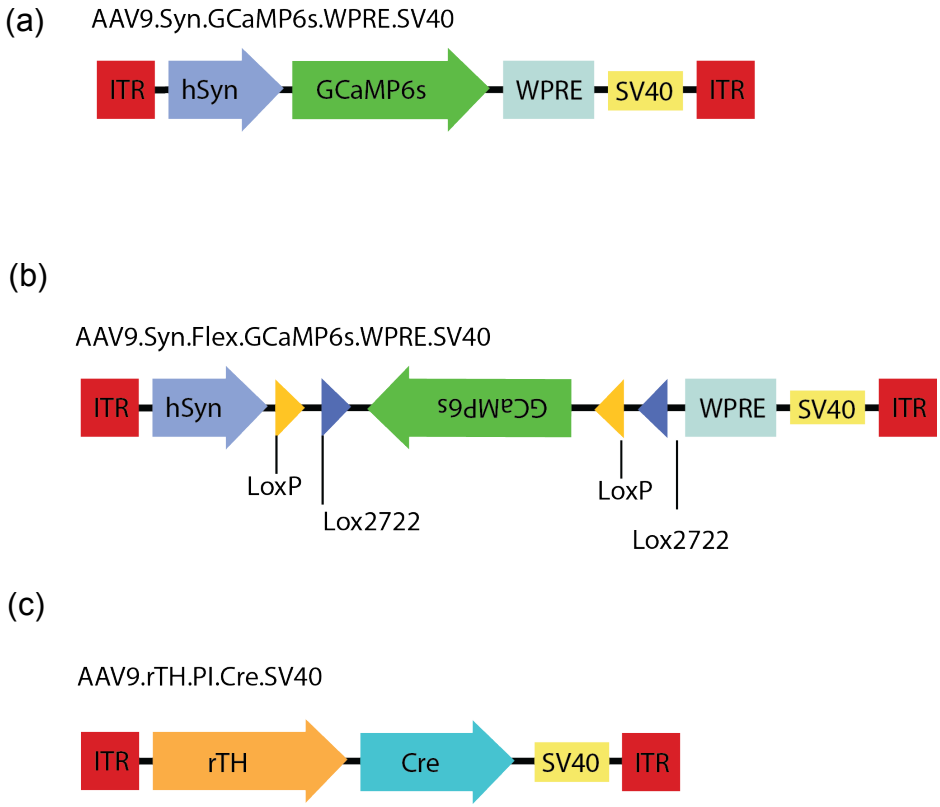


Figure 6.1 – *Three viral constructs used in photometry and histology experiments*

(a) Non-specific adeno-associated virus for the expression of GCaMP6s in all neuronal cell types **(b)** Cre-dependent GCaMP virus, expression of calcium indicator GCaMP6s is contingent upon Cre-recombinase **(c)** TH Cre-inducible virus, this was used in a combinatorial viral strategy with (b) to infection dopamine neurons specifically.

6.3.4 Immunohistochemistry

Brains were perfused with paraformaldehyde and sliced to 40 μm thick slices containing the VTA. As previously described (Chapter 4), slices were stained for GFP and TH using the primary antibodies and fluorescent secondaries. Following staining, slices were mounted, cover-slipped and imaged before staining was quantified. Cells were manually counted using Image J by selecting regions of interest (ROIs) where GFP positive neurons were observed. Within these ROIs TH positive neurons were counted and the extent of co-localisation was calculated. Specificity was calculated as the total number of GFP positive neurons divided by the number of co-localised (GFP+TH positive) neurons. This is expressed as percentage, showing the specificity of each viral strategy in infecting dopamine neurons.

