The Ventral Tegmental Area and Mesolimbic Dopamine System: Contribution to Thermoregulation Assessed Through Novel Neuromodulatory Techniques

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ABSTRACT

This thesis examined the contribution of dopaminergic neurons of the ventral tegmental area (VTA), a crucial component of the mesolimbic dopamine system, to the process of thermoregulation.

The control of body temperature, as a physiological regulator in response to environmental factors or emotional triggers, is vital to the survival of all animals. However, very little is understood in regard to the neurotransmitters, brain areas and neural pathways that are involved in its regulation. As such, many autonomic conditions, such as Parkinson's disease and psychogenic fever, which exhibit thermoregulatory symptoms are poorly understood and consequently undertreated.

Dopamine is a neurotransmitter known to be involved in the regulation of central bodily cognitive and motor functions. Recent findings have indicated that it also plays an important role in body temperature. However, its central mechanisms of action are unknown. One of the major nuclei in the brain that contains dopamine-producing neurons is the VTA, which is approximately 30-70% dopaminergic. Thus, the present study aimed to elucidate the specific role of VTA dopamine neurons in the control of thermoregulatory outputs.

Within the past decade new techniques, such as chemogenetics, have emerged which allow for the specific study of neurotransmitter function in defined brain regions. Hence, this study utilized, the designer receptors exclusively activated by designer drugs (DREADDs) system, in conjunction with specifically modified transgenic animals and specially designed ligands to investigate the effect of temporary neuronal population activation on the common thermoregulatory response, known as emotional hyperthermia. Targeted toxins were also applied to determine the effect of dopamine signalling reduction on various thermoregulatory parameters. All biological parameters were assessed in conscious, freely moving animals through the chronic implantation of thermistor probes, locomotor behaviour tracking and food consumption monitoring.

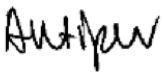
The results presented in this thesis conclusively demonstrate the vital contribution of dopaminergic VTA neurons to the process of thermoregulation; as well as their impact on physiological processes – such as feeding and locomotion. VTA dopaminergic enhancement was found to substantially increase the baseline temperature of animals. This was simultaneously accompanied by changed feeding patterns and locomotor hyperactivity. Specific dopamine

receptor influence and feeding behaviour alterations were assessed as a potential contributing factor to the observed temperature elevation. This facilitated the validation of D_1 dopamine receptor involvement in the process of body temperature control. Lastly, the influence of the mesolimbic dopamine system, originating in the VTA and projecting to the nucleus accumbens, was assessed. Although this pathway has been implicated in locomotion, feeding and emotional processing behaviours - making it a potential candidate for the control for thermoregulation, it was determined that it is likely not the primary pathway responsible for thermoregulation.

Taken together, the evidence provided in this investigation adds fundamental biological information to the field of integrative neuroscience, which helps the understanding of connections between brain regions and physiological responses. Furthermore, the research conducted validates the use of modern genetic techniques in conscious animal physiological research. Importantly, the identification of a thermoregulatory brain region, as well as the receptor type involved in its function, may assist in the development of new, more effective, and targeted treatments for those suffering with thermoregulatory diseases.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: 

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ARTICLES PUBLISHED DURING CANDIDATURE

- Brizuela M, Antipov A, Blessing W, Ootsuka Y. Activating dopamine D2 receptors reduces brown adipose tissue thermogenesis induced by psychological stress and by activation of the lateral habenula. Scientific Reports, 2019;9(1)19512. https://doi.org/10.1038/s41598-019-56125-3
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LIST OF ABBREVIATIONS

BRAC – basic rest- activity cycle

CNS – central nervous system

TRP – transient receptor potential

POA – preoptic area

BAT – brown adipose tissue

VTA – ventral tegmental area

IML – interomedial nucleus

ATP – adenosine tri-phosphate

L-DOPA – dihydroxyphenylalanine

DAT – dopamine transporter

SNc – substantia nigra pars compacta

PBP – parabrachial pigmented nucleus

PN – paranigral nucleus

GABA – gamma-aminobutyric acid

PFC – pre-frontal cortex

LHb – lateral habenula

NAc – nucleus accumbens

6-OHDA – 6-hydroxydopamine hydrobrimide

PPI – Prepulse inhibition

DREADDs – designer receptors exclusively activated by designer drugs

CNO – clozapine-N-oxide

AAV – adeno-associated virus

FLEX – flip-excision

hM3Dq – human muscarinic Gq-coupled receptor

hM4Di – human M4 Gi-coupled receptor

RRRC – Rat Resource & Research Center

Anti-DAT-SAP – Anti-DAT Saporin

N-Des – N-desmethylclozapine

C21 – compound 21

PCR – polymerase chain reaction

DMSO – dimethyl sulfoxide

PBS – phosphate buffered saline

TH – tyrosine hydroxylase

NHS – normal horse serum

AC – adenylyl cyclase

cAMP – cyclic adenosine monophosphate

SCH-23390 – R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5 tetrahydro-1H-3 benzazapine

THC – tetrahydrocannabinol

ADHD – attention deficit hyperactivity disorder

mPFC – medial prefrontal cortex

KORD – kappa-opioid receptor

5-HT – 5-hydroxytryptamine/serotonin

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CHAPTER 1: INTRODUCTION

1.1 The homeostatic state and the importance of thermoregulation

Thermoregulation and the ability to maintain internal body temperature are vital to an animal's survival. In order to understand the process of bodily homeostasis, as well as the wide variety of physiological problems that can arise from its dysfunction, it is imperative to know and recognise the contribution of body temperature regulation. This understanding, in turn, can have a vast range of clinical applications.

Mammals, including humans, are known as homeothermic organisms – that is, they have an almost constant body temperature, with only a very narrow range of temperature deviation (1). Excluding some exceptions, such as hibernation, the limited variation of body temperature ensures optimal cellular and tissue function. Humans, for example, typically have a body temperature of around 37°C, and any significant deviation from this value (hypothermia – core temperature < 35°C and hyperthermia – core temperature > 40.5°C) is considered to be a medical emergency (2). Both severe hypo- and hyperthermia can result in cardiac arrhythmias, muscle dysfunction and altered states of consciousness, which require immediate medical intervention (3-5).

Despite these strict parameters, there are several naturally occurring fluctuations in temperature that are recognised; including circadian variations and ultradian rhythms. Circadian rhythms occur over a 24-hour cycle and are based on light-dark exposure, resulting in a nearly 1°C sinusoidal fluctuation of temperature with elevated temperatures during the 'day' and a reduction of temperature at 'night' (6-8). Ultradian fluctuations occur in a similar manner, but rather than over a 24-hour period, temperature fluctuates throughout the day in what is known as the basic rest-activity cycle (BRAC) (9-12). This cycle occurs approximately every 1-2 hours. Body temperature in the active phase has been reported to increase by over 0.5°C and is commonly accompanied by increases in heart rate, metabolic rate and arterial blood pressure (13). Further, as first noted by Richter in 1927, there is also a marked increase in behavioural activity that is shortly followed by the onset of feeding (14). This is likely preparing the body for the process of food ingestion; however, this has not yet been proven and the exact relevance of this process is still not understood (11). Importantly, in both circadian and ultradian rhythms, parameters return to baseline levels before the cycle begins another repetition.

Given the need of humans and other mammals to regulate their core body temperature in such strict confines – they can survive in exceptionally extreme conditions. In order to enable survival and support optimal physiological functioning, animals must be able to preserve their core body temperature in such a way that body heat gain must equal body heat loss (15). As such, a considerable proportion of metabolic output is consumed by temperature homeostasis, which requires control by an elaborate system of regulatory mechanisms.

In the last few decades, there have been many advances in understanding the central circuitry that is involved in thermoregulatory control. Such developments have particularly highlighted the relationships between thermal sensors, neural networks and effectors.

1.2 Regulating temperature - central mechanisms and sensing changes

Homeostatic control of body temperature involves variations in both heat production and net heat transfer between the individual and the environment; both processes are regulated by central nervous system (CNS) circuits (16).

When referring to 'body' temperature there are two main factors to consider: the core (central) and shell (peripheral) temperature (15). The core temperature refers to the temperature in deep body tissue (around organs with a high basal metabolism), whilst the shell temperature usually refers to the skin. Typically, the shell temperature is approximately 4°C lower than core temperature (15). In warm environments the difference between the core and shell temperature decreases as the skin approaches ambient temperature; conversely, in cold environments there is a decrease in shell temperature as conservation of core heat is prioritised.

All regulation of body temperature relies on two major mechanisms: the processing of afferent thermal inputs from the core and skin, as well as the effective and deliberate control of thermo-effectors by the nervous system (17).

Temperature changes are first detected by two distinct subtypes of peripheral and central thermoreceptors: cold and warm responding (15). Endothermic organisms, such as mammals, primarily rely on their own metabolism as a source of heat. However, due to the significant temperature gradient between core temperature and the environment, the majority of heat is quickly dissipated to the animal's surroundings (18). Because of this, cold-sensitive thermoreceptors are predominantly located on the skin, or immediately beneath the epidermis. The neuronal cell bodies of these thermoreceptors are located in the dorsal root ganglia, and via

Aδ and C fibres, project to the Rexed lamina I, and some layers of lamina II, in the spinal cord - as demonstrated by Light et al (1979) (19), Craig & Dostrovsky (2001) (20), and others (21-24). Although present, warm sensing peripheral receptors are less common and are typically found deeper in the dermis. These receptors convey their signal through unmyelinated C fibres (25, 26). Together, both sets of peripheral receptors produce a distinct phasic response that simultaneously discerns between temperature sensations and initiates the first stages of temperature control.

Since the core body temperature of mammals is within only a few degrees of the survival limit, the core is specifically vulnerable to temperature changes – in particular overheating. As such, central thermoreceptors are primarily warm-sensitive and are found in the brainstem, preoptic area (POA) of the hypothalamus, as well as within the spinal cord (27-31).

The thermosensitive properties of thermoreceptors are a result of alterations in transient receptor potential (TRP) channels. These ion channels are located in the plasma membrane of cells and are activated by cation influx, which itself results from changes in intracellular metabolic reactions due to temperature (32-34). There are over thirty TRP channel types, nine of which have been identified as being temperature sensitive. Each has a limited range of temperature activation, as shown in Figure 1, with TRPV1-4, M2, M4 and M5 being heat-activated, and TRPM8 and A1 being cold-activated (35, 36). It should be noted that there is some overlap between channel ranges and research into their exact mechanisms of function is still ongoing. Given that a wide range of temperatures is detectable via these TRP channels, it is likely that they are most important for the perception of shell temperature (27).

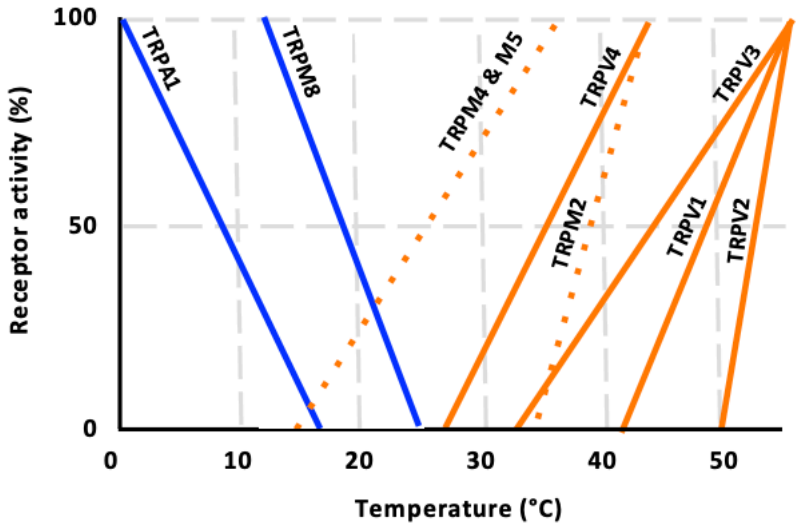


Figure 1: Representation of TRP channel sensitivity and activity level at various temperatures. Cold-activated channels shown in blue, heat-activated channels shown in orange. Diagram modified from Romanovsky, 2007 (27). Reprinted with permission from American Physiological Society.

Once the temperature change has been detected, the information stimulates primary somatosensory afferents in the skin, which relay the information via neurons in lamina I of the dorsal horn of the spinal cord. From here, the information reaches medullary, pontine and midbrain neuronal groups (37). Crucially, signals are passed to the hypothalamus, which is currently believed to be the central coordinator of thermoregulation (28). In 2008, Nakamura and Morrison demonstrated the vital role of glutamatergic neurons of the lateral parabrachial nucleus in transmitting signals to the POA (38). Further research has supported this, as well as the importance of projections to the thalamus, peritrigeminal and paratrigeminal nuclei (31). It is currently understood that these pathways will initiate either heat loss or gain mechanisms.

Once the medial portion of the POA has received input from the brainstem nuclei, thermosensitive neurons within the POA display spontaneous membrane depolarisation (39, 40). These neurons are warm-sensitive and, therefore, their increased activity leads to heat-defence responses. Conversely, decrease in activity triggers cold-defence responses (41).

It was previously thought that thermoregulation involved a comparison of the integrated thermoreceptor signal to an internal 'set' point (42, 43). If there was a significant discrepancy between these two temperatures then appropriate heat loss or gain mechanisms would be triggered. However, the process of integrating the various thermoreceptor signals was unclear (44). As a result, a new theory emerged, championed by Kobayashi and colleagues, which proposed that a singular 'set' point was not significant and that thermoregulatory responses were not controlled by a central neuronal network (45). Rather, it was suggested that central and peripheral temperature influenced individual effector circuits independently, through thermo-effector loops that connected sensors directly to their effectors.

1.3 How the body responds – maintaining control over temperature

Each thermo-effector loop is dependent upon the activation of its appropriate sensor, which occurs when the specific activation temperature is reached (46, 47). The overall thermoregulatory response is, therefore, a result of the integrated effect of many such thermo-effector loops (27).

Current understanding suggests that shell thermosensors regulate behavioural responses, whereas core receptors are responsible for autonomic changes (48-50). Behavioural changes are seen as anticipatory and often precede autonomic responses that, due to their energetically expensive qualities, occur if behavioural responses are inadequate and result in altered core

temperature. As a result, cold responses are more sensitive to changes in skin temperature, whereas responses to heat are primarily triggered by changes in core temperature (15, 51).

In cold environments, the skin plays a significant role in temperature regulation. Skin blood flow is reduced through the process of vasoconstriction, resulting in decreased shell temperature and subsequent conservation of heat in the core (52, 53). Heat can also be generated via increases in skeletal muscle activity, such as through shivering or exercise. The latter is also assisted by the mentioned vasoconstriction that allows for greater cardiac output to active muscles (54, 55). Endothermic homeotherms also generate their own body heat through the process of non-shivering thermogenesis, enabled by brown adipose tissue (BAT) (56, 57).

Conversely, in warm environments the principle response is blood vessel dilation. This mechanism facilitates increased heat dissipation from the body, due to the proximity of cutaneous blood vessels to the surface (15). For humans, sweat production is also increased to allow cooling by evaporation. This is the only mechanism available when ambient temperatures exceed internal body temperature and heat dissipation can no longer occur (58). Various behavioural changes can also be employed, such as seeking shelter or consuming additional liquids.

Distinct mechanisms regulate each of the main thermoregulatory effector pathways: shivering thermogenesis, non-shivering thermogenesis and skin vasomotor tone (30, 59). Importantly, all of these pathways have been shown to be inhibited by warm-sensitive neurons receiving inhibitory inputs from the POA. Changes in cutaneous vascular tone are regulated by the ventral tegmental area (VTA) and the periaqueductal grey (60). The raphé and peripery midal area is the next step in mediation, with the VTA activating and the periaqueductal grey inhibiting neurons respectively (61). Serotonergic and glutamatergic neurons synapsing in the interomedial nucleus (IML) then control skin vasomotor tone (62-64).

Both shivering and non-shivering thermogenesis are regulated by the dorsomedial hypothalamus and its projections to the rostral raphé pallidus, the peripyramydal area and the raphé magnus (65). Shivering thermogenesis is then triggered by input to γ-motorneurons in the spinal cord, which results in overall increase in muscle tone and activation of the stretch reflex (66, 67). The raphé spinal fibres controlling non-shivering thermogenesis project to the IML, where they target neurons mediating BAT (68, 69).

1.3.1 Brown Adipose Tissue (BAT)

BAT was originally thought to only be present in small mammals and neonates; however in recent years BAT has also been found in adults (70, 71). The principal deposit of BAT within the human body, and the bodies of most mammals, is found in the interscapular region (72). Its primary role is in facultative, non-shivering, thermogenesis (56, 57).

In addition to the POA, the dorsomedial hypothalamus and raphé peripyramidal nuclei are involved in its regulation; and there is further association between non-shivering thermogenesis and the activation of neurons in the ventromedial and paraventricular nuclei of the hypothalamus. Madden and Morrison, however, disputed this in 2009 suggesting that the paraventricular nucleus is inhibitory of non-shivering thermogenesis, as its disinhibition results in decreased activity of interscapular BAT (73). The rostral periaqueductal gray, along with the retrobral field, is also known to be a source of inhibition of BAT activity (74). In contrast, the caudal periaqueductal gray contributes to BAT activation, presumably through stimulation of raphé /peripyramidal neurons (75).

Within BAT itself, thermogenesis occurs due to a 'proton leak' across the mitochondrial membrane of BAT adipocytes. The large amount of uncoupling protein-1 in BAT mitochondria enables this, resulting in oxidative metabolism being uncoupled from adenosine tri-phosphate (ATP) production and thus, expending energy and producing heat (9, 72, 76). This process is effectively summarised by Cannon and Nedergaard (2004) (76). Therefore, in essence, this type of tissue increases metabolic rate. The heat that is produced is then able to supply heated blood to rostral parts of the body (9).

There has now been conclusive demonstration of BAT activation via the sympathetic nervous system. In response to cold exposure, sympathetic nerve activity has been demonstrated to increase, stimulating BAT thermogenesis (72, 77). However, it is now also known that non-shivering thermogenesis is also regulated via non-thermal factors. For example, significant increases in BAT temperature have been noted during exposure to stress (56). This process in particular is believed to be driven by the hypothalamomedullary pathway, where stress activated monosynaptic glutamatergic excitatory neurotransmission occurs from the dorsomedial hypothalamus to sympathetic premotor neurons in the rostral medullary raphé, which then drive BAT thermogenesis (56).

Whilst the basic process, and regulation, of BAT thermogenesis is understood, the exact circuitry and brain regions that are involved in its mediation remain under investigation.

1.3.2 The significance of 'emotional' hyperthermia

In addition to ambient environmental temperature changes and increased muscle activity, it is also well known that psychological stress results in, amongst other responses, an increase in body temperature (78, 79).

The term 'stress' was first coined by Hans Selye in 1936 who described it as '*the non-specific response of the body to any demand for change'* (80). Although it is essential for adaptation and survival, 'stress' is still generally associated with negative experiences (81).

Today, whilst there is no single agreed upon definition, psychological stress is commonly described as mental strain that is experienced when there is an awareness of possible danger, pain or discomfort. Consequently, the temperature increase that is experienced is referred to as 'stress-induced' or 'emotional hyperthermia' (9, 82, 83).

In periods of stress, it has been shown that body temperature can increase up to 2°C (78, 84, 85). Acute emotional hyperthermia is typically seen as being beneficial due to its ability to increase both neural and physical performance by warming the CNS, as well as the muscles of the body to prepare the animal for a 'fight or flight' response (9, 17, 56, 86, 87).

However, prolonged exposure to such stress and the associated temperature increases can be extremely harmful and result in severe illness (81, 88). Notably, chronic temperature increases known as 'psychogenic fever', have been reported (56, 89). Whilst the presence of psychogenic fever has been observed as early as the twentieth century (90), it has only been recently recognized as a major psychosomatic symptom – largely due to ever increasing stress levels in modern society and the fever's extremely debilitating nature (78). Indeed, studies by Oka (2015) have revealed that emotional hyperthermia is experienced not only prior to exceptionally significant events, but also in common everyday situations, such as when going to school or work (78). This almost regular exposure to stress greatly increases the likelihood of psychogenic fever development, as well as the occurrence of its associated conditions, such as major depressive disorder (91).

Renbourn's observations in 1960 were the first conclusive demonstration of emotional hyperthermia. He noted that the body temperature of young boxers was higher prior to a match

than prior to a corresponding period of exercise (92). This finding led to the idea that the temperature increase experienced in emotional hyperthermia was not a pathological fever, and was distinct to what was experienced in illness. This, therefore, also prompted the idea that this type of hyperthermia could be mediated by different mechanisms than those that are seen during infection (93). During inflammation and infection induced fever, there is significant involvement of proinflammatory factors, such as prostaglandins and proinflammatory cytokines (94). These factors act on neural endings, which then transmit the inflammatory signal to the POA that increases temperature through regulation of the dorsal medial hypothalamus and rostral medullary raphé (78).

Psychological stressor induced temperature increases, in contrast, do not involve the presence of inflammatory mediators (95). This has been conclusively demonstrated through the use of knockout mice, which lack the prostaglandin E receptor. When exposed to stressful stimuli, these mice experienced the same hyperthermia as the experimental controls (96). Other studies have also supported this finding; such as when prostaglandin-blocking agents were administered a reduction of infection-induced fever was noted, with no effect being observed on emotional hyperthermia (93). Similarly, research that has been conducted in recent decades, demonstrates that treatment of psychogenic fever with common fever reducing agents, such as antipyretics or cyclooxygenase inhibitors, is ineffective (56, 78). Consequently, there are currently a very limited number of treatment options available to those that are affected.