6.4 RESULTS

6.4.1 Specificity of different viral strategies

Cell counts of GFP positive cells and TH positive cells as well as those double-stained with GFP and TH were used to calculate specificity (the number of GFP cells that were also TH positive). This is expressed as a percentage value. One-way ANOVA was used to compare the specificity of the three viral strategies, the non-specific GCaMP, the combinatorial viral approach of TH Cre virus in combination with Cre-inducible GCaMP and the Cre-inducible GCaMP virus delivered in transgenic, TH::Cre rats. There was a significant main effect of virus with different specificities in each of the three groups ($F(2,31) = 120.48$, $p < .001$)

Bonferroni corrected t-test showed that there was no difference in specificity between the non-specific virus (M = 7% , SEM = 1%) and the combinatory viral method (M = 5%, SEM = 0.2%, $p = 1.0$) suggesting that in fact this strategy of did not result in specific expression of GCaMP6s in dopamine neurons in the rats we tested.

However, the Cre –inducible GCaMP virus delivered in TH::Cre rats did prove to be more specific. There was greater co-localisation of GFP and TH staining in this group (M = ,51% SEM = 4%) than compared to both the TH Cre virus ($p < .001$) and the non-specific GCaMP6s ($p < .001$). **Figure 6.2** shows histological examples of co-localisation in the TH::Cre animals.