It has now been demonstrated that, by the use of in situ thermistor probes, BAT thermogenesis largely contributes to the temperature increase seen in emotional hypothermia (85). This finding has also been confirmed in humans with Robinson and colleagues (2016) demonstrating the involvement of BAT in anticipation of psychological stress via infrared thermography (97). Therefore, the association between stress and the brain centres that regulate bodily functions is of great interest; and it is vital to uncover the regulatory mechanisms and pathways that control the physiological stress response. Having this understanding will, in the future, lead to development of new treatments and therapeutic strategies.

The significant role of BAT in thermoregulation is becoming more recognized, and the links between abnormal thermoregulation and states of illness are becoming clearer. As such, BAT and its regulatory pathways are beginning to be considered as potential sites for new drugs that have the potential to alter and regulate energy expenditure. However, rather than regulating the direct

issues that arise from stress, such as psychogenic fever, these treatments will likely target secondary, related issues.

What has become clear in recent decades is that the process of thermoregulation is complicated and involves many areas that contribute to its modulation. However, what is known is that these regulating mechanisms are caused primarily by the action of various neurotransmitters that can control and promote nerve cell activity (within the brain). Various reports have identified the main neurotransmitters to be serotonin, noradrenaline and dopamine (3, 98-100). This thesis will focus on investigating the influence of dopamine.

1.4 Dopamine

Investigation of the dopaminergic system in relation to thermoregulation has been an area of great interest in recent years. The majority of current research has largely built on the foundation of previous studies, such as those conducted by Lee (1985) that were the first to demonstrate that dopamine, and its receptors, are involved in temperature regulation (98).

The independent role of dopamine has only recently been distinguished from its intermediate role in the biosynthesis of epinephrine and norepinephrine. Since dopamine was first recognised, and investigated, as an important monoamine catecholamine neurotransmitter in its own right during the mid-1950's, it has been found to be involved in various functions throughout the CNS (101). Having influence in arousal and regulation of sleep, feeding, memory and cognition, as well as pain and emotional processing; dopamine has also been demonstrated to regulate functions outside of the CNS such as modulation of blood flow and kidney function (102, 103). Of these functions, the two major areas of dopamine action are presently recognised as the regulation of movement and the control of reward related processing (101, 104, 105). As part of its role in guiding appropriate action selection, dopamine is also involved in salience signalling, associative learning and motivation (106, 107).

Importantly, despite its significant contribution to many vital functions, the activity (or lack thereof) of dopamine has also been linked to several states of physical dysfunction – including implications in psychiatric illnesses, such as addiction, depression, Parkinson's disease and schizophrenia (108-110); as well as metabolism-related disorders such as obesity, type 2 diabetes and hypertension (111-113). However, the extent to which changes in dopamine activity contribute to these dysfunctions is not yet fully understood.

Dopamine itself is synthesized from tyrosine, a common amino acid that is consumed as part of a normal diet or created from phenylalanine by enzymes in the liver or within dopamine neurons (114). As shown in Figure 2, once taken up into dopamine neurons via a series of transport processes, the enzyme tyrosine hydroxylase (TH) converts tyrosine into dihydroxyphenylalanine (L-DOPA) (114). This is the rate-limiting step in dopamine production. As such, if required, it is more effective to increase dopamine levels *in vivo* directly by increasing the amount of downstream products, rather than increasing tyrosine levels (114). L-DOPA is subsequently converted into dopamine through the action of aromatic amino acid decarboxylase.

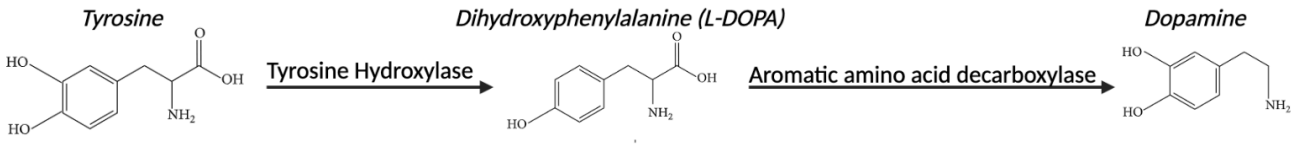


Figure 2: Synthesis pathway of dopamine, showing component structures and enzymes involved. Diagram adapted from Meiser (2013) (463)

Once synthesised, dopamine is packaged into synaptic vesicles by the vesicular monoamine transporter-2, and is stored in axon terminals in preparation for exocytotic release into the synaptic cleft (115). However, there is evidence now available that suggests dopamine release does not occur exclusively at neuronal terminals, but is also present around the cell body of the neuron through the process of somatodendritic release (116). This release has been found to be a crucial part of vital autoinhibitory mechanisms of the dopamine neurons that, through high-affinity D_2 receptors on the soma and dendrites, regulate the cells' firing (117, 118). The secondary major mechanism of self-regulation is released dopamine re-uptake with the assistance of the dopamine transporter (DAT). Following re-uptake, the dopamine is either broken down by monoamine oxidase, or is repackaged into presynaptic vesicles (119). Further discussion of dopamine function, specifically in relation to the various classes of dopamine receptors, can be found in Chapter 3.

Within the brain there are several small dopamine neuron loci, denominated A8-14. The A8 and A9 areas characterise the substantia nigra regions, whilst the A10-14 regions make up the ventral tegmental area (VTA), posterior hypothalamus, arcuate nucleus, zona incerta and the periventricular nucleus, respectively (120). Of these, the two principal regions of dopamine

production are accepted as the substantia nigra pars compacta (SNc) of the basal ganglia, and the VTA of the midbrain (121).

Although dopamine is now one of the best-known neurotransmitter systems in the brain, an incomplete understanding of its circuits and the systems in which they participate remains, presenting a major obstacle to future research. Consequently, investigating the anatomical locations of dopamine neuron's synaptic inputs and outputs may be key to determining its properties and regulating actions.

1.4.1 The Ventral Tegmental Area (VTA)

Previously known as the 'ventral tegmental nucleus', the first anatomical description of the VTA in literature was by Tsai in 1925 based on a Gogli and Nissl preparation (122).

The VTA is located on the floor of the midbrain and encompasses several nuclei that are generally accepted to be the parabrachical pigmented nucleus (PBP), paranigral nucleus (PN), caudal linear nucleus, interfasicular nucleus, parainterfasicular nucleus and the rostral linear nucleus of the raphé, as shown in Figure 3 (123). However, some controversy regarding its exact constituents still remains.

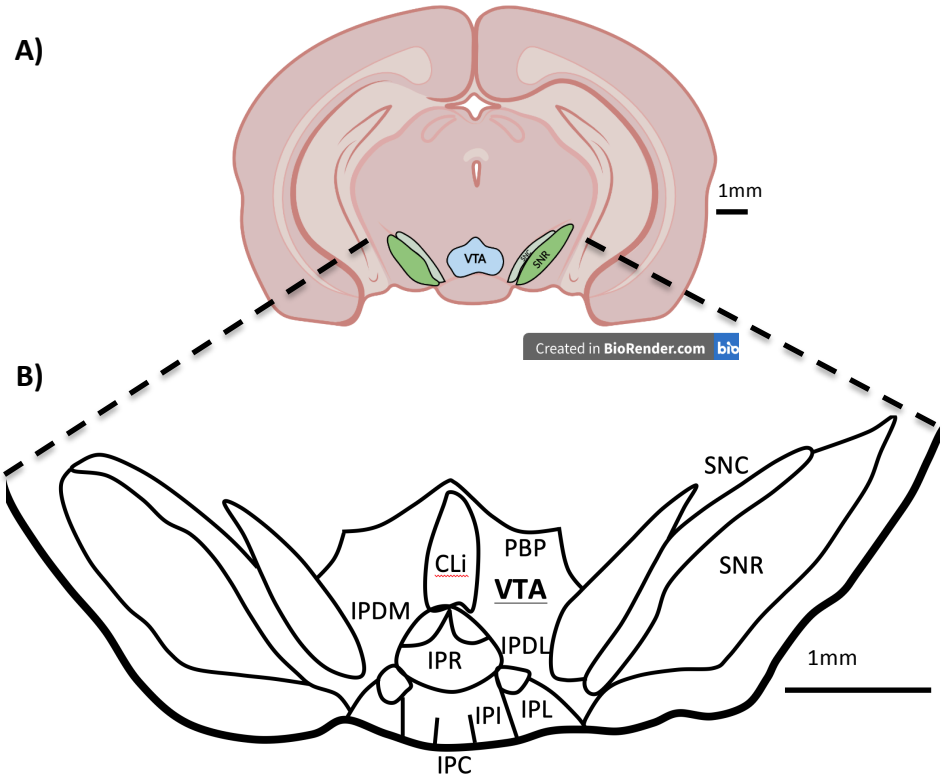


Figure 3: Representative image of **A)** VTA location in the rat brain at 6.04mm caudal to bregma and **B)** its constituting areas: dorsomedial interpeduncular nucleus (IPDM), rostral interpeduncular subnucleus (IPR), caudal interpeduncular subnucelus (IPC), intermediate interpeduncular subnucleus (IPI), lateral interpeduncular subnucleus (IPL), dorsolateral interpeduncular nucleus (IPDL), parabrachial pigmented nucleus (PBP), ventral tegmental area (VTA), causal linear nucleus (CLi), substantia nigra reticular (SNR), substantia nigra pars compacta (SNC). Diagram adapted from Paxinos & Watson (1997) (382)

Whilst there are differences in brain organisation between species, the substantial similarities amongst mammals greatly benefits neuroscientific research as well-established models, such as rodents, can be effectively used to investigate VTA structure and function.

Analysis of the rat VTA has revealed approximately 45,000 TH+ neurons. In comparison, it is estimated that there are between 400,000 and 600,000 TH+ neurons in the human midbrain (124-126).

When studying the VTA, it is of critical importance to note its proximity to the laterally located SNc, and the fact that there are no clear borders or separation between the two areas. Despite this, the SNc and VTA exhibit differing properties (genetic, morphological and electrophysical) and serve distinct functions (127). This thesis focuses specifically on the VTA.

The cellular composition of the VTA was first suggested by Johnson and North (1992) (128). Based on their examination of electrophysical and pharmacological differences, two major types of neurons were described as part of the heterogenous population. Spontaneously firing neurons that displayed long-duration action potentials were classified as dopaminergic, whilst those with opioid sensitivity and short action potentials were identified as gamma-aminobutyric (GABA)ergic. Later investigations, by Hur and Zaborszky (2005), were also able to identify glutamatergic constituents (129). Now, neurons have even further been separated based on tonic (basal) and phasic (in response to stimulation) properties. Although this is a very simplistic classification of VTA neuronal populations, the neurochemical composition is currently accepted as ~60% (ranging between 30-70%) dopaminergic, ~25% GABAergic and 2-15% glutamatergic (130, 131). Further adding to the distinction of neuronal populations in the VTA, in 2008 Nair-Roberts and colleagues found higher concentrations of dopamine neurons in the lateral areas of the VTA (PBP and PN), whilst glutamatergic neurons were located proximity to the midline (130). It has also been shown that these neuronal populations have intrinsic connections within the VTA itself; with both GABAergic and glutamatergic interneurons synapsing onto dopamine neuron dendrites. As such, any investigation into the VTA must consider the action of these diverse subpopulations and their distinct neuronal networks.

Major extrinsic inputs to the VTA are both glutamatergic and GABAergic. Glutamatergic inputs arise from the prefrontal cortex (PFC) (132), subthalamic nucleus (133), mesopontine tegmentum nucleus (134), lateral habenula (LHb) (135) and periaqueductal gray (136). These connections are thought to stimulate dopamine neuron firing, however, there are also connections from these

regions to non-dopaminergic neurons in the VTA (132). GABAergic inputs most significantly project from the nucleus accumbens (NAc) - to non-dopamine neurons (137), from the ventral palladium - to both dopaminergic and non-dopaminergic neurons (138), as well as from the rostromedial mesopontine tegmental nucleus. This latter area is often referred to as the tail of the VTA, and forms inhibitory synapses on ~80% of dopaminergic VTA neurons (139). Together with the glutamatergic and GABAergic projections, there is significant input from both the serotonergic and noradrenergic system to both dopaminergic and non-dopaminergic neurons of the VTA; originating from the dorsal raphé nucleus and the medial raphé nucleus respectively (116, 140, 141).

The variety of intrinsic and extrinsic inputs to the VTA suggests differential roles of potentially independent circuits. A further layer of complexity to the structure and function of the VTA is then added when the location and nature of axon projection targets are considered. In regards to glutamatergic outputs, there appears to be a degree of projection isolation as inputs from the PFC synapse on dopaminergic neurons that project back to the PFC, but not dopaminergic neurons that project to the NAc (142, 143). This theory of individual VTA neuron projection is supported by multiple studies (108, 144, 145). As such, one of the major projections areas of the VTA is the PFC, which primarily (~60%) consists of GABAergic projections (142) as well as limited dopamine (~25%) input (146). The other significant pathway from the VTA is commonly referred to as the mesolimbic system and targets the NAc. Consisting of both dopaminergic and non-dopaminergic projections, this pathway has been implicated in motivation and the processing of emotion (104, 105). A representation of some of the main VTA pathways is presented in Figure 4. Further discussion of the mesolimbic system, its target, and function can be found in Chapter 4.

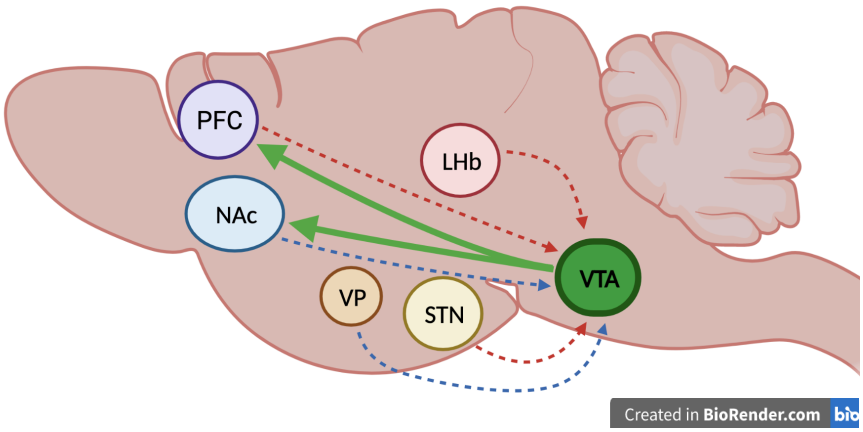


Figure 4: Representative image of some major inputs and outputs of the VTA. Glutamatergic – red, GABAergic – blue, dopaminergic – green. Prefrontal cortex (PFC), nucleus accumbens (NAc), subthalamic nucleus (STN), lateral habenula (LHb), ventral palladium (VP). ventral tegmental area (VTA). Not to scale. Modified from Olive (2011) (464)

The VTA has been demonstrated to be involved mainly in goal-directed and reward-related behaviours; however, it's exact actions remain poorly understood (147-149). It has been shown to regulate reward prediction errors in response to salient stimuli and, as shown by self-administration studies, stimulation of the area is highly rewarding (150, 151). Further demonstration of VTA involvement in reward processing and motivated behaviours is the expression receptors for feeding hormones in the area, which adjust their firing rate accordingly in response to hungry and fed states (152-154). Interestingly, Nagashima (2000) (30) and Romanovsky (2007) (27) both implicated the VTA as a potential area important for thermoregulation.

1.5 Current state of research

1.5.1 Thermoregulation and the involvement of BAT

Investigations of central thermoregulatory pathways, especially those controlling BAT, have in the past largely focused on responses during cold-exposure. However, since increased BAT thermogenesis is now known to contribute to emotional hyperthermia, research has nowadays largely shifted to identifying the pathways that regulate this process through stress.

Many animal models, such as mice, rats and rabbits, have been used with a variety of approaches to evoke a stress response. Shibata et al (1982, 1984) was one of the first to demonstrate the temperature increases associated with emotional hyperthermia (87, 155). In these studies the restraint model was used, and to this day, this model remains one of the most common. Borsini et al (1989) corroborated these findings with the use of the group-housed removal model (84). Since that time, many other models have been developed to study stress such as foot shock tests, altering-stimuli tests, cage-switch paradigms and social defeat models to name a few (9, 78, 86, 156, 157). These new studies have found that temperature increases that are experienced are comparable between animals that are kept in thermo-neutral and cold environments (1). Studies conducted by Blessing et al (2013), have also demonstrated that the temperature increases during stress are very similar to the spontaneous increases seen in the active-phase of BRAC (10, 11, 13). This suggests that this specific response in stressful conditions is not secondary to increased metabolism in skeletal and cardiac muscle, but it is in fact under actively initiated control (9, 158).

In 1984 Shibata and Nagasaka used the disruption of sympathetic innervation to conclusively demonstrate that BAT is under central control and is important for heat generation in the maintenance of body temperature (155). This pivotal study not only confirmed that BAT

temperature increased during immobilization stress, but this temperature increase was approximately 0.5°C greater than colonic temperature measured under the same conditions. Further, comparable temperature increases were recorded following 10 minutes of cold (5°C) exposure. Their investigation also employed 6-hydroxydopamine hydrobrimide (6-OHDA) – a catecholaminergic neurotoxin that functions through the same transport system as dopamine and noradrenalin (159, 160). The autooxidation of 6-OHDA, resulting in the formation of reactive oxygen species, and inhibition of the mitochondrial respiratory chain consequently destroys the aforementioned neuron types (161). Thus, following a sympathectomy via a single intraperitoneal injection 6-OHDA, Shibata and Nagasaka demonstrated that there was no increase in BAT temperature observed following stress, although it remained significant in cold exposure (0.31°C) (155). Thus, these results indicated that stress-induced BAT thermogenesis may be mainly under sympathetic nervous control, whilst BAT thermogenesis occurring as a cold defence is also likely to be partly controlled by additional hormonal factors.

As such, although the hypothalamo-medullary pathway is still believed to be the main regulator of stress-induced BAT thermogenesis, it is unlikely that this is the only pathway that is involved; or there may be additional regions involved in its control. For example, the LHb is a region of the dorsal diencephalon that is activated by negative salient events (162-164). In 2017 Brizuela and Ootsuka demonstrated that activation of the LHb causes BAT thermogenesis (165). The LHb also sends downstream projections to the VTA, and there is strong potential that the LHb, via the VTA and one of its rostral projections, relays in the hypothalamus before descending to the medullary raphé.

This theory is supported by recent discoveries, such as the demonstration that outputs from the LHb activate GABAergic neurons in the tail region of the VTA (139, 166, 167). This region is known to inhibit dopamine cells, in what is known as the ‘dopamine brake’, which thereby reduces activity in the mesolimbic reward system (167-170). This thereby enables responses to life-relevant events in the external environment by facilitating a redirection in attention (171). These responses include an increase in body temperature.

Recently, Brizuela et al (2018) supported this understanding by showing that focal microinjections of muscimol, a $GABA_A$ agonist that inhibits VTA neurons, powerfully activates BAT sympathetic nerve activity and BAT thermogenesis (172).

1.5.2 Involvement of dopamine in thermoregulation

Primarily pharmacological evidence suggests that dopamine is one of the neurotransmitters involved in thermoregulation. It has been found that the systemic, intraventricular or intracerebral administration of dopamine D_2 receptor agonists, such as apomorphine and quinpirole, cause a significant fall in body temperature. This is due to changes in metabolic rate and increased heat dissipation via cutaneous circulation (98, 173-176). There has also been limited investigation of D_2 antagonist administration, such as with the antipsychotic agent haloperidol; however, the findings of this research have been mixed. In rabbits haloperidol administration was found to increase temperature (177), whereas Lin and associates (1979) found that it lowered body temperature in rats at thermoneutral ambient temperatures, and also prevented cold-induced thermogenesis (178). Although it is vital to consider that the dose used in Lin's study (2 mg/kg) was approximately 50 times greater than the therapeutic dose used in humans. Research that has utilised the equivalent of clinically therapeutic doses has failed to find any acute impact on body temperature (179, 180). Comparatively, relatively low doses of D_2 agonists, that are in the same order as clinical therapeutic doses (158), are required for major biological effects. Thus, it appears that dopamine enhancement results in greater impacts than dopamine blocking, and it has also been suggested that dopamine may elicit inhibition of cold-sensitive cell activity – thereby, supressing heat production. Further, the discrepancies in results could potentially also be explained by possible differences between species in terms of the effect of dopamine on body temperature. It should also be noted though that the action of clinical agents such as apomorphine and haloperidol may be mediated by other neurotransmitter systems, such as noradrenaline and serotonin (168); as such, further investigation of the actions of dopamine in thermoregulation is necessary.

Importantly, these pharmacological agents have been shown to specifically act within the brain and CNS. Administration of peripheral dopamine antagonists fails to prevent the cooling action of the agonist (173-175, 179, 181-184). However, when an antagonist that crosses the blood-brain barrier is administered, the hypothermic effects are reversed.

Further evidence has been provided through demonstration that heat production in BAT, in animals is exposed to cold environments, is reduced by the systemic administration of quinpirole (175). Measuring BAT sympathetic nerve activity in anesthetized rats and directly administering quinpirole has also supported the action of these agonists in the CNS (175).