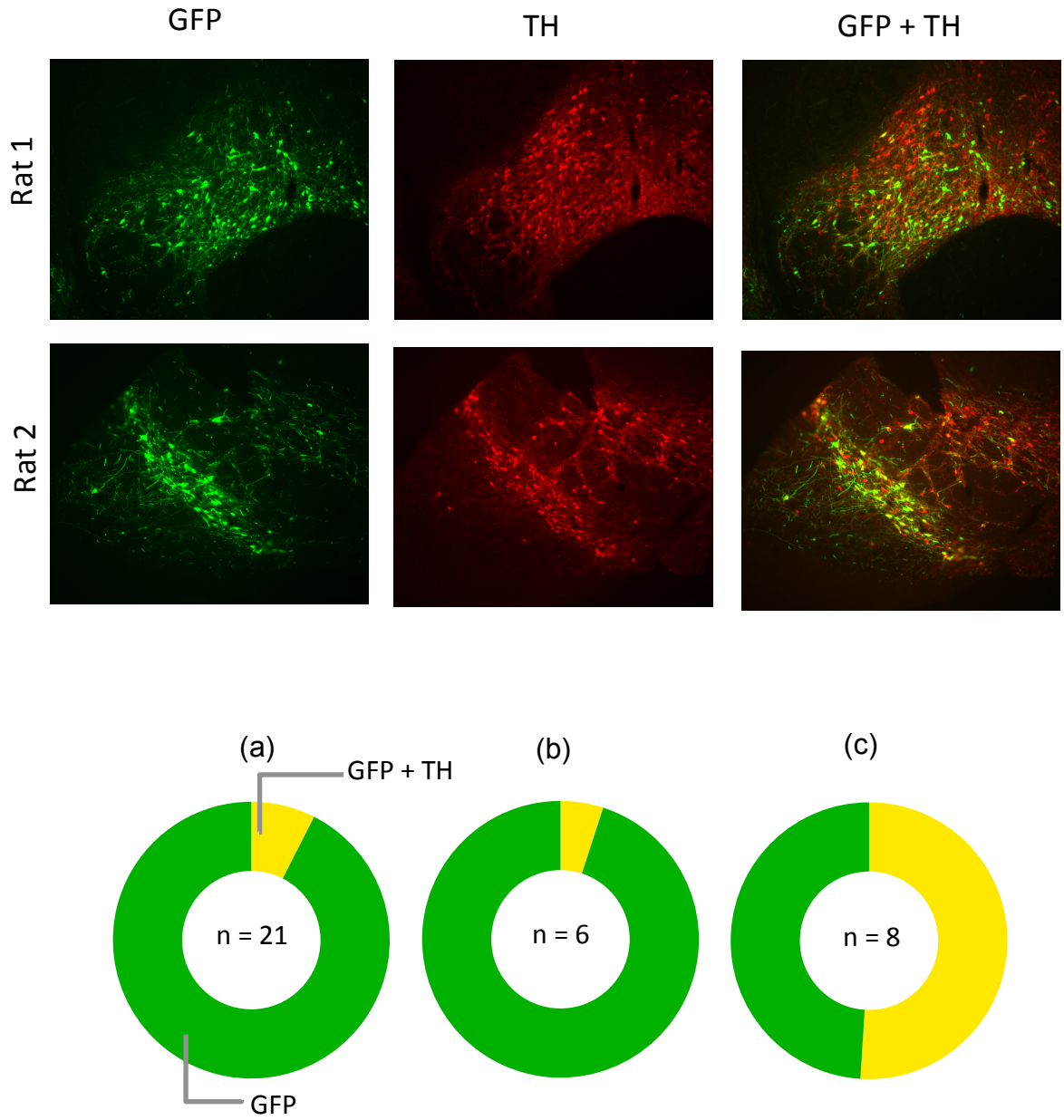


Figure 6.2 – *GFP and TH staining in VTA slices and quantification of virus specificity*

GFP and TH staining in TH::Cre transgenic rats infected with a Cre-inducible GCaMP virus. To panel shows viral expression in VTA neurons, GFP (green) shows the calcium indicator expression, TH (red) shows dopaminergic neurons and the merged images show doublestaining. Three representative slices from separate animals are shown. Lower diagrams show specificity of different viral strategies**, (a)** non-specific GCaMP **(b)** TH Cre-inducible virus in combinatorial viral strategy **(c)** Cre-inducible GCaMP virus in TH::Cre rats (the most specific strategy here)

6.4.2 Fibre photometry recordings

Due to unexpected variation in the skull thickness of transgenic TH::Cre animals there were substantial misplacements of all photometry fibres (**Figure 6.4**). Unfortunately this resulted in a lack of signals in these animals. The viral expression was successful (and the amount of co-localisation is reported above), however, the fibre tips were not positioned in close enough proximity to the expressed calcium indicator to reliably measure GCaMP6s fluorescence.

Figure 6.3 shows the averaged photometry recordings from three of these animals during licking and distractor presentations. The peaks are difficult to distinguish above noise and therefore no further analysis was undertaken.

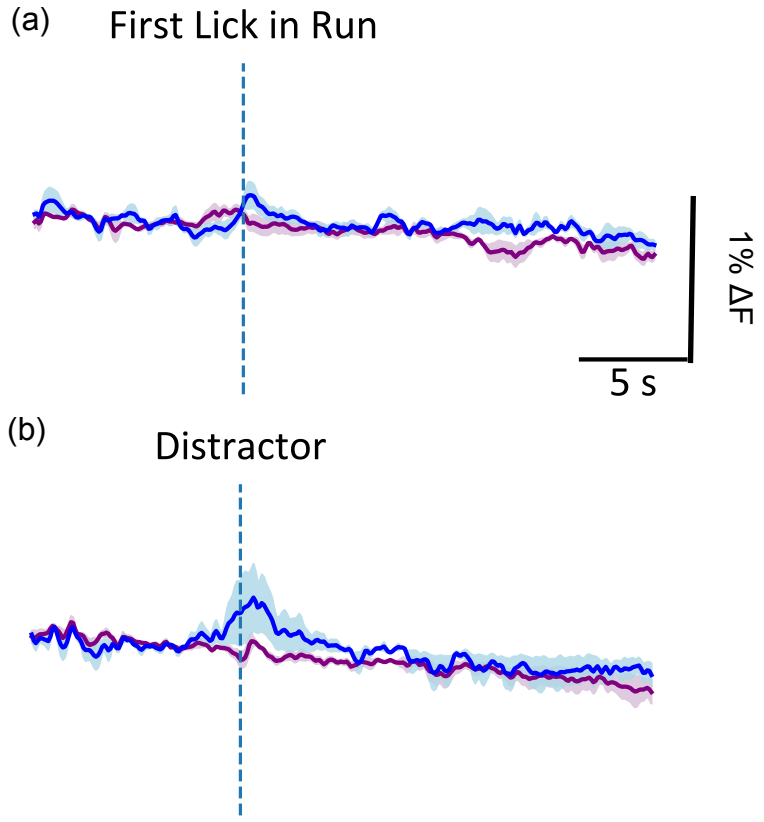


Figure 6.3 – *Photometry responses in the VTA of TH::Cre rats to bouts of licking and in response to distractor presentations*

VTA neural activity following **(a)** the initiation of licking runs, **(b)** distractor presentations. Peaks are small and difficult to discern above noise, this is likely due to the distance of the photometry fibres from the virus injection and expression site.

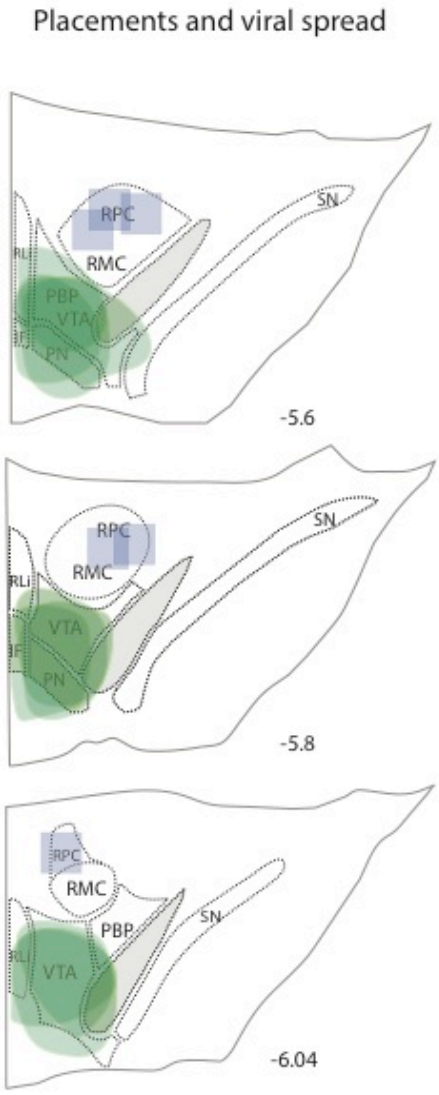


Figure 6.4. – *Misplaced fibres in TH::Cre rats*

Figure shows placement error in TH::Cre cohort, fibres are placed too dorsally missing the VTA subnuclei, the majority of fibres are placed in the red nucleus whereas the viral expression is localised to the VTA. Blue squares show fibre placements and green densities show viral spread (GFP positive stained neurons) in all rats. Atlas images are modified from Paxinos and Watson (2005). VTA subnuclei are labelled as follows: VTA (ventral tegmental area), PBP (parabrachial pigmented area), PN (paranigral nucleus), RII (rostral linear nucleus), IF (interfascicular nucleus). The red nucleus subdivisions are laballed: RPC (red nucleus parvocellular part) and PMC (red nucleus magnocellular part)

6.5 DISCUSSION

Our findings suggest that the combinatorial strategy was not specific in infecting dopamine neurons. Contrary to other reports of successful dopamine specific expression of GCaMP using this viral strategy (Gompf et al., 2015; Stauffer et al., 2016), we did not see significantly higher specificity than a non-specific GCaMP virus. The virus expression data show clear GFP staining, suggesting that there was recombination, however this was not located in dopaminergic neurons exclusively (or even preferentially).

Given the large dopaminergic content of the VTA it is surprising that the non-specific virus labels so few dopamine neurons also. However, there is substantial non-dopaminergic population in the VTA (40% of VTA neurons are not dopaminergic, (Fields et al., 2007)) so this is not impossible. The TH promoter in the TH Cre virus, however, is designed to target only dopamine neurons and should only express Cre in those TH containing neurons. It is possible that there are serotype differences, Stauffer et al (2016) report that the AAV5 version shows greater specificity for dopamine neurons. Furthermore, they used monkeys and there may be species differences in rodents. Alternatively there may be an issue with the staining for TH or some promotor competition. It is possible that the amount of TH present in these brains may be lower due to the ongoing production of Cre in these neurons.