Mice with reduced dopamine synthesis have increased BAT thermogenesis, as well as increased energy expenditure and consequently decreased body weight (185, 186). This suggests that increased BAT thermogenesis may contribute to weight loss. This theory coincides with observations in human patients with Parkinson's disease, who have weight loss that is associated with increased basal metabolic rate (187-189). It is, therefore, noteworthy that their elevated energy expenditure is reduced by dopamine replacement therapy (190).

1.5.3 Thermoregulation and Parkinson's

Parkinson's is the most prominent neurological disorder arising from disruptions in the dopamine system, with over 7 million people currently suffering from the disease worldwide (191). Characterised by a loss of dopamine producing neurons in the VTA and substantia nigra (192-194), some of the major and most worrying symptoms are thermoregulatory in nature; including abnormal temperature sensation, fever and sweating (195, 196). Patients also exhibit higher brain temperatures and low hand temperatures, which is consistent with decreased heat loss and increased heat production (197-199). Despite being so prominent, there are currently only very limited treatment options available; the majority involving pharmacological dopamine replacement therapy, which itself involves many unfavorable side effects such as nausea, low blood pressure, hallucinations and poor impulsive behaviour control (191).

1.5.4 Importance of dopamine in salience perception

Thermoregulation and emotional processing are critically linked. As such, whilst the term emotional hyperthermia is usually used in a context that suggests an abnormal change in the animal's physiology, the increased body and brain temperature are more likely to serve a vital biological purpose (158). Synaptic processing may be enhanced by moderate increases in brain temperature, enabling the complex cognitive and emotional processing that is required to successfully manage stressful or challenging interactions (9). Indeed, in the context of disorders where there is dysfunction between the two systems, such as in schizophrenia, the integration of thermoregulation and salience perception becomes apparent (200, 201). Although only limited studies to date have investigated the connection, unmedicated patients with schizophrenia, who have inappropriate attribution of salience to insignificant stimuli, have been reported to have elevated body temperature and increased thermoregulatory vasoconstriction (202, 203).

Dopamine has also been shown to be an important regulator in salience processing. Indeed, activation of central dopamine D_2 receptors reduces responses to perceived threats, as

demonstrated through the study of prepulse inhibition (PPI) of the acoustic startle response in both rats and humans (204, 205).

1.6 The development of new techniques – finding solutions to the problems of previous research

The vast majority of research on dopamine and its functions has utilized pharmacological stimulation that increases post-synaptic dopamine typically by either blocking reuptake or enhancing pre-synaptic dopamine release. Depending on what chemicals are used, this may affect the duration of activity on synaptic signalling, which in turn may account for discrepancies in observation. Further, systemic administration of drugs affects dopamine functioning throughout the entire brain, making it hard to establish in what specific areas it is acting, and thus, what brain areas are involved in physiological regulation. Due to this, there are many regulatory areas whose function remains incompletely understood or under contention.

As such, large gaps in understanding present a huge obstacle to the development of new, novel, and most importantly, effective treatments for the multitude of disorders that are related to dopamine dysfunction.

In order to properly understand the functions of dopamine, it is important to enhance endogenous neuronal activity and to be able to anatomically restrict effects of neuron activation.

1.6.1 Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Chemogenetics is a new technique that is quickly growing in popularity, especially in the field of neuroscience (206, 207). Its primary use is to uncover the brain circuitry in the regulation of various bodily functions (208).

In recent years the Bryan Roth lab (University of North Carolina, Chapel Hill, USA) has developed this novel tool for the investigation of altered neuronal activity (209). Designer receptors exclusively activated by designer drugs (DREADDs) are a mutated form of the human muscarinic receptor that can no longer be activated by acetylcholine (their natural endogenous ligand) (210). They are G-protein coupled receptors that couple to Gq and Gi proteins and that can only be activated by the administration of otherwise inert “designer drugs” such as clozapine-N-oxide (CNO) (211). Using non-toxic adeno-associated viruses (AAVs), these DREADDs can be transduced into specific cell populations.

The AAV system itself is combined with the flip-excision (FLEX) switch method to introduce the selected receptor exclusively to cells comprising the circuit of interest (212). In summary, the system operates through a viral vector that contains two pairs of heterotypic, oppositely oriented loxP sites that are cloned with an inverted coding sequence. Specific cell-type promoters, such as hSyn, are also used to limit viral expression to neurons within the circuit of interest (213).

There are currently two main groups of DREADDs that are available: human M3 muscarinic Gq-coupled receptor (hM3Dq), which is excitatory and human M4 muscarinic Gi-coupled receptor (hM4Di), which is inhibitory (209, 211). Therefore, when they are activated, they can either enhance or reduce neuronal activity. Figure 5 presents an overview of the DREADD system process and the function of the two receptor variants (hM3Dq and hM4Di).

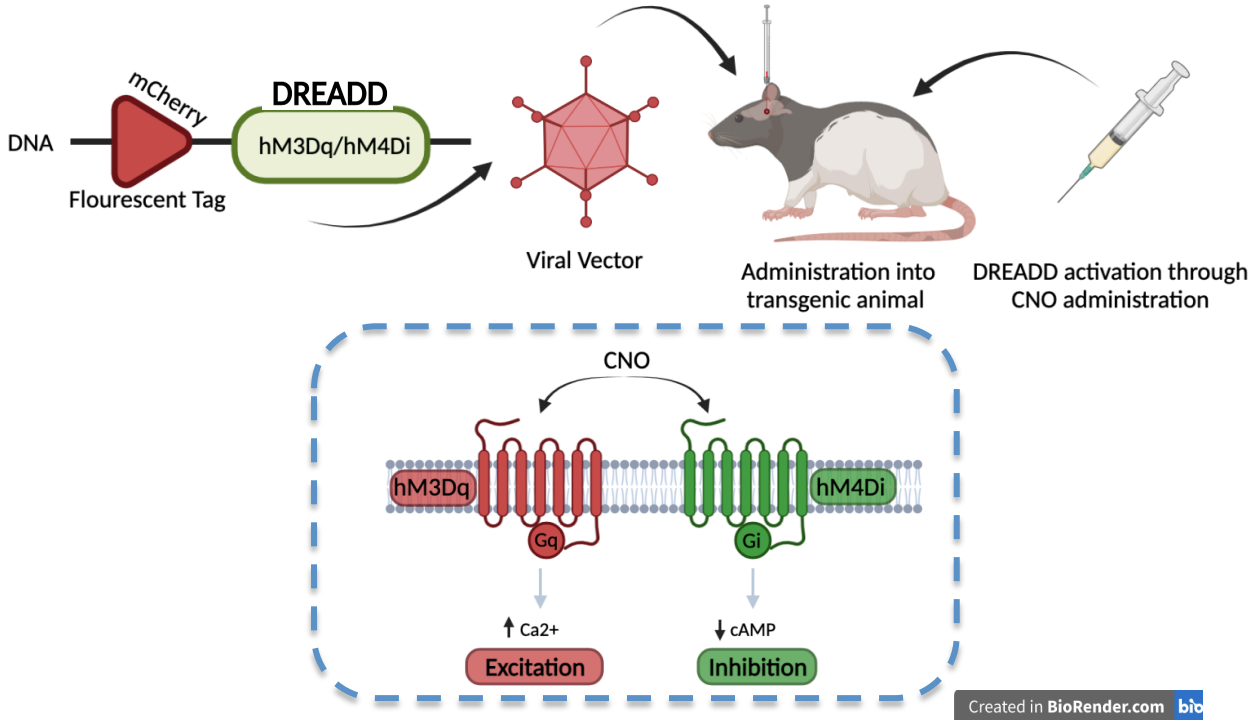


Figure 5: Overview of the DREADD system process and function of two receptor variants (hM3Dq and hM4Di). Diagram modified from Ju (2018) (465)

Chemogenetics allows for the temporary and direct manipulation of specific neural populations. Compared to techniques that have been used in the past; such as lesioning, or electrical and pharmacological stimulation, this technique possesses several important advantages. In particular, it allows for the manipulation of neurons of a specific cell type and the activity of DREADD expressing cells is unaffected in the absence of CNO (209). In contrast to techniques that involve the implantation of chronic catheters or optical fibers, chemogenetics is also relatively easily applied and non-invasive. Therefore, chemogenetics allows for repeated assessments and within

subject comparisons (with and without neuronal activation). This is something that is not always possible in techniques involving lesions or specific transgenic animals (209), in which variations between animals may make results difficult to interpret, and the exact effect of a particular manipulation hard to determine.

Chemogenetic manipulation also allows for the control of neuronal activity in freely moving animals (207). This, therefore, allows for the simultaneous regulation of neuronal activity and the observation of any resulting changes in behavioural activity (214). Once the neurons have been activated by chemogenetic means, the effects can typically be observed for multiple hours, which is a time period that is comparable to traditionally used pharmacological manipulations (214, 215).

Largely, this new technique has confirmed many ideas that have previously been hypothesized and tested with various approaches. Presently, it is beginning to affirm that modulation of neural activity, and its resulting effects, are similar to prior post-synaptic manipulations, specifically regarding activity (214, 216, 217). However, there has also been evidence to suggest that there are significant differences in the effects produced by pharmacological and chemogenetic techniques (214).

1.6.2 Genetic modification

DREADD viruses are also beneficial because they can be designed to be Cre-dependent. Cre (**c**yclization **re**combinase) is a tyrosine site-specific recombinase, originally isolated from bacteriophage P1, that recognizes the specific loxP (**l**ocus **o**f **x**-over, **P**1) DNA fragment (refer to Section 1.6.1) (218). As such, it can facilitate excision, inversion and integration as dictated by the orientation of loxP sites, relative to each other (219, 220). The Cre-loxP system has now become a widely used tool for mammalian gene editing, and has been employed in the investigations presented in this thesis. When DREADD viruses are Cre-dependent, the Cre-recombinase enzyme is necessary for the expression of the receptor. This Cre-recombinase is not endogenously present in animals; however, it can be introduced using transgenic animals (214).

The genetically modified DAT::Cre strain of rats (line: LE-Tg(DAT-iCre)6Ottc) was created in 2016 (Rat Resource & Research Center, RRRC). The transgenic construct is depicted in Figure 6. In this strain, Cre is expressed in dopamine cells that contain the dopamine transporter (DAT). Therefore, by introducing a Cre-dependent virus, containing either hM3Dq or hM4Di, into the brain of DAT::Cre rats, dopaminergic neurons can be specifically targeted and either enhanced or reduced.

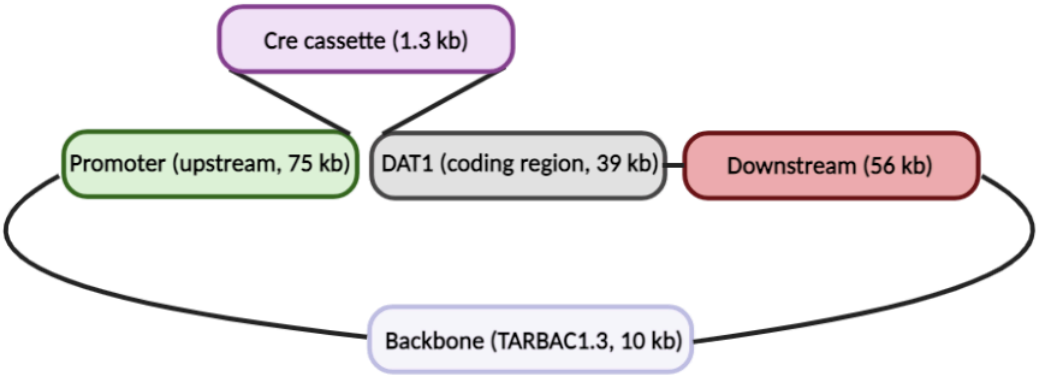


Figure 6: Schematic of DAT::Cre transgenic construct. Modified from the Transgenic Rat Project (LE-Tg(DAT-iCre)6Ottc), National Institute on Drug Abuse Intramural Research Program.

The Cre-recombinase itself is not known to affect dopamine function, and therefore transgenic rats are phenotypically normal (221).

Rats have been used for the study of brain circuitry for many decades, as early as the 1850's – as such, their physiological responses are very well documented and understood. It has also been suggested that rats more accurately reflect human physiology compared to mice (222, 223). Further, their larger size, compared to mice, minimises any potential damage to surrounding brain tissue that may result from viral vector administration. For these reasons rats were chosen for the experiments presented in this thesis instead of DAT::Cre mice.

It should further be noted that only male rats were used as estrous-related temperature effects in females complicate the interpretation of experimental results.

1.6.3 Saporin

A second approach that we have utilized in this study is Anti-DAT-Saporin (Anti-DAT-SAP). This is a targeted saporin - conjugated to antibodies against DAT, which is used as a tool for abolishing dopaminergic cells.

Saporin, a ribosome-inactivating protein from the seeds of the *Saponaria officinalis* plant, is bound to a targeting agent that is recognized on the cell surface (224). Once it is internalized the Saporin separates from the targeting agent and inactivates the ribosomes. This, in turn, causes protein inhibition leading to cell death (225). Any cell that does not possess the target cell surface marker remains unaffected. Anti-DAT-SAP is highly specific for cells that express DAT; therefore, the ability to specifically eliminate DAT expressing dopamine cells is incredibly useful for studying the role of dopaminergic neurons (224, 226-230).

1.7 Details of the present study

The brain is the most complex organ of our body, and we have only just begun to understand all the intricacies of its anatomical and chemical composition. If we don't understand the fundamental functionality of the brain, this severely hinders our ability to provide improved, target-specific and personalized treatments for neurobiological disorders.

Whilst new advances in neuro-imaging techniques have provided major insights into the networks and functions of many brain circuits, including sensory, motor and cognitive; a plethora of brain regions that contain many cell types, all with differing properties, continue to remain obscure (214).

The midbrain dopamine system is known to be important in both psychological and physiological function. However, there remains much that is missing in our understanding.

In order to fully understand the circuitry and brain regions underlying normal, and abnormal behaviours, specific regions and their cell types must be investigated. The focus of this thesis is a small, but significant area of the brain – the VTA, its dopaminergic neuronal population and its connections.

There has been little attention given to the role of the VTA in the regulation of physiological responses to a behavioural stimulus, and its potential role in linking emotional and physiological function. There has also been limited investigation of the VTA in relation to its potential role in thermoregulation, as a response or otherwise.

The aim of this thesis was to investigate the dopamine system and elucidate the role of the VTA, and the mesolimbic dopaminergic pathway to the NAc, in the regulation of aspects of the autonomic physiological stress response, as well as in the process of thermoregulation. It was hypothesized that the dopaminergic VTA neurons that project to the NAc play an important role in maintaining normal thermoregulation, and in regulating the temperature increases seen in emotional hyperthermia, as well as other physiological responses to stress. The experiments conducted investigated how dopamine-synthesizing neurons in the midbrain, via control of the sympathetic nervous system, regulate BAT thermogenesis, body temperature, behavioural activity and food consumption.

The most basic approach used to investigate brain mechanisms is to activate or reduce cell activity and observe the resulting effects on bodily function. To achieve this purpose, modern approaches were applied, including the implementation of chemogenetic DREADD technology, transgenic rats and a targeted toxin to activate and inhibit dopamine neurons in the VTA. This was used to determine the effects on neuronal modulation on a variety of physiological stress responses - including cold ambient temperatures and psychological stress, as discussed in the next chapter.

CHAPTER 2: THE ROLE OF THE VTA IN THE AUTONOMIC STRESS RESPONSE AND ANALYSIS OF NEW DREADD AGONIST EFFICACY

2.1 INTRODUCTION

As mentioned in Section 1.5.1, there is an abundance of models available to experimentally evoke autonomic stress responses in animals. However, a substantial limitation of these techniques is the involvement of physical contact. This presents a particular challenge when investigating the regulatory mechanisms of emotional hyperthermia. When stressogenic tests, such as foot shock or restrain tests, are conducted on conscious animals it becomes difficult to differentiate whether any reaction that is occurring is due to a physical or psychological stimulus. Because of this, limited attention has been paid to identifying the mechanisms, development and regulation of emotional hyperthermia that is distinct from responses evoked by physical stimulation. Further, it is difficult to draw comparisons between tests due to their differing intensities and durations.

Indeed, it has previously proven difficult to find a suitable model for the testing of emotional hyperthermia. Even when the stressor is psychological, such as disturbance stress when entering a room or novel cage stress, selecting appropriate methodology for recording temperature increases has been challenging. For example, rectal probe thermometry is still commonly used to measure core body temperature, especially in rodents and when studying arousing stimuli, such as in the case of emotional hyperthermia (231). However, rectal probe thermometry has been demonstrated to be stressful to animals and can result in long-lasting elevations in body temperature (231-233). Additionally, this technique adds confounding factors, especially to pharmacological studies, as the effects of many drugs depends on the basal temperature of animals (234).

It should also be noted that rectal measurements are less accurate and less reliable compared to other methods involving surgical implantation of temperature recording devices (235). This was confirmed by Bae and colleagues (2007) who indicated that temperature measurements varied up to 0.6°C between rectal and implanted electrode readings (231).

Technology has significantly advanced since the first studies examining emotional hyperthermia, and telemetric setups have now enabled the continuous registration of body temperature and facilitated many advanced investigations. It is now common to measure body temperature either

through telemetric probes in the peritoneal cavity, or using infrared tomography (236, 237). However, these methodologies are accompanied with their own limitations – such as sensor location affecting temperature read-out, the need for expensive equipment and most importantly, the constraint of only measuring one variable at a time. Consequently, many studies choose to focus on one physiological variable or strictly behavioural monitoring. As a result, when examining emotional hyperthermia, the contribution of BAT is often neglected and overlooked. Measuring multiple variables in conscious animals requires multiple sophisticated techniques and, to date, only a handful of studies have successfully recorded both BAT and body temperature following stress-inducing stimuli. These studies, however, retained the use of physiological stressors.

In order to overcome the issues previously faced, the Ootsuka/Blessing laboratory (Flinders University, South Australia, Australia) has developed a model utilising a caged-intruder. This model eliminates any physical contact between the freely moving, conscious, experimental animal (resident rat) and the response eliciting, stressor animal (intruder rat) that is confined to a see-through cage (9). Such a model, therefore, ensures that any emotional hyperthermia response that is experienced is solely due to a psychological stimulus. Along with this, there are other advantages to the model in that it is easily controlled and eliminates any potential interference from uncaged freely moving animals - such as contact with cables and recording devices. Utilizing this model along with the chronic implantation of small thermistors to measure both BAT and body temperatures, allows for the specific and accurate assessment of BAT thermogenesis contribution to the process of psychologically induced emotional hyperthermia. The simultaneous monitoring of other thermoregulatory factors, such as feeding behaviour and locomotor activity, is also made possible using this model.

It was the aim of this study to employ the caged-intruder paradigm and determine whether VTA dopamine neuron activation, as facilitated by hM3Dq DREADD expression in DAT::Cre transgenic animals, reduced the process of emotional hyperthermia. Based on the previous evidence that is available, it was hypothesised that activation of VTA dopaminergic neurons would attenuate the stress-induced hyperthermic response.

Concerning the usage of DREADDs in transgenic animals, it should also be noted that whilst the use of chemogenetics is becoming more common, there has been very limited investigation in regards to how the activation of dopamine neurons, via the use of this technique, acts *in vivo*. Several studies have investigated neuronal activity *in vitro*, however, in an intact organism, these

same neurons are under the regulated control of many inhibitory and excitatory inputs. This most significantly affects tonic and phasic firing, which themselves have been proposed to be implicated in differing functions.

Further, despite the well-recognised benefits of the DREADD system that have already been discussed (refer to Section 1.8.2) there are some limitations to this methodology. The first DREADD ligand, clozapine-N-oxide (CNO), a derived metabolite of the atypical antipsychotic clozapine, was long believed to be inert (210, 238). Few studies conducted during the time of its original introduction failed to find any off-target effects. However, a study by Gomez et al. (2017) cast doubt on this notion when it was demonstrated that CNO is readily back-metabolized to clozapine and *N*-desmethylclozapine (*N*-Des) in rodents, guinea pigs and humans (239-241). Further, it was suggested that the activation of DREADD receptors was in fact due to clozapine and not CNO, as a result of its extremely poor penetration of the blood-brain barrier (242). Indeed, clozapine shows a significantly greater affinity towards DREADD receptors than CNO. Worryingly, Gomez et al. also determined that the behavioural effects seen in DREADD experiments following 10 mg/kg CNO injection were comparable to effects seen when a 100-fold lower dose (0.1 mg/kg) of clozapine was used to activate the receptors instead (238, 240).

Since then studies on the behavioural effects of CNO back-metabolism have increased; however, the exact pharmacokinetics remain unknown, and studies often differ in regards to the amount of back-metabolised clozapine that is measured. It appears that amount of clozapine detected following CNO administration depends on a variety of factors, including strain, dosage, and sample measurement. This can be seen through studies of Jann (1994) (241) and Lin (1996) (243) respectively; Jann, was unable to detect clozapine in the plasma of Wistar rats (following 1 mg/kg CNO administration), whilst Lin documented their presence in the urine of Lewis rats.