Using a transgenic approach with TH::Cre positive rats and the FLEX virus we did observe significant specificity. The transgenic animals expressing Cre in dopamine neurons (TH specific) showed much higher co-localisation of GFP and TH with a specificity of over 50%. However, this co-localisation is still lower than expected. One possible explanation for this low expression of GFP in TH neurons (with all three strategies) is our histological method. It is possible that the staining for TH positive neurons was not optimal, our immunohistochemical approach may not be sensitive enough to detect all TH neurons and thus may underestimate co-localisation. Alternatively there may be an effect of neuronal cell death in the TH neurons. Dopamine neurons are especially sensitive to oxidative stress (Pacelli et al., 2015) and it is possible that some of the neuronal

population recorded have died during viral expression or over long term recordings. Alternative methods such as in situ hybridisation could be used to further assess the expression of TH and to assess co-localisation perhaps with greater precision.

Unfortunately there were substantial methodological problems with the fibre photometry recordings in these animals, most detrimental were incorrect fibre placements which meant that the viral expression of GCaMP6s was not effectively measured using the photometry probes. The strongest hypothesis we have currently is that the thicker skulls in TH-Cre rats (which are on a Long-Evans background and get far larger than Sprgaue-Dawleys) prevented the optic fibres from being lowered into close proximity with the location of infected GCaMP-expressing neurons.

There were three rats with some detectable signal (likely due to small variations where these had higher viral expression and/or fibres slightly closer to the virus expressing neurons) which enabled small changes in signals to be detected. However, even in these rats the signal was negligible and precluded further analysis. With the insight gained from these preliminary experiments further experiments should ensure the correct placement of fibres in TH::Cre rats and measure photometry responses in dopamine neurons specifically.

CHAPTER 7

General Discussion

7. GENERAL DISCUSSION

7.1 Summary of findings

These experiments assessed the ability of distractor stimuli to interrupt ongoing licking for saccharin and investigated VTA signalling during this task. Building on the work of O'Connor et al (2015) we developed an assay to induce and quantify distraction during saccharin consumption whilst measuring licking microstructure (Davis and Smith, 1993). An initial pilot experiment characterised male Sprague Dawley rats' responses to distractors. This experiment demonstrated that rats will often pause their consummatory behaviour in response to distractors, a behaviour not observed during normal consumption (lick training). Furthermore, animals habituated to distracting stimuli after a single session and, as such, repeated testing resulted in a decrease in percent distracted as rats stopped pausing following distractor presentations. Finally, amphetamine enhanced distraction reversing the habituation effect without affecting licking, implicating catecholamine transmission in the pausing response to distractors during licking.

Further experiments used this assay in PCP pre-treated rats under a subchronic regime, which models some aspects of schizophrenia. The aim was to assess the potential increased propensity to distraction in these rats. Evidence from Neill et al (2010) suggested that some of the deficits seen in the subchronic PCP model may be underlined by a disruption in attention and an increase in distractibility (Grayson et al., 2014). We found no clear evidence of increased distraction in this model in either male or female rats as well as no difference in habituation and no enhancement of the amphetamine effect. However, as discussed in the relevant chapters, we also report a lack of NOR deficit in these animals. As the NOR test is the primary means of verifying the success of PCP treatmen,t a lack of deficit indicates that PCP treatment may have been ineffective. This suggests that rather than an inability of the distraction assay to find the expected differences or in fact a lack of such difference in distraction in PCP animals, there were fundamental issues with the subchronic treatment and this model itself. Unfortunately, we were therefore unable to fully assess the utility of this distraction assay in this particular model.

Importantly, this may highlight some significant shortcomings of the subchronic PCP model specifically and, more fundamentally, this highlights several issues of modelling psychiatric conditions more generally.

Fibre photometry recordings in the VTA and NAc were used to assess the activity of neurons originating in the VTA at both their cell bodies and in NAc terminals during consumption and distraction. We found transient changes in calcium signals at the onset of licking bouts in addition to responses following distractor stimuli. These changes appear to have different rates of decay, with distractor responses generally briefer whereas some responses seen during consumption are often followed by a significant suppression of activity. Interestingly, such suppression was not observed in the NAc terminals of VTA neurons but only in the cell bodies. Although this could be because of small signals seen in the terminals or as a result of local circuit modulation, it is also possible that the neurons recorded at the cell bodies are a different population to those from whose terminals we recorded.