Clozapine, being an atypical anti-psychotic agent, is well known to interact with a variety of neurotransmitter systems, including histamines, noradrenaline, serotonin, dopamine and acetylcholine. Particularly it is known to affect levels of these neurotransmitters in the striatum and medial prefrontal cortex (244, 245) (238, 246). As such, systemic administration of therapeutic doses of clozapine (1-10 mg/kg), and doses as low as 0.05-0.1 mg/kg, are known to decrease spontaneous locomotor activity and cognitive flexibility in rodents (244, 247), whilst simultaneously increasing anxiety behaviours. Interestingly, it appears that clozapine does not act equally on all systems as these same low doses failed to have any effect on working memory or

social interaction. Importantly, Blessing et al (2017) have previously confirmed that 2 mg/kg doses of clozapine robustly reduced body temperature in rats, a finding also previously reported in humans (158). This reduction in body temperature likely arises from clozapine's ability to alleviate vasoconstriction of cutaneous artery beds, and its ability to assist in the reduction BAT thermogenesis – both factors playing major roles in increases of body temperature, especially in emotional hyperthermia (9).

In order to combat the uncertain off-target effects of CNO, a new synthetic ligand, Compound 21 (C21), was developed as an alternative by Chen et al in 2015 (248). Due to its relatively recent introduction, *in vivo* characterisation of its properties is currently lacking. In 2018 Thompson et al stated that low doses of C21 (< 3 mg/kg) successfully altered the behaviour of DREADD-expressing animals without any off-target effects (249). This finding was supported by Jendryka et al in 2019, who conducted pharmacokinetic and pharmacodynamics experiments with the same dosage in mice (250). However, similarly to CNO, it appears there is emerging controversy regarding the potential off-target effects of C21. In male TH::Cre rats, it has been shown that a 1 mg/kg dose of C21 strongly increases nigral neuron activity in control animals, a finding that has also been supported by other researchers (242).

It should be noted that, despite research into the effects of CNO and C21 increasing, the majority of studies focus on behavioural aspects, and to date there has been no study investigating the physiological impacts of either ligand.

Therefore, as a secondary goal, this study aimed to compare the efficacy of CNO and C21 in the activation of VTA dopamine neurons, and their physiological effects in wild-type animals. It was hypothesised that, when using the DREADD system, there would be no difference in effects seen following CNO or C21 administration. However, it was anticipated that C21 in wild-type animals would have fewer physiological effects compared to CNO.

2.2 METHODS

All viral vector experiments were performed in transgenic DAT::Cre Long-Evans rats (319 – 536 g) (221, 251).

Animals were initially obtained from the Rat Resource & Research Center (Missouri, USA), and were then bred at the Flinders University College of Medicine and Public Health Animal Facility. The breeding colonies were maintained by crossing DAT::Cre rats with normal Long-Evans rats. Each rat was then identified as transgenic or wild-type by genotyping using Polymerase Chain Reaction (PCR) prior to experimentation with R730F (5'- GTT CTG CCG GGT CAG AAA GAA TGG T-3') and R730R (5'- GGC TGG CAA CTA GAA GGC AC-3') primers.

All experimental procedures were conducted at Flinders University and received ethical approval from the Flinders University Animal Welfare Committee (ethics approval number: 940/17). Experimental protocols were in compliance with the Australian code for the care and use of animals for scientific purposes (8^{th} edition).

All surgical procedures were performed under general anaesthesia (2% isoflurane in 100% oxygen, 0.8 L/min) (Veterinary Companies of Australia, Kings Park, NSW, Australia). Analgesia (Carprofen 5 mg/kg s.c) (Norbrook Laboratories, Melbourne, Australia) and antibiotics (Baytril 0.1 mL s.c) (Bayer Aust.,Pymble, NSW, Australia) were administered prior to surgery. During the surgical procedures, body temperature was maintained at a constant level via a heating pad, and a lack of hind limb withdrawal reflex was confirmed to ensure no pain perception prior to the start of surgery. At the completion of surgery, 5 mL of saline solution (0.9 %w/v sodium chloride for irrigation, s.c) (Fresenius Kabi, Australia) was administered, and oxygen flow was maintained until recovery. Animals were kept warm under a lamp and were closely monitored for an hour post-surgery.

Aseptic technique was utilised during all surgical procedures. This was maintained by using sterile instruments and consumables, sustaining an aseptic working environment by cleaning surfaces with 70% ethanol prior to and during procedures, and swabbing shaved incision sites (pre-surgery) with Betadine antiseptic solution (Sanofi-aventis Australia pty ltd., NSW).

Following recovery from anaesthesia, the rat was returned to the animal holding room and individually housed in an open top cage for a minimum of a one-week period prior to any additional surgery or experimental recording (252). Additional analgesia (3.3 mg/50mL) was provided in *ad-libitum* drinking water for the first two days of the recovery period.

2.2.1 Viral Vector Administration

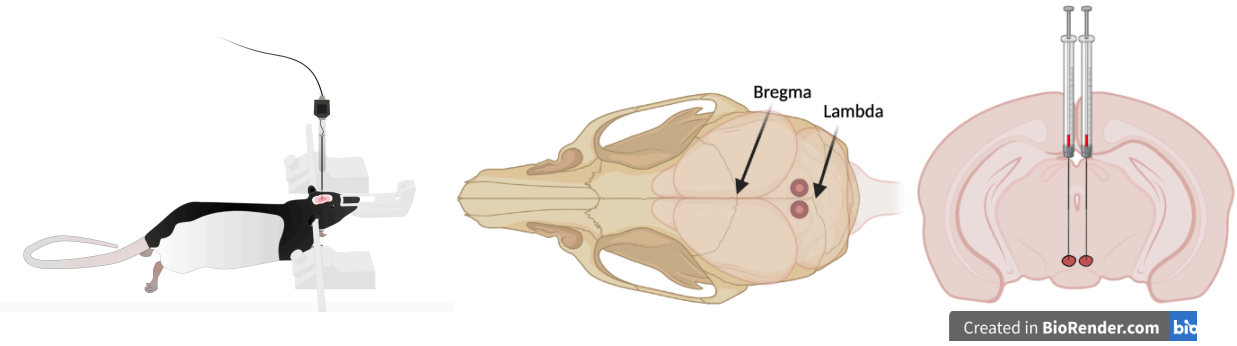


Figure 7: Schematic representation of stereotaxic set-up (left), burr-hole location with respect to bregma and lambda on the rat skull (middle) and viral vector administration sites in the VTA (right).

In preparatory surgery to inject Adeno-associated agents into the brain, rats were mounted onto a stereotaxic frame, as shown in Figure 7. A 2.5 cm midline incision was made along the back of the head, the skin retracted and the bregma-lambda plane was aligned for a flat-skull configuration. Periosteal fascia over the sagittal skull suture was removed using forceps and a scalpel. A burr-hole craniotomy was performed, in which two holes were drilled through the skull to the dura (the location of which is shown in Figure 7) in order to allow for chemical administration. A long-shanked 5 µL glass micropipette, connected to a syringe to allow for pressure injection, was then used to administer AAV-hSyn FLEX hM3Dq mCherry (serotype DJ; 1×10^{13} copies/mL) (produced by Professor Akihiro Yamanaka -Nagoya University, Japan) bilaterally into the VTA (6.8mm caudal from bregma, 0.5 mm lateral from midline, 6.5 and 6.7 mm deep from the cortex surface) (172). Each side was injected with a total of 500 nL: 250 nL at each depth, over a period of 10 minutes. Bone wax (Ethicon, US) was applied over the drill sites to close the holes at the end of the procedure.

2.2.2 Thermistor and Catheter Implantation

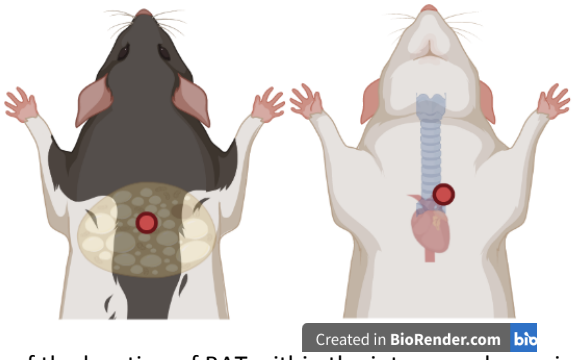


Figure 8: Schematic representation of the location of BAT within the interscapular region in rats, and BAT temperature probe location (left) as well as location of body temperature probe implantation site (right). Probes are indicated by red dots.

Two weeks following viral vector administration, animals underwent another preparatory surgery to implant a catheter and temperature probes to facilitate the measurement of biological parameters.

All temperature probes were made manually from thermistor sensors (NTH5G10P, Mu-rata, Kyoto, Japan) that were sealed with silicone (RTV 3-1744, Dow Corning, Midland, MI, USA). The complete assembly process for the temperature probes is outlined in the Appendix.

The precalibrated probes were implanted into the interscapular BAT region near the vein of Sulzer (BAT temperature) (10) and into the anterior mediastinum, ventral to the trachea (body temperature) as illustrated in Figure 8. The insulated wires from each of the probes were subcutaneously passed to the head of the rat, where they were attached to a head socket that was screwed to the skull and reinforced with dental cement. To facilitate drug administration, a catheter was implanted into the intraperitoneal cavity. The catheter tubing was also subcutaneously passed to the head and attached to the head socket with dental cement (252).

2.2.3 Recording of Physiological Parameters

The complete experimental set-up is shown in the Appendix.

One day prior to experimentation, the virally injected 'resident rat' was set-up in a plastic open roofed 'home cage' (35 cm wide x 40 cm long x 45 cm high) that was located within a modified, ventilated, commercial freezer (Biomedical Engineering, Flinders University). This chamber provided isolation from any external stimuli or disturbances, and also allowed for the control of the ambient temperature, which was pre-set and maintained at 24-26°C.

Food was available to the rat *ad libitum* and was suspended in a hopper on a high-frequency response gauge. This allowed for the monitoring of meal timings, as well as the observation of the amount consumed. Fresh water was also always freely accessible.

The head socket, via a flexible 'gooseneck' cable, was connected to a counter-balanced swivel device (SL12C, PlasticOne, Roanoka, VA, USA), located on top of the 'home cage', which in turn was connected to several recording devices. BAT and body temperature signals were passed to a bridge amplifier (Biomedical Engineering, Flinders University) and digitized (1Hz) with PowerLab (ADInstruments, Castle Hill, NSW, Australia). Behavioural locomotor activity of the 'resident rat' was recorded with a pyroelectric passive infrared sensor (NaPiOn, AMN1111, Panasonic, Osaka, Japan), located above the 'home cage'. Behavioural activity signals were digitized at 100Hz and

expressed as the total amount of active time (sec) per minute (sec/min). To observe the rat's behaviour and interactions with the 'intruder rat', a camera was used on occasion to record short videos.

The catheter was connected to tubing that was accessible from outside of the freezer-unit, allowing for drug administration with minimal interference to the animal. Prior to set-up, this tubing was flushed with iso-propyl alcohol and 0.7mL Ringer's Solution (Baxter Healthcare Pty Ltd., NSW, Australia), to account for dead-space.

The 'resident rat' was kept in the 'home cage' for the entire experimental period. The cage was kept under a 12-h/12-h reverse light-dark cycle (lights off at 0700 hours and lights on at 1900 hours). Light intensity was recorded with PowerLab, allowing for the documentation of the exact start of the intrusion period.

Prior to experimentation, each 'resident rat' showed typical ultradian increases in both BAT and body temperature, with episodes occurring at approximately 1-2 hour intervals as shown in Figure 9. Observation of these rhythms facilitated the selection of a suitable baseline, therefore, drug administration occurred during the dark phase between ultradian episodes, when BAT and body temperature were near baseline level for each particular animal. Given that rats are nocturnal, testing was performed in the active dark phase instead of the inactive light phase in order to enable the results to reflect the natural conditions of the animal. This also allowed for interpretations to more accurately reflect 'emotion hyperthermia' that would experienced in standard everyday situations; whilst the light phase would provide a more stable baseline, the temperature increases experienced would not reflect a 'real life' occurrence and may instead present an exaggerated result due to disturbance of the animal resting.

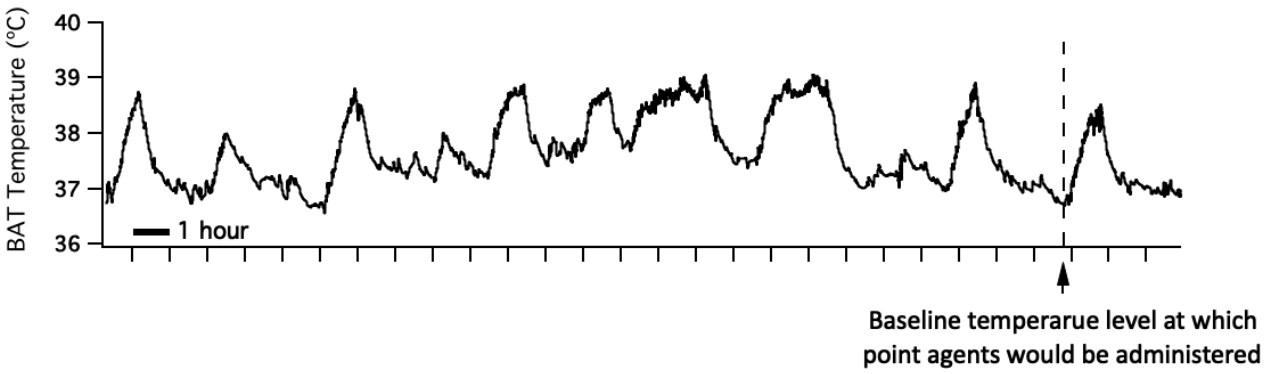


Figure 9: Representative image of ultradian rhythms observed over a period of time in the 'resident rat' prior to experimentation. Scale bar represents 1 hour. An example of a suitable baseline temperature level, at which point agents would have been administered, is indicated with a dashed line.

2.2.4 Drug Administration and Intruder Experimental Design

In order to determine the effects of chemogenetic dopamine neuron activation on the baseline condition of the animal DREADD agonists were initially administered without any 'intruder' stimulus.

Prior to administration, a 2 mg/kg dose of CNO (Tocris Bioscience, Bristol, UK) was weighed out on a microbalance. This was then firstly dissolved in 490 µL water for injection (Fresenius Kabi, NSW, Australia) and then 10 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to make up a total 0.5 mL volume for injection. This was injected into the implanted catheter and a further 0.7 mL water for injection was added to flush the tubing.

Two days following this experiment, the procedure described above was repeated with 2 mg/kg C21 (HelloBio, Bristol, UK), and also with vehicle (water for injection & DMSO) to serve as a control. All injections were given in a counterbalanced rotating order (similar to the example described by Brizuela et al (252)).

Drug doses were obtained from literature (239) and prior pilot experiments.

For the investigation into emotional hyperthermia, DREADD agonists were prepared as discussed above. One hour after drug administration, the freezer was opened and a second male wild-type Long-Evans rat (intruder rat), confined to a small plastic and wire-mesh cage (19 x 29 x 12 cm), was placed into the 'home cage' and the freezer lid closed. Experimental parameters were recorded for a further thirty minutes, and the intruder rat was then removed.

2.2.5 Cell Activation c-FOS study

At the conclusion of the experimental period, all animals were euthanized with Lethabarb (pentobarbitone sodium 180 mg/g i.p) (Virbac (Australia) Pty Limited, Milperra, NSW, Australia) and transcardially perfused with 0.5 M phosphate buffered saline (PBS), followed by formaldehyde fixative solutions containing 10% formaldehyde in 0.4 M phosphate buffer, and 10% formaldehyde with 20% sucrose in 0.4 M PBS respectively.

In order to test whether DREADD agonist administration indeed activated dopamine neurons, a c-FOS (marker of neuronal excitation) expression study was conducted. In this set of experiments, either 2 mg/kg C21 or vehicle was administered to the 'resident rat', which was perfused, using the method described above, 90 minutes following drug administration.

For all experimental rats, following perfusion, the brain was removed for histological confirmation of viral vector injection. Serial sections (50 µm) were cut with a cryostat (Cryocut 1900, Leica Microsystem Pty Ltf, North Rude, NSW, Australia) the following day after perfusion. The visualization of fluorescence from the acquired brain sections was conducted using the Invitrogen EVOS FL Fluorescent microscope. The procedure below was followed to confirm activation of dopamine neurons.

2.2.6 Immunohistochemistry

For fluorescent staining of tyrosine hydroxylase (TH), sections were washed in ImmunoBuffer (Tris-PBS with Triton-X 100 (ChemSupply, SA, Australia)) three times, with each wash being 10 minutes; and were then left to incubate in 10% Normal Horse Serum (NHS) (Gibco, Lot #2128900) (diluted with 0.05 M TPBS (Trizma Base (MERK), 0.4 M phosphate buffer, sodium chloride, thimerosal (MERK), distilled water), pH 7.63) at room temperature for 30 minutes. A solution of sheep anti-TH antibody (1:1000) (MERK Lot #3206348) was made up with 10% NHS (in TPBS) and the sections were left, at room temperature on a shaker, for three nights. After the incubation period the sections were washed with TPBS – three washes of 10 minutes each. For the secondary incubation, a donkey anti-sheep antibody (Alexa Fluor 350) (1:100) (Jackson, Lot #96307) solution was made with TPBS, in which the sections were incubated overnight. Following this, the sections were once again washed with TPBS three times as previously described.

The sections were then stained once more to visualise c-FOS. Firstly, they were incubated for two nights in a solution of rabbit anti-c-FOS primary antibody (1:500)(Santa Cruz, Lot #E1606), made up with 10% NHS. Three washes were then done with TPBS, after which the sections were incubated overnight in a solution of donkey anti-rabbit CY5 antibody (1:50) (Jackson, Lot #80443). To conclude the staining process, three final washes with TPBS were conducted, after which sections were mounted onto gelatine coated glass slides with Triton water (distilled water with Triron X-100).

The sections were then imaged using the Olympus VS200 Research Slide Scanner.

Cell counts of the VTA area were done to determine the number of cells that were: mCherry+, TH+, c-FOS+, mCherry/c-FOS+, mCherry/TH+ and mCherry/c-FOS/TH+. These counts were done in three brain slices of each experimental rat, and an average was taken.

2.2.7 Off-target Effect Controls

To investigate whether the DREADD agonists had any off-target effects, 2 mg/kg CNO and 2 mg/kg C21 administration was examined in wild-type animals.

The thermistor implantation procedure described above (see Section 2.2.2) was repeated with wild-type Long-Evans littermates that served as controls. However, these animals did not receive a catheter implantation. Following a one-week recovery period, physiological recordings were set-up (see Section 2.2.3) and CNO, C21 or vehicle were administered. Intraperitoneal injections (0.5mL total volume) were given in a counterbalanced order with two days between each injection (see Section 2.2.4).

2.2.8 Data Recording and Analysis

Physiological signals were recorded with PowerLab and were graphed and analysed with IgorPro (WaveMetrics, Portland, OR, USA). Any occasional artifacts were removed from traces by replacing with values at surrounding time points just before or after the artifact.

Group data was calculated as mean ± SEM for each experimental condition. Baseline temperature values were calculated from the average of the 5 minute period prior to drug administration or intruder introduction. All summary graphs were created in Prism 9 (GraphPad Software LLC, SanDiego, CA, USA). All statistical analyses were performed in SPSS (IBM Corp, version 23).

In order to investigate the effects of drug administration alone, the 55-65 minute time period was examined, for BAT and body temperature as well as behavioural activity. This time period was chosen as it was observed to be the point of maximal temperature increase.

In this series of experiments, feeding behaviour was also examined. A 7 hour period following drug administration was examined, where the following factors were considered: total number of interactions with the food hopper, average duration of each interaction, and the total amount consumed over the 7-hour period. The definition of a significant 'interaction' was set as difference of 0.3 g or over between pre- and post-interaction weight, based on previous reports.

To establish the effects of intruder introduction on BAT and body temperature values of the 5 minutes prior to intrusion were compared to values at the 18-28 minute period post intruder introduction. Due to interanimal variation, this longer period of time was selected. The rate of temperature increase was also investigated by examining the slope of the BAT or body temperature trace, respectively, for the first 5 minutes following intruder introduction. The total

amplitude of temperature increase was calculated by comparing the 18-28 minute period post-intruder introduction to the 5 minute period prior to initial drug administration. To examine behavioural activity, the mean value was calculated as a percentage of the initial 5 minute-post intruder period.

In wild-type animals the effects of CNO and C21 administration were compared to vehicle at 10-minute intervals. Behavioural activity was examined for the 0-120 minute period post-injection, as described for the virally injected transgenic animals.

In some cases the number of recordings between recorded parameters was not the same due to signal failure in probes. Due to this, one-way analysis-of-variance (ANOVA) was used to determine if experimental parameters, following drug administration and intruder introduction, were different for the various experimental conditions. Pre- and post- intruder temperatures were compared using a paired *t*-test.

The Fischer's least significant difference (LSD) was used for post hoc analysis. In order to determine significant difference in the c-FOS study, an independent *t*-test was performed. The level of statistical significance was set at $P < 0.05$.

2.3 RESULTS

2.3.1 Effect of DREADD agonist administration on baseline thermoregulatory variables

In order to examine the effect of VTA dopamine neuron activation on baseline thermoregulatory variables vehicle, CNO and C21 treatments were given without the introduction of an intruder animal.

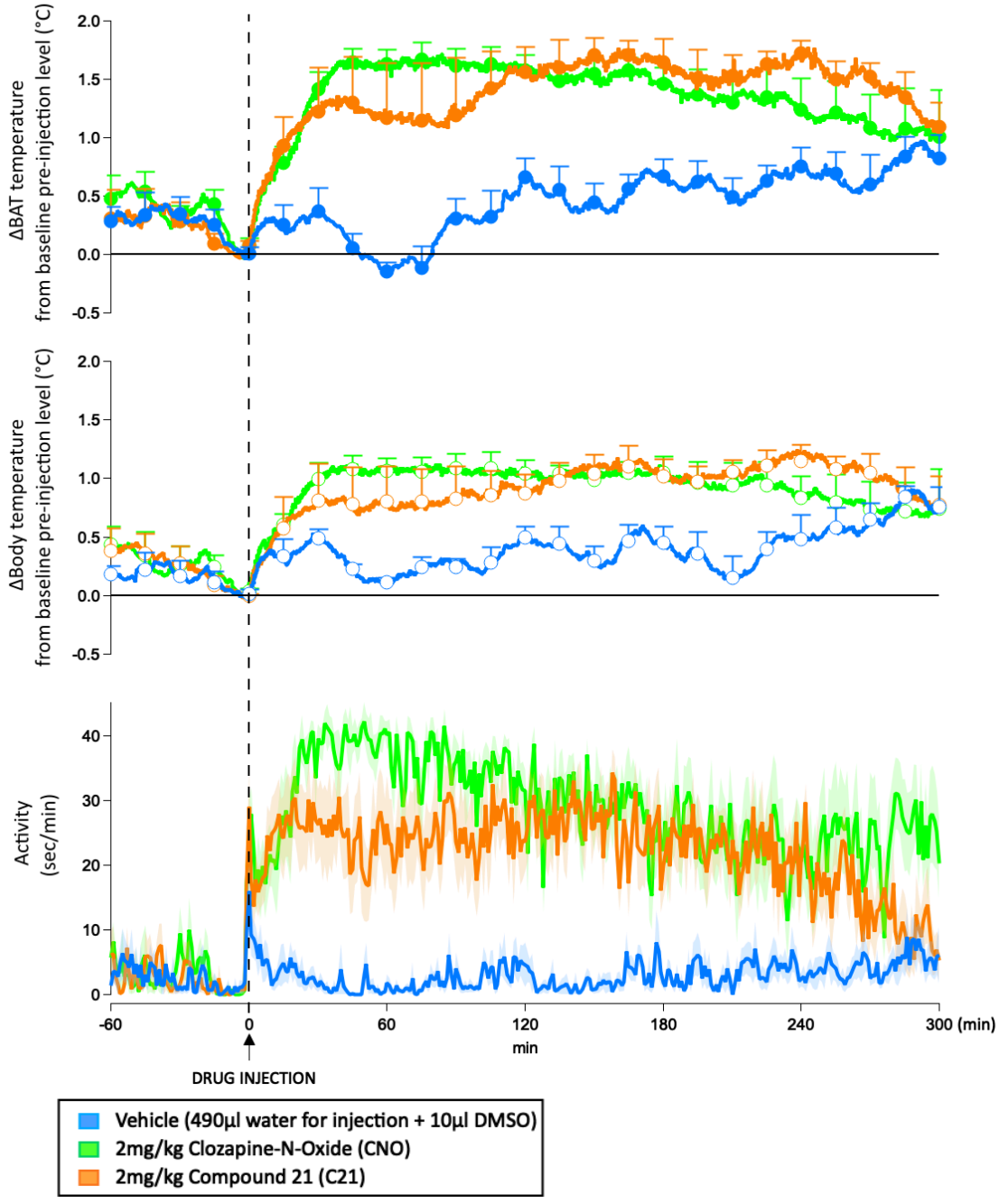


Figure 10: Group data (mean ± SEM) following VTA dopamine neuron activation. Averaged experimental record from the 'resident rat' after vehicle (BAT n=8, Body n=7), CNO (BAT n=7, Body = 6) or C21 (n=6) treatment showing BAT and body temperature, and behavioural activity levels. Drug administration was made at time 0.

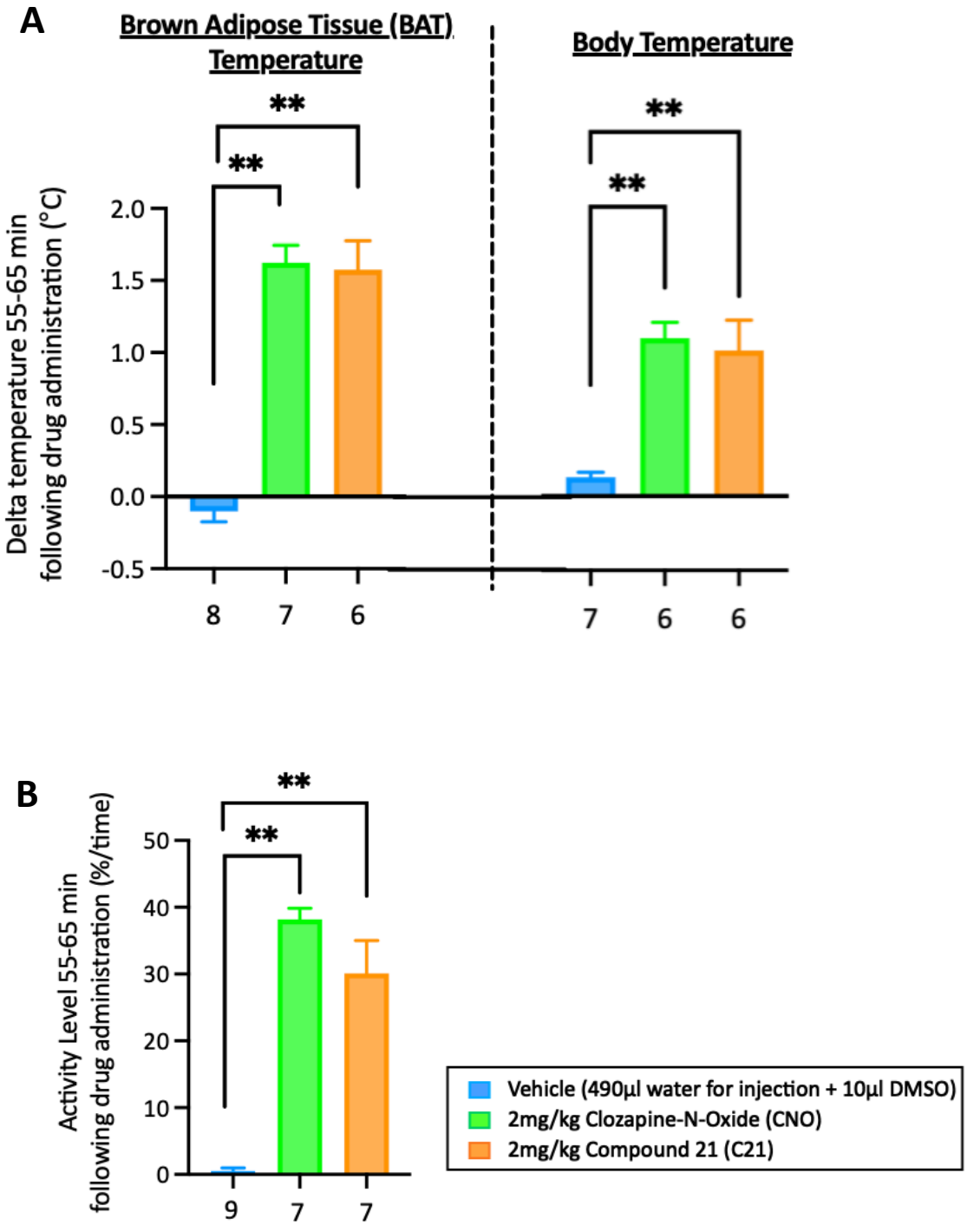


Figure 11: Bar graph showing group results (mean ± SEM) of the effect of vehicle or DREADD agonists (2 mg/kg ip CNO or C21) on baseline thermoregulatory variables. **A** Asterisks indicate significant change in BAT and body temperature recorded in the 'resident rat' 55-65min following drug administration $^{**}P < 0.001$. **B** Behavioural activity level 55-65min following drug administration (percent total time). Asterisk indicate significant change compared to vehicle treatment, **P < 0.001. Numbers below columns indicate number of rats in each experimental condition.

Effect of VTA dopamine activation on baseline BAT and body temperature

The activation of dopamine neurons in the VTA triggered a substantial increase in both BAT and body temperature, as shown in Figures 10 & 11.

The effect of neuron activation following DREADD agonist administration was long lasting, as seen in Figure 10. The increases in BAT and body temperature occurred immediately following administration and lasted approximately 5 hours. There was no significant difference between the average duration of CNO (5.5 ± 1.9 hours) and C21 (5.3 ± 1.3 hours) activation ($P > 0.05$).

It should be noted that in one case of CNO administration, there appeared to be several bouts of activation that followed the initial activation period. In the case of C21 administration there was also one case that experienced a one-and-a-half hour delay between time of administration and observed activation.

The group results for changes in BAT and body temperature measured at 55-65 minutes after vehicle and DREADD agonist treatment are shown in Figure 11. Following vehicle administration, BAT and body temperature did not change significantly. However, DREADD agonist administration significantly increased baseline temperature values, resulting in a temperature level of approximately 1°C higher than baseline. For BAT: $F(2,18) = 60.829$, $P < 0.001$, and body: $F(2,16) = 17.330$, $P < 0.001$.

Interestingly, for CNO administration, BAT temperature increase was higher than for body, $t(5) = 8.453$, $P < 0.001$. But this was not seen following C21 administration, $t(4) = 2.370$, $P > 0.05$.

However, there were no significant differences between the observed effects following CNO and C21 treatment ($P > 0.05$) in relation to total temperature increases.

Effect of dopamine activation on thermoregulatory variables (locomotor activity and food consumption)

Activation of VTA dopamine neurons via DREADD agonist administration also significantly affected other thermoregulatory variables, including locomotor activity. As shown in Figures 10 & 11, there was a robust increase in activity level compared to vehicle pre-treatment: $F(2,20) = 56.241$, $P < 0.001$.

Similarly to what was observed for the temperature recordings, there was no difference between activity levels following CNO and C21 administration ($P > 0.05$).

Interestingly, it was observed that following VTA dopamine neuron activation, the 'resident rat' spent the majority of time near the food hopper. Analysis of feeding behaviour revealed that after CNO and C21 administration there was a drastic change in the feeding patterns of the 'resident rat', as shown Figure 12.

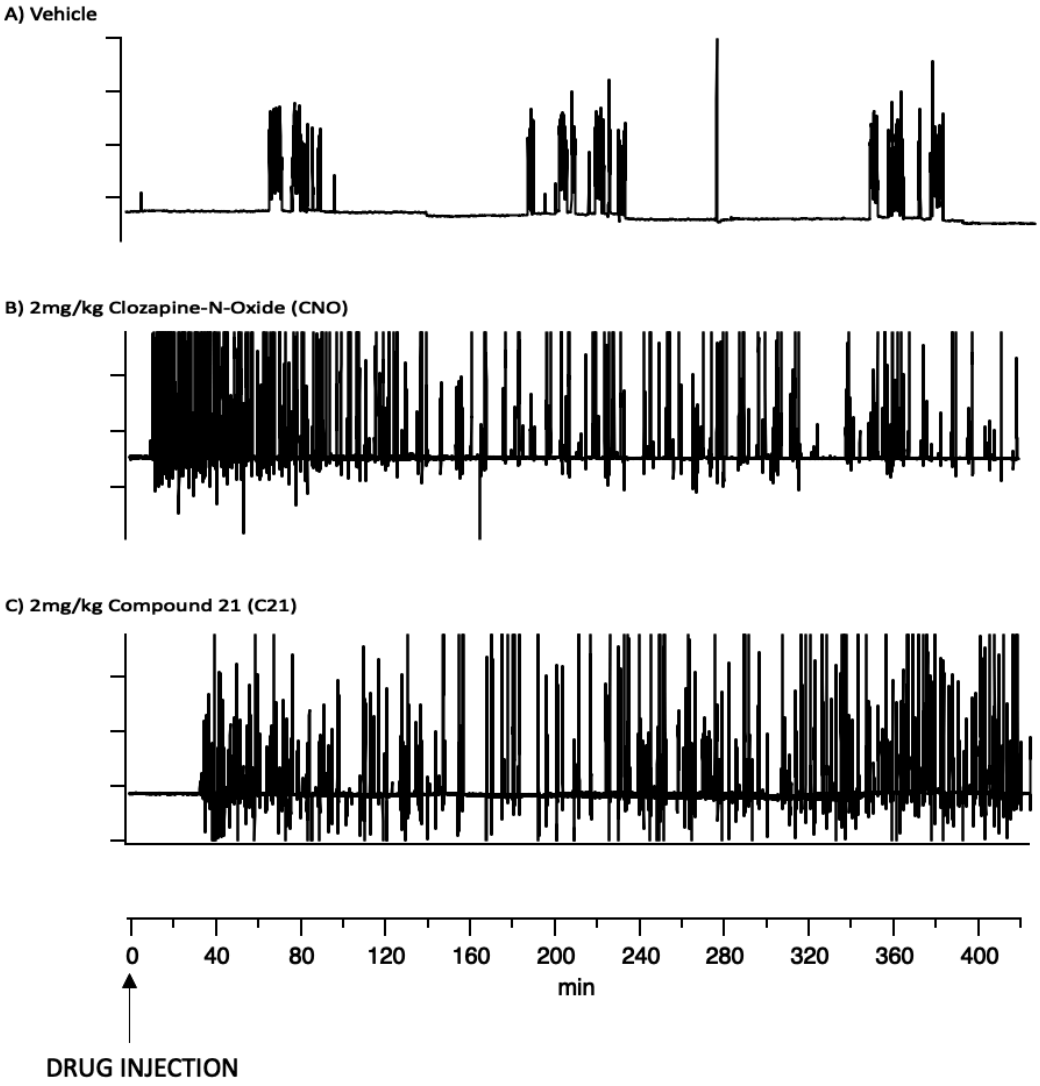


Figure 12: Representative traces from an individual 'resident rat' showing interactions with the food hopper following vehicle (A), CNO (B) and C21 (C) (2 mg/kg i.p) administration. The drug injection was made at time 0. Weight of the food hopper is not indicated, but axes for all treatments are comparable. An artificial 'upper' weight limit was placed on CNO and C21 traces.

It is clear that following VTA dopamine neuron activation, there was almost constant interaction with the food hopper. Taking this into consideration, it is possible that the increases seen in behavioural activity could be in part caused by these interactions.

Figure 13 shows an in-depth analysis of feeding microstructure following vehicle, CNO and C21 administration.

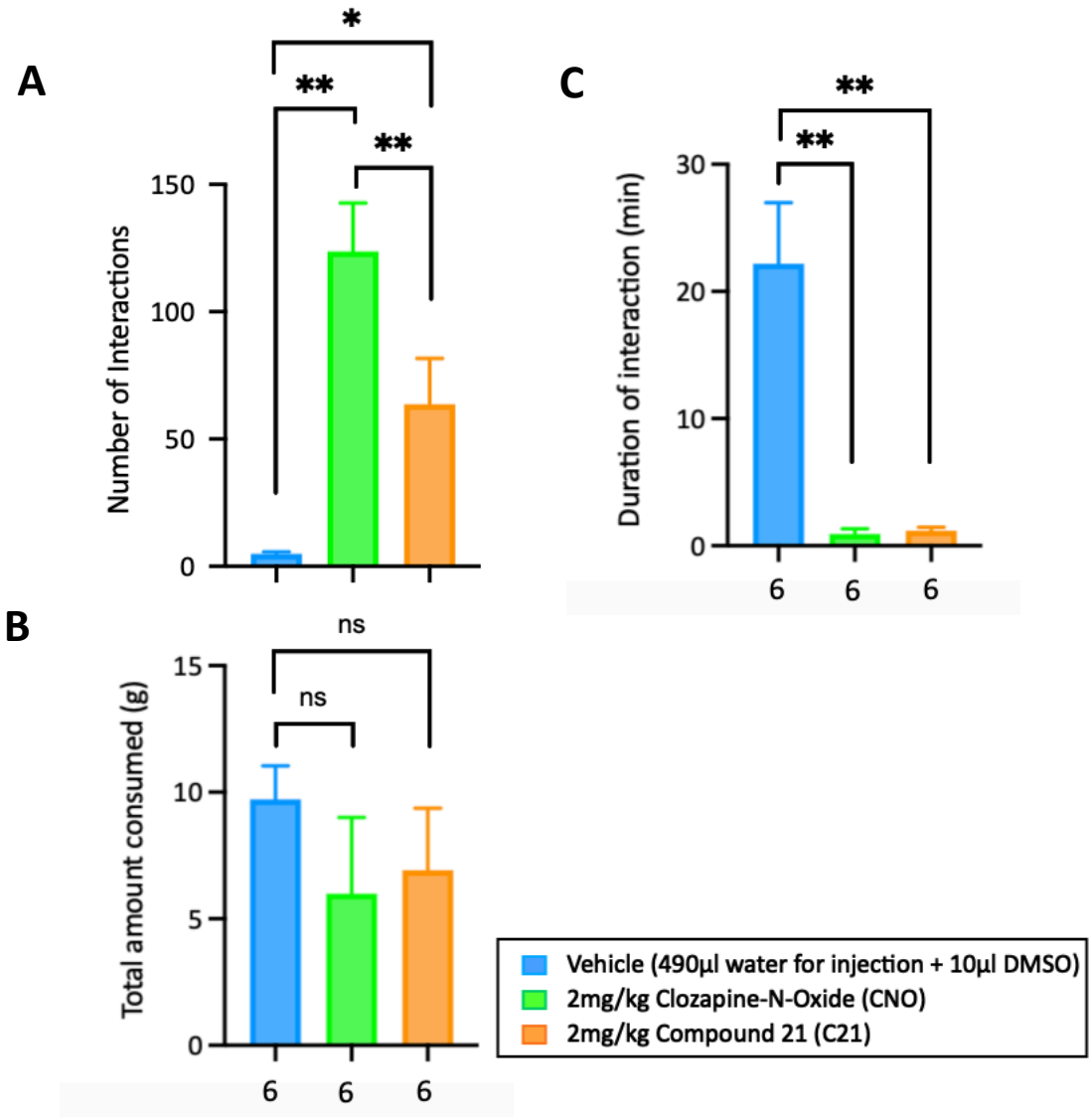


Figure 13: Bar graphs showing group results (mean ± SEM) of the effect of vehicle or DREADD agonists (2 mg/kg i.p CNO or C21) on feeding behaviour. **A** Total number of interactions with the food hopper in a 7 hour period. Asterisk indicates significant difference: significant different between number of interactions following vehicle and C21 treatment (*$P < 0.05$) and vehicle and CNO treatment ** $P < 0.001$. There was a significant difference between CNO and C21 treatment (** $P < 0.001$) **B** Total amount consumed in a 7 hour period post drug administration. *ns* no significant difference from vehicle treatment. **C** Average duration of each meal (min). Asterisk indicates significant difference from vehicle treatment ** $P < 0.001$. Numbers below columns indicate number of animals in each experiments condition.

Indeed, DREADD agonist administration significantly changed the 'resident rat's' feeding behaviour, as seen in Figure 13. There was a drastic increase in the number of interactions had with the food hopper following VTA dopamine neuron activation with CNO and C21,

$F(2,15)=14.610$, $P < 0.001$. However, the duration of each interaction was also significantly reduced compared to vehicle treatment $F(2,15)=16.693$, $P < 0.001$.

Despite the greater number of interactions with the food hopper following CNO and C21 administration, the total amount of food consumed over the 7-hour post-drug administration period was not significantly different to vehicle treatment ($P > 0.05$).

It is interesting to note that there were more interaction with the hopper following CNO administration then there were following C21 treatment ($P = 0.05$). This is likely due to secondary activation of the DREADD receptors via metabolised CNO.

2.3.2 Effect of dopamine neuron activation on intruder-elicited thermoregulatory variables

Intruder elicited change after pre-treatment with vehicle, CNO or C21

Despite the baseline condition of the animal being significantly affected by chemogenetic dopamine neuron activation it was still of interest to determine how a stressful stimuli affects body temperatures in the presence of an elevated baseline.