In fact, our immunohistochemistry results suggest that those neurons recorded in the VTA using a non-specific GCaMP6s virus, were not primarily dopaminergic. It is possible that this neuronal population is made up of other cell types such as GABAergic neurons (Morales & Margolis, 2017). We know that around 60 % of VTA neurons are actually non-dopaminergic (Swanson, 1982), and of the other cell types that make up this heterogeneous population, GABA neurons are the most abundant (Carr and Sesack, 2000). Projection neurons from the VTA to the NAc however are more likely to be dopaminergic with estimates ranging from 65 – 85 % of these neurons as dopaminergic (Morales & Margolis, 2017). It is possible these divergent signals come from distinct neural populations. Although speculative, perhaps the suppression following licking bouts seen in the VTA and not the NAc is a result of greater GABA contribution to these signals providing an inhibitory component. Mirroring the behavioural habituation effect seen in the distraction task, as percent distracted decreased over days so did the peak neural responses observed. Calcium changes were smaller on the second and third days of distraction

testing. Peak responses were also larger when the rat was distracted compared to when they continued to lick for saccharin and were not distracted.

To determine the contribution of dopamine neurons to the VTA signal, further experiments assessed the ability of two viral strategies to target dopamine neurons preferentially, with the aim of recording from dopamine neurons only in future experiments. First, we tested a TH (dopamine) specific virus combined with a Cre-inducible (FLEX) virus to induce cre-dependent expression in TH positive neurons. Our preliminary immunohistochemical evidence suggests that the TH specific virus was, in fact, not effective in targeting dopamine neurons specifically and did not lead to dopamine neuron specific expression of GCaMP6s in the VTA. However, a transgenic approach was also tested, with the use of the same FLEX virus applied in transgenic rats, which were TH::Cre positive. In this case, we did observe a good level of specificity with over 50% of GFP expressing neurons also showing markers for dopamine. Fibre photometry recordings in the VTA were undertaken in these animals, however, substantial methodological issues – specifically poor fibre placements – hindered these recordings such that no clear conclusion can be made from this experiment. However, with the information we have gleaned from the immunohistochemistry, future experiments can assess the specific contribution of dopamine to the signals we have observed by implementing this transgenic and viral strategy and overcoming the methodological issues this current study had.

Together these experiments demonstrate that the distraction assay we developed produces a behavioural response, which can be quantified with percent distracted, and that this is accompanied by neuronal changes in calcium within the VTA. Rats pause ongoing licking for saccharin in response to distractor stimuli and VTA signals accompany both licking initiations and distractions. This test did not prove effective in assessing distraction in the subchronic PCP model, however, this highlights important methodological considerations when using animal models of psychiatric conditions which are further discussed below.

7.2 Modelling schizophrenia in rodents

It is disappointing that we were not able to describe the expected differences in distraction in subchronic PCP treated animals. It is unusual that we did not observe a NOR deficit in these animals. Previous experiments in this lab and others have reported this deficit consistently in multiple strains and sexes. NOR is the most commonly used means to verify the success of PCP treatment. Deficits in NOR are presumed to show significant impairments in visual recognition memory and novelty preference which reflect a core deficit in schizophrenia. This test has been assessed for its translational relevance and is high face validity. However, it is unclear what a lack of the NOR deficit means for the subchronic treatment regime. Relying on this single task to assess the success or failure of treatment does not seem robust enough. The ambiguity that arises from a lack of NOR deficit shows a need for other ways to validate the subchronic PCP treatment. Some have used a PCP challenge, where acute administration of PCP leads to increased locomotor activity; this is heightened in subchronic PCP treated animals as they show a locomotor sensitisation effect. However, the presence of this effect simply shows that they had received the drug previously, and not necessarily that the treatment is effective in modelling neurochemical, neuroanatomical and behavioural features of schizophrenia.

It is possible that strain differences may have contributed to the lack of NOR deficit that we observed. Although the NOR deficit has been reported with subchronic PCP treatment in the strain that we used, the model is more commonly used in female Wistar rats (Neill et al.., 2010). However, if this is the case, then it is possible that our animals still display some ‘schizophrenic’ phenotype, i.e. deficits in memory, attention and information processing, but that this is subthreshold for the NOR assay. If these experiments were repeated we could use multiple behavioural assays to verify that PCP treatment was effective, such as the five choice serial reaction time test. However, the aim to assess distraction in PCP animals using this assay was to avoid complex tasks with extensive training. Thus, it might be more appropriate instead to use the distraction test in a different model of schizophrenia such as social isolation, maternal deprivation or ventral hippocampal lesion models.

However, the translational relevance of rodent models to human conditions is often questioned. This is particularly pertinent with psychiatric conditions such as schizophrenia, which often presents in patients with symptoms that are uniquely human. For example, complex delusions often involve the patient's specific belief systems and personal experiences. Furthermore, these are only revealed by self-report measures, which cannot be used in rodents. Animal models simply cannot recapitulate such human, self-referential and complex experiences, which are central to schizophrenia. It is necessary to reduce models to core features and symptoms that we can model. This is why NOR and other tests are used, and why we aimed to assess distraction in this very specific way. However, how much do these and other models really capture of schizophrenia versus general dysfunctions that are not disease specific? Cognitive disturbances and attentional dysfunction are features of many psychiatric conditions including ADHD, addiction, depression, mania and other distinct forms of psychosis. This does not mean we should not study such cognitive impairments but caution should be taken when referring to these deficits as modelling schizophrenia and in the interpretation of behavioural and neurochemical findings.

The CENTRICs initiative (Carter and Barch, 2007; Barch et al., 2009) which aimed to identify the most translationally relevant tests for cognitive deficits and to develop models that have construct, face and predictive validity in modelling and assessing cognitive impairments in schizophrenia is over a decade old now. This initiative was started alongside efforts in human studies to greater understand cognition in schizophrenia with a goal to accelerate our understanding of cognitive symptoms and enhance models so that new pharmacological therapies would be devised. However, in the 10 years that have passed since these publications the landmark developments in our understanding of schizophrenia and in pharmacotherapies have not been delivered. Cognitive symptoms are still poorly understood and no new treatments are available. It is perhaps time to reconsider the heterogeneity of schizophrenia and re-evaluate how we model aspects of this condition.

7.3 The many roles of the VTA

Although we were not able to provide evidence for increased distraction in a model of schizophrenia, we did gather interesting data from fibre photometry experiments in control rats, which have implications for the role of VTA neurons in multiple behaviours. The responses we report here support other evidence for the varied rols of VTA neurons in encoding diverse information to guide motivated behaviour. We report calcium responses in the VTA to both licking and distractors, which differ temporally, as well as differential responses on distracted versus non-distracted trials. We provide evidence for the VTA as an important site for the integration of multiple behaviourally relevant inputs. Our findings support results that already demonstrate a role for the VTA → NAc connection in feeding and motivation (Roitman et al., 2004; Bassareo & Di Chiara, 1999), and our findings are consistent with a role for the VTA in monitoring potentially important external stimuli and helping to orient attention to such stimuli. Further studies characterising the VTA responses to a variety of stimuli and contexts are needed to help us understand how this circuitry can process such complex and divergent information to inform adaptive and motivational behaviours.

7.4 Conclusions

This thesis investigated distraction; we developed a novel assay exploiting rats self-paced licking behaviour, applied this assay to a model of schizophrenia and carried out fibre photometry recordings to examine mesolimbic neural activity. The findings presented here implicate the VTA and mesolimbic circuitry in both ongoing consummatory behaviour and in monitoring environmental stimuli. These results, particularly the lack of effects in the subchronic PCP model raise important questions about modelling psychiatric conditions in rodents. Future experiments will determine the contribution of dopamine to the signals within the VTA to determine whther this transmitter is the substrates for these behavioural results.

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