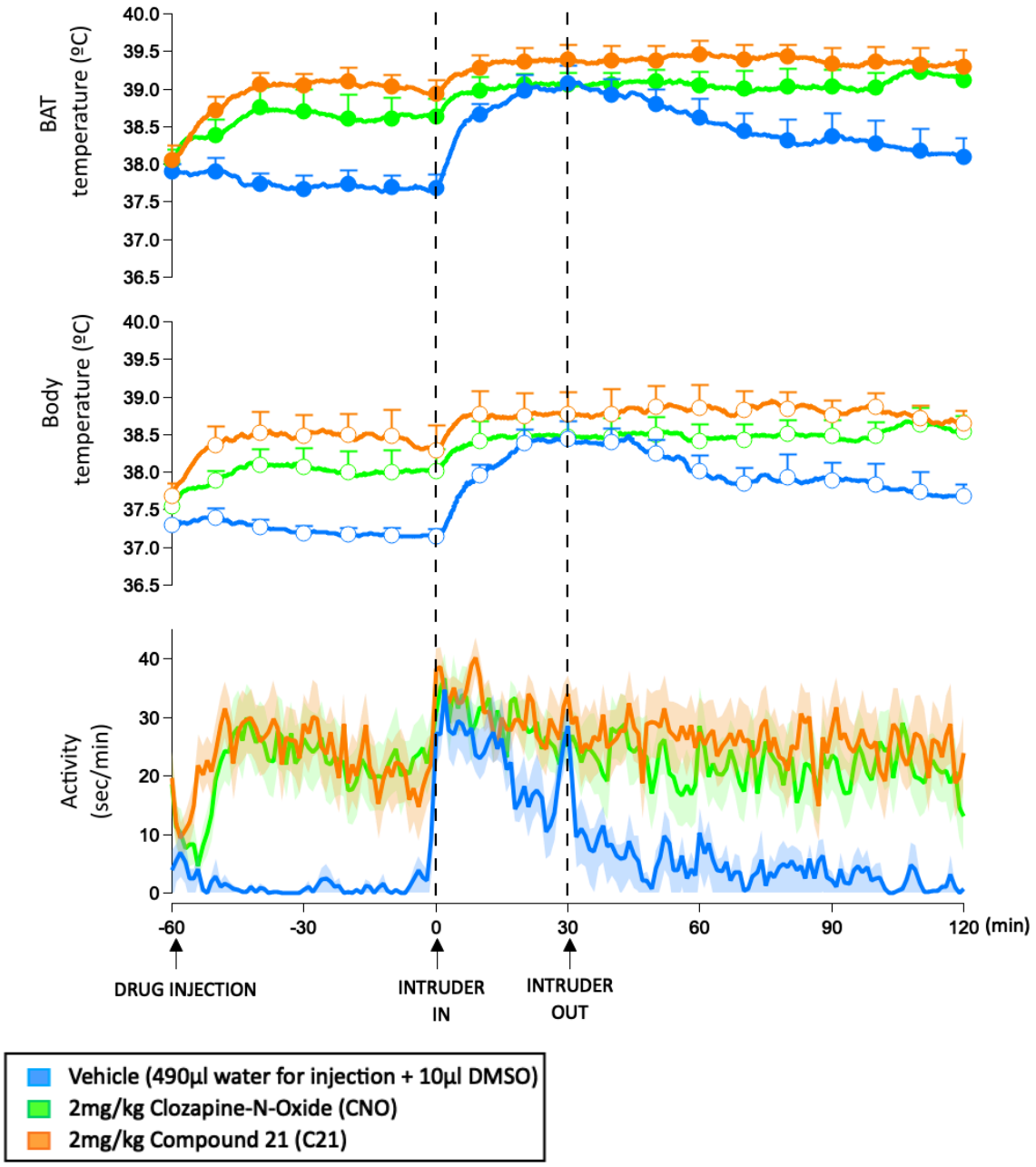


Figure 14: Group data (mean ± SEM) from intruder response experiment following VTA dopamine neuron activation. Averaged experimental records from 'resident rat' after vehicle (BAT n=8, Body n=6), CNO (n=7) or C21 (BAT n=7, n=6) treatment showing BAT and body temperatures, and behavioural activity level. Administration (time -60) was made 1 hour prior to introduction of the intruder (time 0). The intruder was introduced for 30 min and then removed.

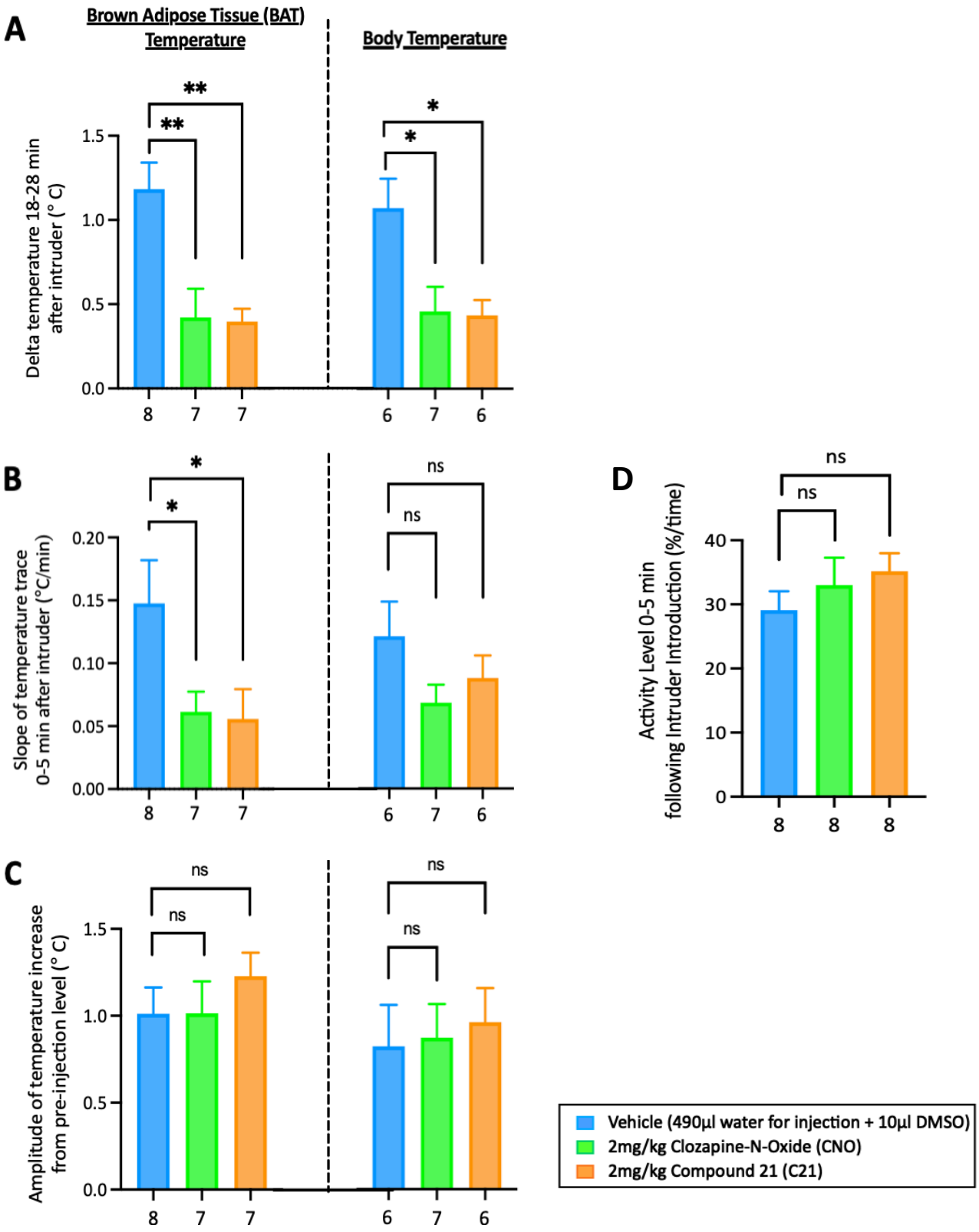


Figure 15: Bar graphs showing group results (mean ± SEM) of the effects of vehicle or DREADD agonists (2 mg/kg i.p CNO or C21) administration on intruder-elicited thermoregulatory events. **A** Asterisks indicate significant changes in BAT and body temperature recorded in the 'resident rat' 18-28 min after introduction of the intruder rat * $P < 0.05$, ** $P < 0.01$. **B** *ns* slope of 0-5min body temperature signal is not significantly different than following vehicle administration. Asterisks indicate significant different in the slope of BAT temperature signal recorded for the 'resident rat' during the first 5min after introduction of the intruder rat, *$P < 0.05$. **C** *ns* the total amplitude of temperature increase from pre- DREADD agonist administration level to 18-28min following intruder rat introduction is not significantly different than following vehicle administration. **D** Behavioural activity during 0-5min after introduction of the intruder rat (percent total time) *ns* behaviour following intruder introduction after DREADD agonist administration was not different compared to vehicle treatment. Numbers below the columns indicate number of rats in each experimental condition.

Comparison of intruder-elicited increases in BAT and body temperature prior to and following VTA dopamine cell activation with DREADD agonists

The effects (group data) of introducing the intruder rat following vehicle administration are shown in Figure 14 and 15. BAT and body temperature both increased rapidly following introduction of the intruder after pre-treatment with vehicle.

Following pre-treatment with DREADD agonists, CNO or C21, the intruder-elicited increases in BAT and body temperature were significantly reduced. For delta BAT temperature: $F(2,19) = 10.079$, $P < 0.001$, for delta body temperature: $F(2,16) = 6.222$, $P < 0.01$.

Similarly, activation of VTA dopamine neurons also decreased the 0-5 minutes post-intruder introduction slope of BAT temperature: $F(2,19) = 3.977$, $P < 0.05$. However, the post-intruder introduction slope of body temperature was not found to be statistically significant, compared to the corresponding values for vehicle pre-treatment.

Interestingly, following DREADD agonist administration emotional hyperthermia was still observed, this was marked by significant increases in both BAT and body temperature compared to pre-intruder temperature. For CNO BAT: $t(6) = 2.463$, $P < 0.05$ and body: $t(6) = 3.118$, $P < 0.05$ and for C21 BAT: $t(6) = 5.184$, $P < 0.05$ and body $t(5) = 4.794$, $P < 0.05$ respectively.

It is important to note that for the total amplitude of temperature increase, from pre-DREADD agonist administration to post-intruder introduction, there was no significant difference between the vehicle control and experimental groups – for either BAT or body temperature ($P > 0.05$). Following vehicle, CNO and C21 treatment the maximum temperature reached for BAT was, on average, 39.0°C and 38.5°C for body temperature, respectively – for both temperatures this level was approximately 1°C higher than baseline resting level.

Comparison of intruder-elicited changes in behavioural activity after pre-treatment with vehicle or DREADD agonists

After vehicle pre-treatment, introduction of the intruder rat caused the 'resident rat' to become very active – investigating and climbing on top of the intruder's cage. Similar observations have previously been reported (9).

Following pre-treatment with CNO or C21, as seen in Figure 14, there was also observed to be a very moderate increase in activity following introduction of the intruder. However, this increase was not found to be significant compared to the vehicle pre-treatment ($P > 0.05$).

2.3.3 Confirmation of viral vector expression and dopamine neuron activation

In order to confirm adequate viral vector expression in the VTA, along with confirming that dopamine neurons were indeed activated following CNO and C21 administration – the brain's of experimental animals were examined and also stained for TH and c-FOS expression. The distribution of viral vector expression for vehicle and C21 treated animals is presented in Figure 16.

Representative images, taken from the c-FOS study, following vehicle or C21 administration, are shown in Figure 17 and Figure 18 respectively.

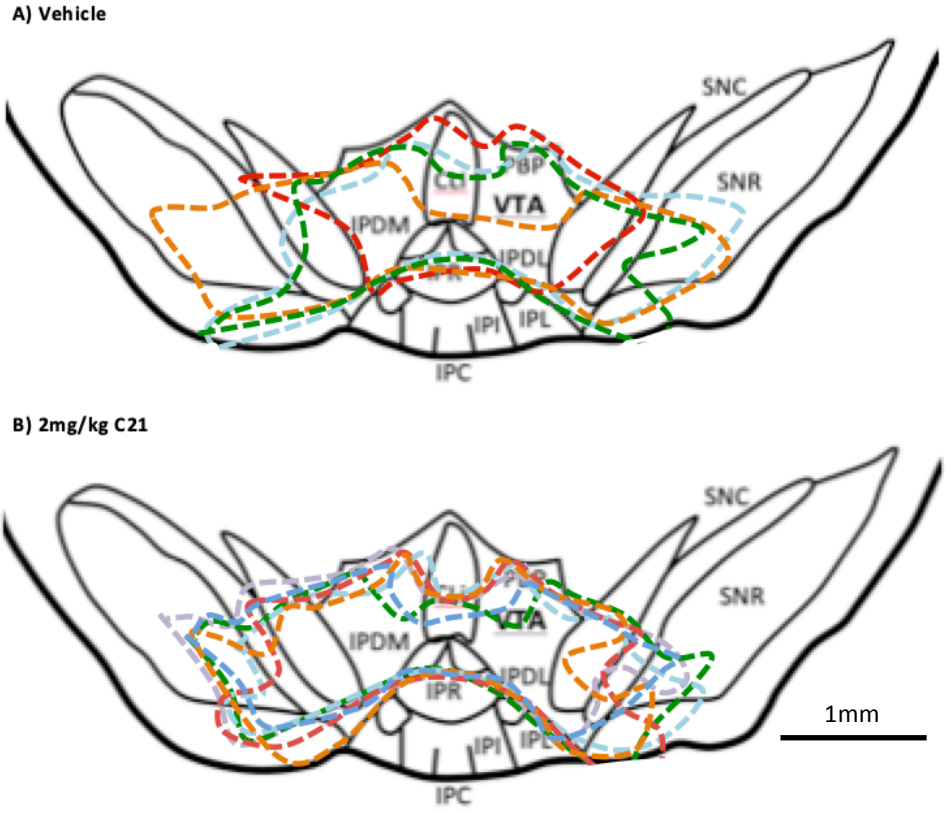


Figure 16: Distribution of viral vector spread for A) Vehicle and B) 2mg/kg C21 treated animals.

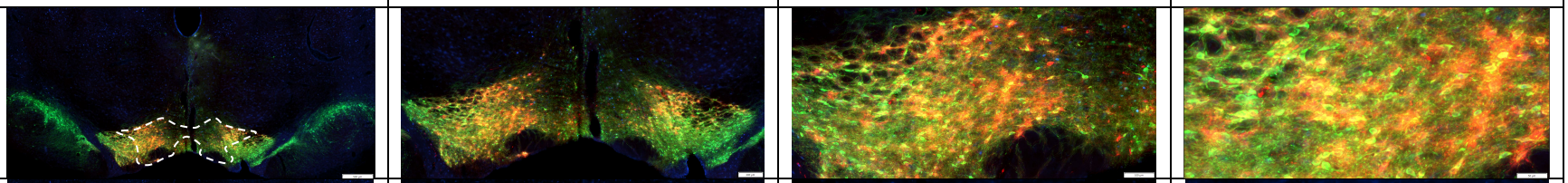
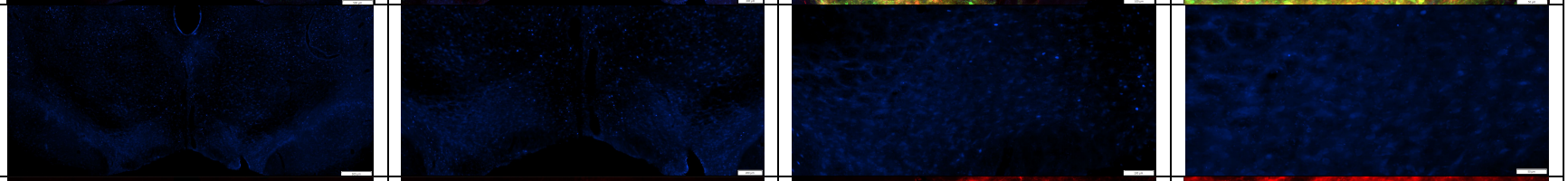
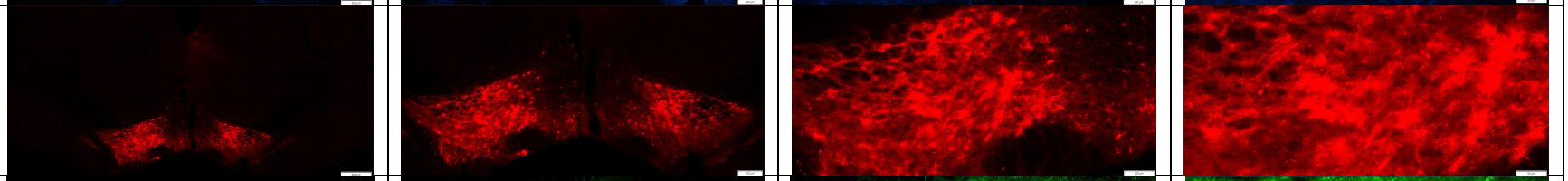
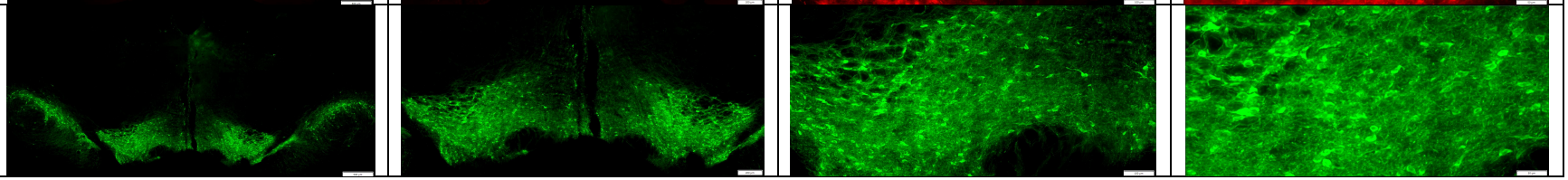
Vehicle Administration	2x	4x	10x	20x
Overlay				
Activated Neurons c-Fos				
Viral Vector AAV-hSyn-FLEX-mCherry hM3Dq				
Dopamine Neurons TH				

Figure 17: Representative immunohistochemical demonstration of activated cells (blue – c-FOS), DREADD expression (red – hM3Dq mCherry) and dopamine expression (green - TH) in coronal sections through the midbrain of a vehicle treated animal. Subregion from which cell counts were taken in outlined in white, first panel. Scale bar is located in the bottom right corner of every image; 2x=500µm, 4x=200µm, 10x=100µm, 20x=50µm.

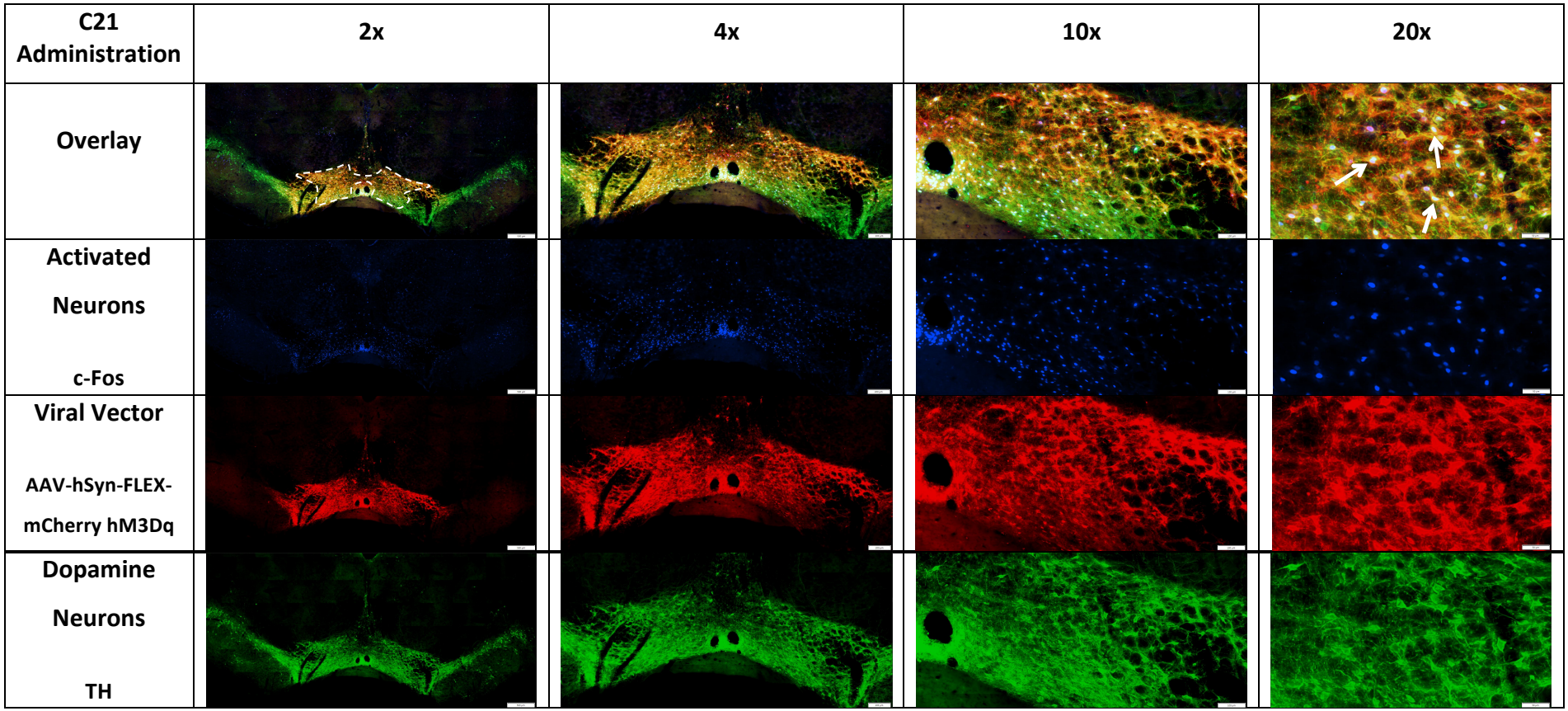


Figure 18: Representative immunohistochemical demonstration of activated cells (blue – c-FOS), DREADD expression (red – hM3Dq mCherry) and dopamine expression (green - TH) in coronal sections through the midbrain of a 2 mg/kg C21 treated animal. Subregion from which cell counts were taken in outlined in white, first panel. White arrows identify examples of typical triple stained neurons (last panel). Scale bar is located in the bottom right corner of every image; 2x=500µm, 4x=200µm, 10x=100µm, 20x=50µm.

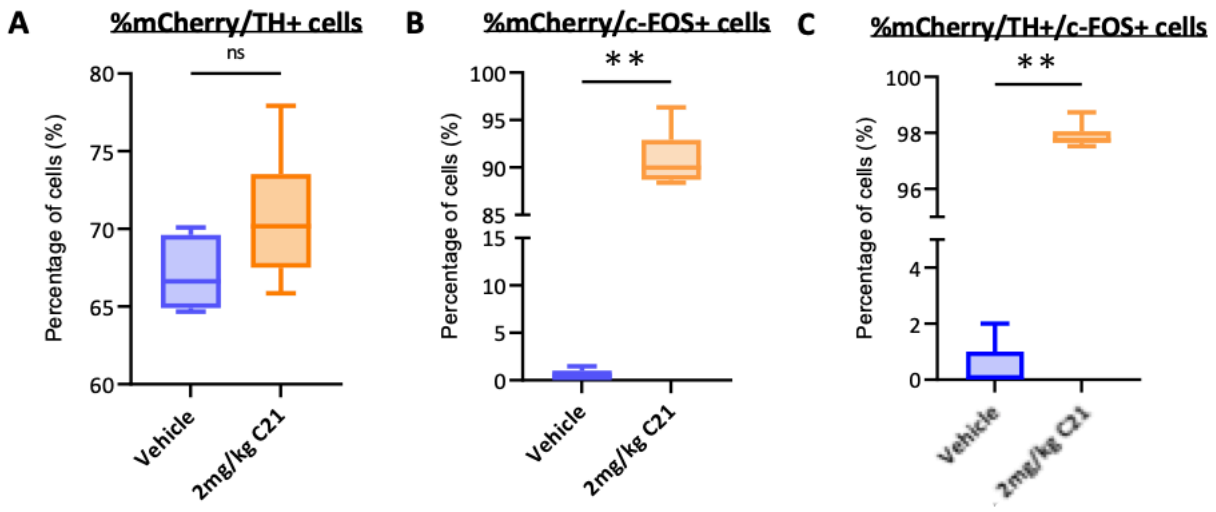


Figure 19: Summary of **A** DREADD expression in dopamine neurons in the VTA, **B** their activation following vehicle or 2 mg/kg C21 administration and **C** percentage of mCherry/TH+ neurons that were activated. *ns* no significant difference compared to vehicle treatment, asterisks indicate significant increase compared to vehicle treatment ** $P < 0.001$

The results from the c-FOS study cell counts are summarised in Figure 19. There was no significant difference between the number of mCherry expressing TH+ cells in vehicle and C21 treated animals ($P > 0.05$). However, as expected, following C21 treatment, the number of mCherry expressing cells that were activated (c-FOS+) was significantly greater than what was seen following vehicle treatment $t(9)=-75.297$, $P < 0.001$.

In total, approximately 70% of mCherry expressing cells were TH+, indicating that there was some off-target viral vector expression in non-dopamine cells. Of the mCherry/TH+ cells, on average 97.9% were activated following C21 administration (Figure 18C).

As can be seen in Figures 17 & 18, there was also a small amount of non-TH mCherry and c-FOS expression in the SNc of some rats. This is important to consider when interpreting results.

The number of mCherry+ cells were also compared to the duration of C21 action, as shown in Figure 20. Duration of C21 action was estimated based on when BAT and body temperatures returned to baseline level.

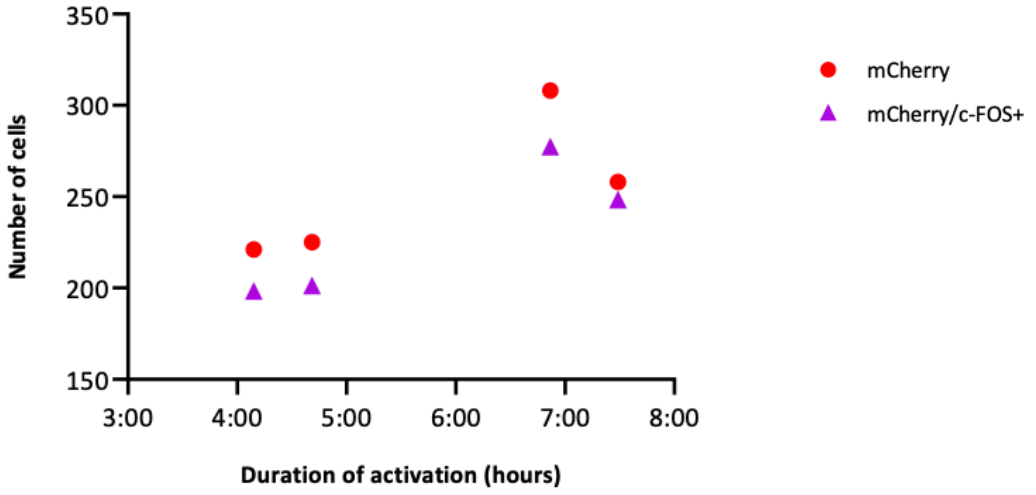


Figure 20: Duration of C21 action (hours) dependent on the total number of mCherry cells in the VTA (red dots) and the number of mCherry cells that were activated (mCherry/c-FOS+) (purple triangle)

There was no apparent correlation found and greater amounts of mCherry expression did not result in longer periods of activation. Linear regression analysis of the total number of mCherry cells: R^2=0.551, $F(1,2)$=2.453, $P > 0.05$, and the number of mCherry cells activated by 2 mg/kg C21 administration: R^2=0.754, $F(1,2)$=6.142, $P > 0.05$, were insignificant.

However, only a small sample size was available as not all animals that were used for the cell activation study received prior long-term recordings. Consequently, the duration of C21 action for the animals used exclusively for the cell activation study could not be assessed.

2.3.4 Effect of DREADD agonist administration in wild-type animals

In order to confirm that the previously mentioned increases in thermoregulatory parameters resulted from activation of VTA dopamine neurons, and were not result of the DREADD agonists themselves, each of the agonists – CNO and C21, were given to wild-type animals to examine any off-target effects.

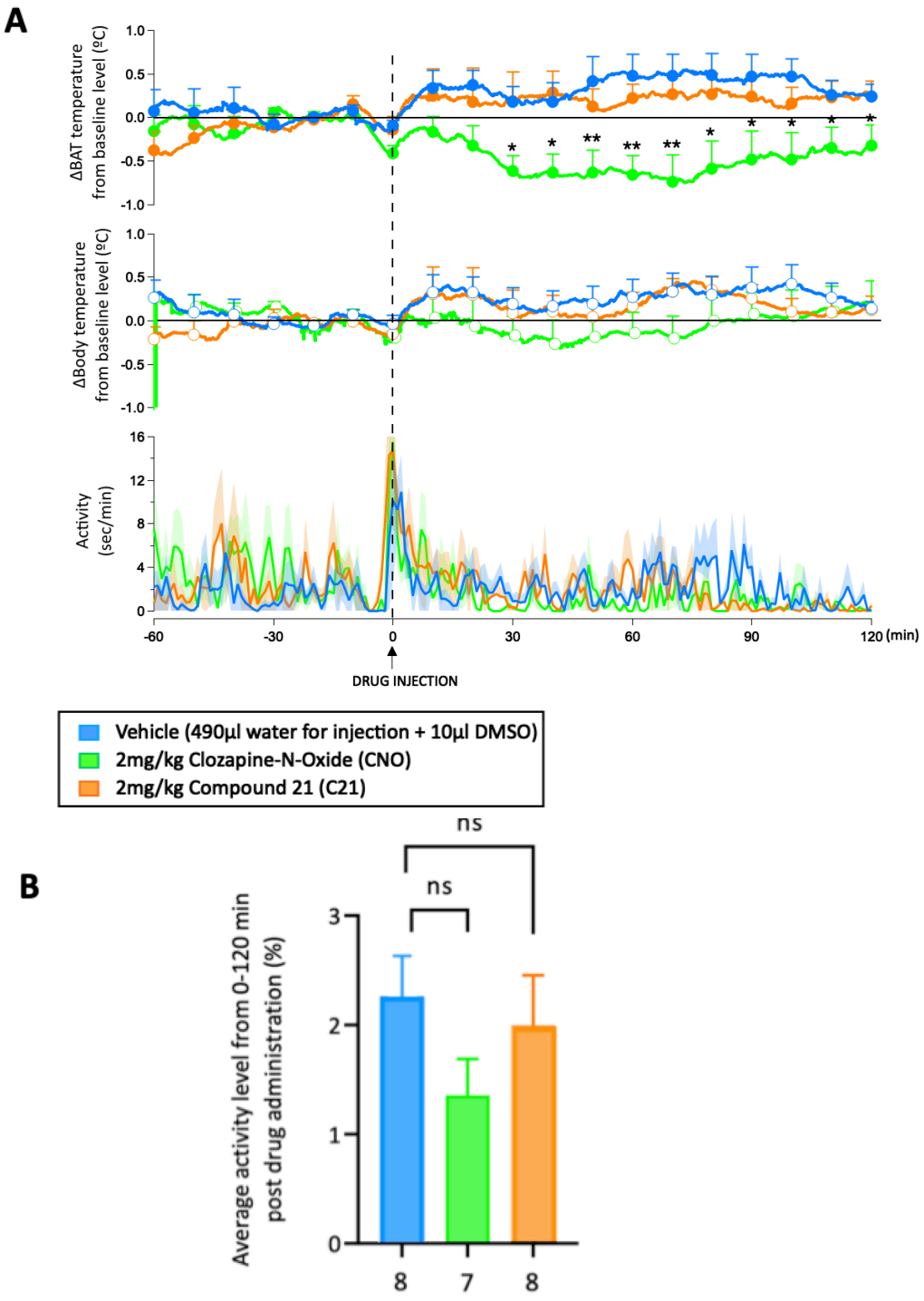


Figure 21: **A** Effect of vehicle (n=7), 2 mg/kg CNO (n=6) and 2mg/kg C21 (n=7) on baseline thermoregulatory variables. Averaged physiological record data (with SEM) showing BAT and body temperature and behavioural locomotor activity. Asterisks indicate a significant difference from baseline value before injection *$P < 0.05$, **$P < 0.01$. **B** Effect of vehicle or DREADD agonist administration on behavioural locomotor activity. Bat graph showing group results (mean ± SEM) for locomotor activity 0-120 minutes following vehicle or DREADD agonist administration. *ns* no significant change compared to vehicle treated animals. Numbers below columns represent number of animals in each experimental group.

Effect of vehicle, CNO and C21 on BAT and Body temperatures

As can be seen in Figure 21, following vehicle and C21 administration, there was minimal change to BAT and body temperature during the 2-hour post-administration recording. No change in temperature was found to be significant compared to pre-injection levels ($P > 0.05$), and there was

no difference between the two treatment groups ($P > 0.05$). The modest rise in temperature seen approximately 50 minutes following vehicle administration is more than likely a continuation of the natural ultradian rhythm, as reflected by the accompanying slight rise in locomotor activity.

CNO administration similarly did not affect body temperature ($P > 0.05$), but did interestingly produce a significant decrease in BAT temperature 30 minutes following drug administration (*$P < 0.05$, ** $P < 0.01$). The greatest decrease in BAT temperature was seen 70 minutes following administration, when the temperature fell 0.7°C from pre-injection baseline level. This significantly lowered BAT temperature remained for over 90 minutes after administration.

Effect of vehicle, CNO and C21 on behavioural locomotor activity

When behavioural activity was examined, it was found that there was no significant difference between vehicle, CNO and C21 treatment ($P > 0.05$).

2.4 DISCUSSION

To the best of my knowledge, this is the first study to examine the resultant effects on body temperature following chemogenetic activation of dopamine neurons in the VTA. Further, the experiments conducted here had the advantage of all behavioural outcomes being assessed within the same animal. This enabled the accurate, in-subject comparison between baseline, or vehicle treated, conditions and enhanced dopamine neuron activation.

2.4.1 Effect of temperature elevation on interpretation of results

Based on the results presented here, it is difficult to definitively conclude whether stimulation of VTA dopamine neurons attenuated the process of emotional hyperthermia. Whilst the response to intruder introduction was reduced following VTA dopamine neuron activation, a small but significant response to intruder stress remained. The fact that the response was still present potentially suggests that VTA dopamine neurons do not, in fact, play a major regulatory role in emotional hyperthermia. It is important to note that both the administration of CNO and C21 also significantly altered the baseline conditions of the animal. Consequently, it is likely that the elevated temperatures following DREADD agonist administration were the primary reason for the reduced temperature increase seen following intruder introduction. Indeed, the total amplitude of temperature increase, from pre-injection level to post-intruder introduction, was the same for both vehicle and DREADD agonist administration. As such, the results of this study support the ideas previously suggested by Vinkers and colleagues (2010), who indicated that the process of stress-induced hyperthermia is limited by a 'physiological temperature ceiling', after which no further stress-induced temperature rise is possible (253). Whilst there has not yet been consensus in the literature in regards to what this value is - as, without doubt, this value varies depending on species and location of temperature measurement – in the present study this value appears to be 39.0°C for body temperature, and 39.5°C for BAT temperature, in rats.

This upper-temperature limit has even previously been suggested for fever regulation and the febrile response (254). As shown in humans, even in extreme cases, a core temperature above 41°C is exceptionally rare (2).

It is important to note, that even following removal of the intruder, in CNO and C21 treated animals the temperature remained significantly elevated. This unquestionably indicates that activation of dopamine neurons in the VTA affects normal thermoregulatory mechanisms.

2.4.2 Potential factors contributing to observed temperature increases

Given the large amount of evidence supporting body temperature decrease following dopamine neuron activation, the resultant temperature increases seen in this study are somewhat surprising. There are several factors that may possibly contribute to this. Firstly, most studies examining temperature involve pharmacological manipulation that affects receptors in large areas, whilst this study utilised a chemogenetic approach to focus on a defined, specific area.

Further, it is vital to consider that dopamine can be both inhibitory and excitatory. As such, its function depends on which receptor it binds to. Previously reported temperature decreases have primarily arisen from observation of D_2 agonist administration, which is known to be inhibitory. Indeed, activation of the D_2 receptor has been shown to attenuate emotional hyperthermia (252). In this study, however, we activated dopaminergic neurons in general, without a specific focus on receptor type. Therefore, it is possible that other dopamine receptor types were activated, resulting in a temperature increase.

Noticeably, activation of VTA dopamine neurons also resulted in increases in locomotor activity and significant changes in feeding behaviour. Both of these aspects must be carefully considered when interpreting the present results, as they are known factors that contribute to effective thermoregulation (255, 256). So, whilst it appears that dopaminergic neurons in the VTA are involved in healthy temperature regulation, it is not currently clear whether this effect occurs due to dopamine neuron stimulation alone, or whether it is a by-product of other effects.

2.4.3 Effect of VTA dopamine neuron activation on locomotor activity

The fact that dopamine neuron activation increased locomotor activity is not surprising. Rodrigo et al (2011) (257), Molloy et al (1986) (258) and Kurashima et al (1995) (259) have all previously demonstrated that activation of D_2 dopamine receptors has excitatory effects on locomotion. Canals and Iversen (216), Ikemoto (260) and Delfs (261) have similarly demonstrated that the administration of psychostimulant drugs that enhance dopamine signalling typically result in hyperactivity. Wang and associates (2013) utilised DAT::Cre transgenic mice and verified that selective activation of dopamine neurons in the midbrain specifically induces hyperactivity (262), which was further supported by Vardy and colleagues in 2015 when they demonstrated that disinhibition of midbrain dopamine neurons, through inhibition of GABAergic neurotransmission, also increased locomotor activity (263). Han (2017) also confirmed that optogenetic manipulation that excited VTA dopamine neurons induced hyperlocomotion (147) and Sun (2017) showed that

activation of VTA neurons increased wakefulness (264). Human clinical imaging studies have similarly reported alterations in dopamine signalling in individuals with hyperactivity, and even over-stimulation of dopamine receptors in over-treated Parkinson patients causes chorea – jerky, involuntary movements (265).

However, in many studies that have examined midbrain dopaminergic activity and locomotion there has not been distinction between the VTA and the SNc neuronal populations (262, 263). This is one of the most substantial factors that must be taken into account for the present study, as there are no distinct borders between the two areas - making it practically impossible to stop the spread of the viral vector and affect only one area without at least some minor influence on the other.

In reference to the results presented in this chapter, it is unlikely that any activation of the SNc had a substantial affect on locomotor activity. Indeed, Boekhoudt (2016) has supplied conclusive evidence that chemogenetic activation of dopamine neurons in the VTA, but not the SNc, induces hyperactivity in rats (266). Of course Parkinson's, a movement disorder, is caused by loss of dopaminergic neurons in the SNc, which implies involvement of both the VTA and SNc in locomotor activity (192-194). However, the areas are likely involved in the regulation of differing aspects of movement; for example, the SNc has more significant effects on motor coordination. The exact 'type' of locomotion was not examined in this study, and therefore, it is an important factor to consider and examine in future experimentation, such as through careful analysis and motion tracking of recorded video.

2.4.4 Effect of VTA dopamine neuron activation on feeding behaviour

As discussed, the current results also presented a noteworthy change in feeding behaviour following VTA dopamine neuron activation. As such, the constant interaction with the 'food hopper' could account for the increased activity levels observed. In support of this, the use of various dopamine antagonists is known to affect and reduce food intake, primarily through changes to locomotor behaviour including alterations to vital aspects of feeding behaviour, such as approach and food handling (267-269). In fact, it is feeding behaviour that is of greatest likelihood to have contributed to the observed elevation of temperature.

Although muscles are intrinsically capable of producing heat, and muscle contractions generate considerable amounts of heat (which is particularly exploited in shivering during cold exposure) (256), Mohammed and colleagues, who previously employed the same intruder-paradigm, showed

that increased locomotion does not substantially contribute to rise in body temperature in this model (9). Unfortunately, the contribution of increased locomotor activity to temperature increase using the current paradigm induced by DREADD activation is difficult to assess without introducing an element of physical restraint.

It is known that dopamine signalling is required to maintain healthy feeding behaviours; however, much debate remains in regards to its exact involvement. Dopamine deficient mice fail to eat, to the point of starvation, without appropriate treatment (270). On the other hand, dopamine reuptake inhibitors and dopamine-enhancing psychostimulants, such as amphetamine, have also been found to result in reduced appetite (271). Interestingly, dopamine-blocking antipsychotic drugs induce weight-gain (272). Further, Davis (2012) (273) and Leibowits (1986) (274) have reported similarities, both behavioural and neurochemical, between obesity and alterations in dopamine signalling caused by drug addiction. These results are likely to be related to, at least in part, the rewarding properties of food and increased dopamine signalling throughout the striatum. The results presented here, however, are not fully consistent with this theory of reward and dopamine signalling increases. Again, the differences in results are likely due to differences between chemogenetic and pharmacological activation, as well as variations in pre- and post-synaptic signalling.

The analysis of the feeding behaviour microstructure is in support of previous work that has specifically examined the effects of chemogenetic midbrain dopamine neuronal activation on free food consumption (275). Without doubt, the current study demonstrated that enhanced VTA dopamine signalling affects normal feeding patterns. Whilst it was observed that meals occurred far more frequently following activation of VTA dopamine neurons; the overall total amount consumed was unaffected. Therefore, rather than a broad increase or decrease in hunger and satiety, it appears that activation of these neurons mediates the initiation and cessation of meals. This is interesting to note as enhanced dopamine signalling has previously been associated with increased food intake and enhanced motivation for food.

Similarly to the locomotor activity increases, it is unlikely that any SNc activation contributed to feeding behaviour. Although feeding hormone receptors are found in both the VTA and SNc, chemogenetic activation of SNc dopamine neurons has no significant impact on normal feeding patterns and behaviours, as demonstrated by Boekhoudt (2017) (275). However, taken together with the increases in locomotor activity, it can be hypothesised that the enhanced activity of

dopamine neurons in the VTA may disrupt sustained attention to tasks and induce short and impulsive actions.

2.4.5 Potential off-target effects of viral vector administration and DREADD agonists

Based on immunohistochemical examination, some off-target, non-dopaminergic transduction of VTA neurons was found. This means other neuronal types were activated in addition to dopamine, which is something that should be carefully considered. Both GABAergic and glutamatergic modulation of thermoregulatory circuits has been reported (276-278). As such, in the current experiments, the potential involvement of these neurotransmitters in the results obtained cannot be ruled out and warrants further investigation in the future.

It was found that VTA dopamine neuron activation with CNO and C21 produced comparable results, indicating that C21 is a suitable substitute to CNO in chemogenetic studies, at least for the current purpose. The fact that for CNO treated animals, BAT temperature increase was higher than for body temperature is not surprising, as BAT thermogenesis is known to significantly contribute to the increase of body temperature. The same result was not seen for C21 administration; however, this can be attributed to slight variations in temperature probe placement, and small sample size.

The investigation of DREADD agonists performed in the wild-type animals confirmed that 2 mg/kg CNO significantly affects BAT temperature, and thus, confirms that CNO is not an inert ligand. This result is not surprising, given the multitude of previous studies that have identified several behavioural non-specific effects following systemic CNO administration. Even CNO doses that are lower than those used in this study, such as 1 mg/kg, were found to reduce aspects such as the acoustic startle reflex (239). The off-target effects are likely due to the back metabolism of CNO to clozapine and *N*-Des. Indeed, clozapine is well known to influence temperature, with doses of 2 mg/kg lowering temperature in both humans and rats (158, 279).

Consistent with the results we observed in this study, previous reports that examined plasma levels of clozapine following CNO administration, also found peak levels 30 minutes after administration. At this point, clozapine levels were at one tenth the dose of CNO originally given (244, 280). This amount is enough to see behavioural effects. Indeed, it is 30 minutes after drug administration that the results showed a significant decrease in BAT temperature, indicating that it is likely the presence of back-metabolised clozapine that is responsible for this fall. When considering this, however, it should be mentioned that the metabolism of CNO to clozapine has

been established to differ considerably between species, and also fluctuates according to sex (238).

Nevertheless, the present wild-type study failed to find any significant effect of CNO on locomotor activity. This is interesting, as if clozapine was the major metabolite present at this time, some changes should have been observed. However, this is also in-line with previous reports that have found no impact on locomotor aspects, such as PPI (239). Interestingly, even higher doses (2 to 5 mg/kg) of CNO were not reported to have effects on spontaneous locomotion, suggesting that clozapine may act on receptors that differ in their control of temperature and locomotion; or that potentially CNO has no single, or clearly predictable effect, on a system, but has multiple different effects on several different systems (239, 281). Similarly, no behavioural changes were found following administration of C21 in wild-type animals, which is once again consistent with what has previously been demonstrated, particularly by Jendryka et al (2019), who also failed to find any nonspecific behavioural effects, as assessed by the 5-choices-serial-reaction-time-task, for CNO or C21 (250). When assessing these findings however, it is important to note that within the research groups that use CNO, there seems to be little consistency in regards to the dosage administered. Most commonly a dosage of over 1 mg/kg is used, with the majority of studies using 3 mg/kg – however, some studies have used doses as high as 10 mg/kg.

It is important to mention that C21 has been shown to have superior brain penetration compared to its DREADD agonist counterparts, and to have a longer lasting presence – with effects being seen, at the latest, 15 minutes after intraperitoneal application (250). Since the current study did not identify any off-target effects of C21 in relation to physiological parameters, it appears to be a more promising and more specific agonist for DREADD studies. However, it is vital to consider that C21 has been demonstrated to have a substantial affinity for several receptor types, including both serotonergic and histaminergic (238). Bonaventura et al suggested that a dose as low as 1 mg/kg may modify brain function in wild-type mice (282, 283). Indeed, blockade of histaminergic receptors, particularly those located on nigral GABAergic neurons, can lead to the disinhibition of nigral dopaminergic neurons, enhancing their activity (242). This must be considered carefully as a previous study by Brizuela and colleagues (2019) demonstrated that activation of D_2 dopamine receptors robustly reduces body and BAT temperature in male Sprague-Dawley rats (252).

At the same time, while C21 seems to be a more suitable ligand to use in order to activate DREADD receptors, compared to CNO; due to the fact that the DREADD system is based on a

modified endogenous receptor and is dependent on the use of pharmacological compounds, it is doubtful that a synthetic ligand can be created that is completely devoid of any off-target effects (238). Even newly designed ligands such as C21, or JHU37152 and JHU37160, remain structurally similar to the originally used CNO, and due to their recent introduction remain insufficiently characterised (282).

2.4.6 Conclusion

In conclusion, the present study shows that C21 is a more suitable ligand to utilise and activate DREADD receptors compared to CNO, in accordance with the hypothesis. This is due to its lack of physiological off-target effects. However, it would be speculative of us to state that C21 is 'safe' and completely inert based on the results these experiments alone. As such, in agreement with the suggestion of other groups who have investigated the off-target effects of DREADD ligands, it is suggested that *in-vivo* and *in-vitro* characterisation of C21 should include appropriate "model", species and strain dependent controls to account for any nonspecific effects in the studied experimental conditions (238). This is vital to the robust and reliable use of DREADDs, however, even in studies administering CNO, very few have previously used control animals that do not express DREADD receptors. Further, at present a wide variety of agonist doses (ranging from 0.2 – 10 mg/kg) are applied, with limited justification as to why the dose was selected. Therefore, it is also suggested that the dose of ligand must also be carefully considered and the lowest effectual dose, which does not result in off-target effects on the studied parameters, should be used.

Whilst, based on the results of this chapter, it could not be conclusively stated that activation of dopaminergic VTA neurons attenuates emotional hyperthermia, it can be confirmed that VTA dopamine neurons are involved in thermoregulation. Further, the DREADD system was validated for use in physiological integrative studies, with C21 being identified as the preferable ligand of use, due to the lack of physiological off-target effects. For this reason, C21 will be used for the rest of this study.

CHAPTER 3: CONTRIBUTION OF FOOD CONSUMPTION AND THE DOPAMINE D1 RECEPTOR IN VTA DOPAMINE NEURON ACTIVATION INDUCED HYPERTHERMIA

3.1 INTRODUCTION

In the previous chapter we showed that VTA dopamine neuron activation resulted in a significant temperature increase, accompanied by a notable change in feeding behaviour.

It is well known that body temperature increases during food intake (284-290). Since as early as 1918, when Benedict and Carpenter proved that heat production sharply increased following a meal (291), numerous studies have reported similar body temperature elevation and have attributed this to an increase in resting metabolic rate (255, 292). However, since Rubner (1902) first expressed the hypothesis that animals eat to keep warm, food consumption and thermoregulation has primarily been studied in the context of investigating the amount of feeding done at various ambient temperatures (293). Critically, in addition to ambient temperature (294), hedonic processes that are associated with the taste of food, along with peripheral satiety also affect food consumption; this is particularly related to dopamine (295-297). Increased dopamine signalling has been associated with amplified motivation for food and overall intake. In concurrence, dopamine deficient mice often fail to eat (270). However, there has been discrepancy regarding the exact actions of dopamine in feeding behaviour, as studies utilizing dopamine reuptake inhibitors have found that this results in reduced appetite. Similarly psychostimulants, such as amphetamine, which enhance dopamine signalling, are also known to reduce appetite and food intake (271, 274).

Regardless of experimental results, it is recognised that the D_1 receptor is the primary dopamine receptor involved in food consumption regulation (298, 299). Further, it has been established that D_1 like receptors are involved in the regulation of gastric acid secretion in the rat (300). Indeed, laboratory observations also strongly implicate the dopamine D_1 receptor in mechanisms of motivation and reward (301).

It is important to note that a few studies have also implicated the D_1 receptor in temperature regulation. There has been demonstration of D_1 involvement in the rise of body temperature in hypothermic reserpinized animals, and the selective D_1 agonist SK&F38393 is also known to induce a significant rise in body temperature, up to 1°C (302). However, the research that has looked at

the D_1 receptor for its role in thermoregulation has been limited. Additionally the majority of the studies took place in the early 1990s, with very few studies conducted after. Therefore, the exact role of this dopamine receptor in temperature regulation warrants further investigation.

It is interesting to note that although dopamine is most commonly associated with hypothermia, this is a result of studies investigating the D_2 dopamine receptor. As such, it has been suggested that pharmacologically different dopamine receptors mediate different effects on body temperature.

There are five types of dopamine receptor, D_1 to D_5, all of which are metabotropic G protein-coupled receptors (303). These receptors can be further grouped into two subtypes - D_1 subtype, which includes the D_1 and D_5 receptor; and the D_2 subtype consisting of the D_2, D_3, and D_4 receptors (103, 304). As shown in Figure 22, the D_1 subtype exerts excitatory actions on the host neuron through coupling with the stimulatory Gs protein and increasing the activity of adenylyl cyclase (AC), a membrane-bound protein that catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP) (119, 305). The cAMP is itself an intracellular signalling molecule, which as part of its role, activates protein kinase A which releases intracellular calcium and opens calcium channels in the cell (103). Conversely, the D_2 subtype is inhibitory through coupling with the Gi protein and decreasing AC activity (103, 119).

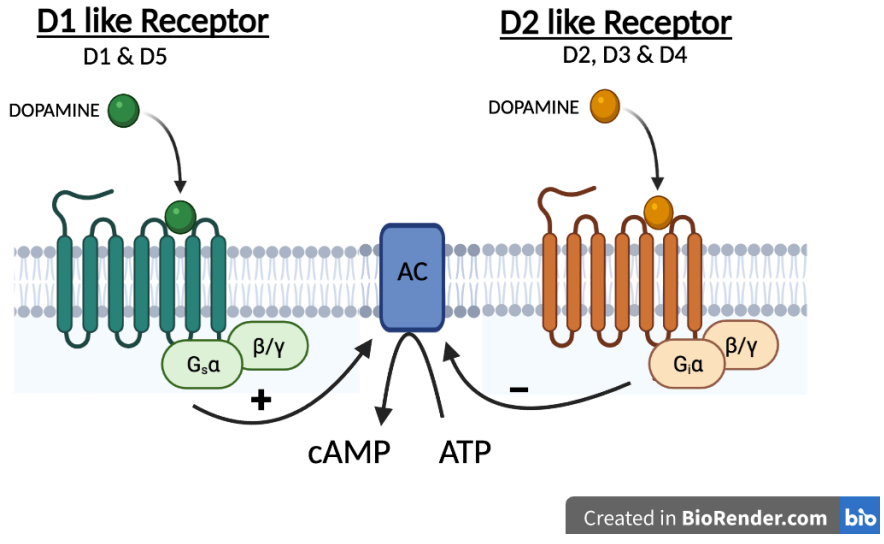


Figure 22: Schematic representation of D_1-like and D_2-like receptor function, highlighting Gs and Gi coupling and the respective impact on adenylyl cyclase (AC) activity. Modified from Bao (2021) (466)

Dopaminergic neurons in the VTA are known to contain high concentrations of D_2 and D_5 receptors, but poor levels of the D_3 receptor. However, the D_1 and D_4 receptors are indistinguishable on the neurons; therefore, their presence is difficult to ascertain (306). Importantly, D_1 receptors are also present on the glutamatergic terminals projecting to the VTA (307, 308) and the highest amount of D_1 receptors is located within in the nucleus accumbens, the primary projection site of VTA dopamine neurons (309) .

The aim of the experiments, described in this chapter, was to determine whether the temperature increases seen following VTA dopamine neuron activation were a result of the stimulation itself, or a by-product of altered feeding patterns – that is, occurring as a result of increased BAT thermogenesis prior to food consumption, as described by Blessing (11) and post-prandial heat generation. There was also an additional aim to determine which dopamine receptor type was responsible for the observed temperature elevation. It was hypothesised that the changed feeding behaviour, at least to some extent, contributed to the rise in BAT and body temperature. Further, due to its known impact on feeding behaviour and demonstrated involvement in thermoregulation, it was hypothesised that the dopamine D_1 receptor of the VTA was responsible for the unexpected results observed in the previous chapter.

In the past the D_1 receptor has been hard to study as the only antagonists available were also D_2 receptor antagonists. However, the halobenzazepine R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH-23390), a highly potent and selective dopamine D_1-like receptor antagonist, now makes the study of this receptor possible (310).

Therefore, in order to conduct the investigation, a food-removal study was utilised - in which the food hopper was removed for 6.5 hours following DREADD agonist induced VTA dopamine neuron activation. A secondary study was also performed, in which hM3Dq expressing rats were pre-treated with the D_1 antagonist SCH-23390 prior to DREADD agonist administration.

3.2 METHODS

All experimental animals were bilaterally injected with AAV-hSyn FLEX hM3Dq mCherry in the VTA, using the procedures described previously in Section 2.2.1.

In order to allow for sufficient DREADD expression, there was a minimum of two weeks between viral vector administration and temperature probe implantation (as described in Section 2.2.2). Following probe implantation, a one-week recovery period was given prior to experimental testing.

Set-up for physiological recordings was performed as described in Section 2.2.3.

At the conclusion of the experimental period for each animal they were humanely euthanized with pentobarbitone sodium (180 mg/g i.p), transcardially perfused (see Section 2.2.5) and DREADD expression was visually confirmed using the EVOS FL Fluorescent microscope.

3.2.1 Food Restriction Testing

Prior to testing, a 2 mg/kg dose of C21 was weighed out on a microbalance and dissolved in 490 µL water for injection with 10 µL DMSO, to make up a total 0.5mL volume for injection (Section 2.2.4).

For the control, 2 mg/kg C21 i.p was administered and the experimental rat was given *ad libitum* access to food. Physiological parameters including BAT and body temperature, locomotor activity and feeding behaviour were recorded.

For food restricting testing, the food hopper was removed from the 'home cage' at the same time as the 2 mg/kg C21 injection was given. The experimental rat was food restricted for 6.5 hours, after which the food hopper was returned and *ad libitum* access resumed. Duration of the food restriction testing was decided upon based on the observations made and described in Chapter 2 - consistent with the length of DREADD agonist action. BAT and body temperature, and locomotor activity were recorded throughout the food restriction period.

3.2.2 Drug Administration

In the second stage of testing, 1 mg/kg R(+)-SCH-23390 hydrochloride (Sigma-Aldrich, St. Louis, USA), was weighed out on a microbalance and dissolved in 0.5 mL Ringer's Solution. Ringer's Solution (0.5 mL) was used as the control for this set of experiments. The dose of SCH-23390 was obtained from pilot studies and previous reports (311, 312).

Drugs were administered intraperitoneally, as described in Section 2.2.3, during the dark phase when BAT and body temperature were close to baseline level for each individual animal.

Fifteen minutes following 1 mg/kg SCH-23390 or vehicle administration, 2 mg/kg C21 (see Section 3.2.1) was administered. Physiological parameters were recorded, according to the description provided in Section 2.2.3.

Single injections of either vehicle (0.5 mL Ringer's Solution) or 1 mg/kg SCH-23390 were also given to each experimental animal in order to observe any effects on resting baseline levels.

There were three days between each experimental treatment, and all treatments were given in a counterbalanced order.

3.2.3 Data Recording and Analysis

All files from PowerLab were imported into IgorPro for graphing and analysis (see Section 2.2.8). Any occasional artifacts were removed from traces by replacing with values at surrounding time points just before or after the artifact.

Prism 9 was used to visualise group data, which was calculated as mean ± SEM for each experimental condition. Baseline temperature values were calculated from the mean of the 5 minute period prior to initial drug administration. Statistical analyses of the data were performed in SPSS.

For food restriction analysis BAT and body temperatures were examined for 6.5 hours following C21 administration at 30-minute intervals. Behavioural activity levels were examined for the periods of 55-65 minutes and 235-245 minutes following C21 injection.

For the 1 mg/kg SCH-23390 pre-treatment set of experiments, delta BAT and body temperature increases from baseline pre- initial injection levels, and behavioural activity levels were compared at 55-65 minutes following C21 administration.

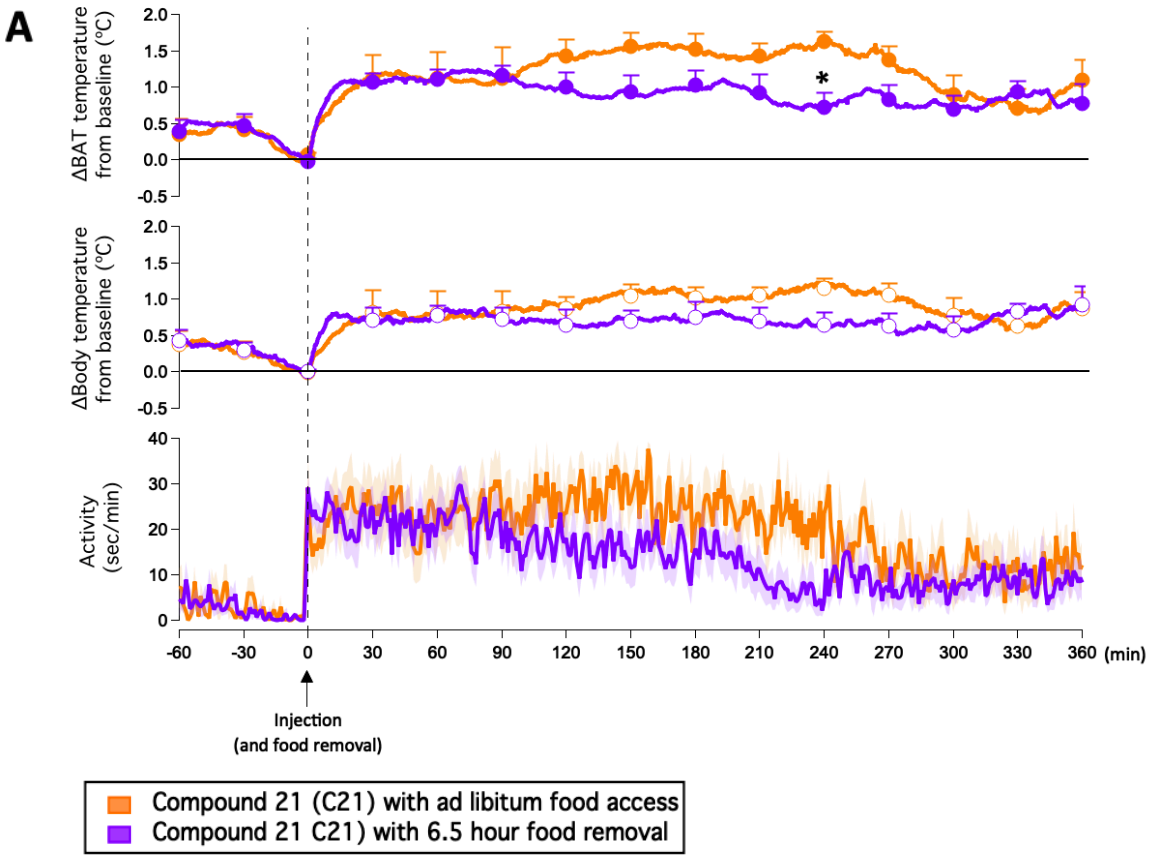
Similarly, in order to examine differences between individual 1 mg/kg SCH-23390 or vehicle injections, delta BAT and body temperature increases from pre-injection baseline level were compared to temperatures 55-65 minutes post-injection. Activity levels at 0-20 and 55-65 minutes following injection were also assessed. Feeding behaviour was also examined, with the following variables considered for a 3-hour period following injection: number of meals, average duration of

meals, the amount consumed per meal and the total amount consumed during the 3-hour period. This time period was chosen based on previous studies (313).

Treatment effects on BAT, body temperature and activity levels were examined using an independent *t*-test. Feeding microstructure was assessed using the Welch Test – this test was selected as the results violated homogeneity. The level for statistical significance was set at $P < 0.05$ for all analyses.

3.3 RESULTS

3.3.1 Effect of food restriction, following VTA dopamine neuron activation, on thermoregulatory variables



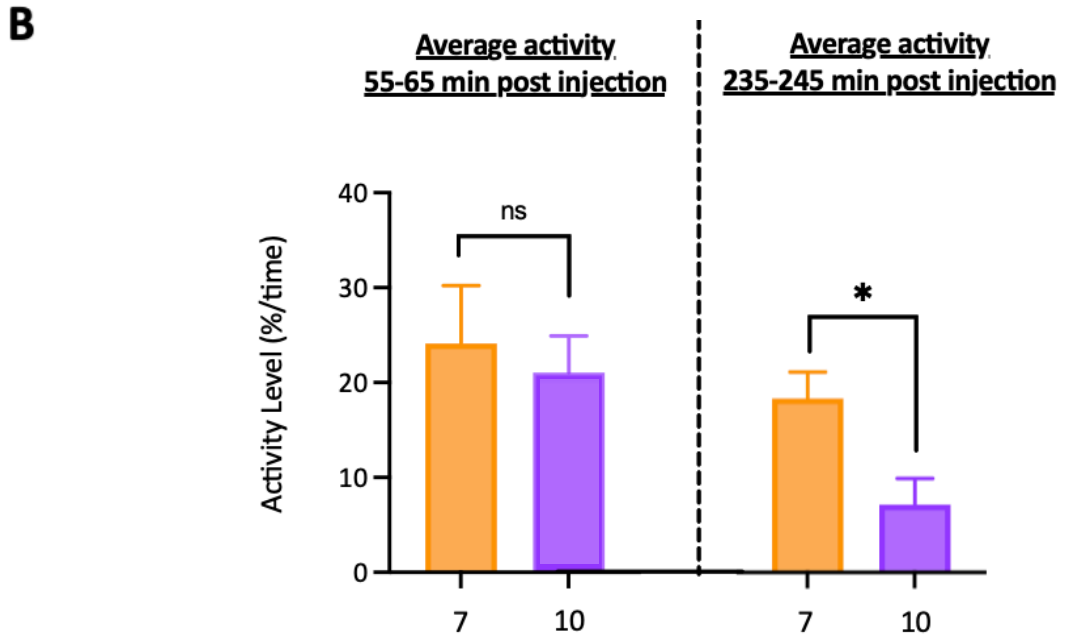


Figure 23: A Effect of food removal, following 2 mg/kg C21 administration, on thermoregulatory variables including BAT and body temperature, and locomotor activity. Drug administration is indicated at time 0. Food was removed at the same time as drug administration. **B** Bar graphs showing group results (mean ± SEM) of the effects of 2 mg/kg C21 administration in *ad libitum* or food restricted conditions for activity levels at 55-65 min and 235-245 min post-injection. *ns* no significant difference compared to *ad libitum* food access, asterisk indicates significant difference compared to *ad libitum* food access * $P < 0.05$. Numbers below columns indicate number of animals in each condition.

Following food removal after 2 mg/kg C21 administration, as can be seen in Figure 23 (A & B), there was minimal change to BAT and body temperatures and activity levels when compared to rats with *ad libitum* food access.

Indeed, when the temperatures were examined, only one point for BAT temperature was found to be significantly different. This time point was 240 minutes (4 hours) following C21 administration, ($t(15)=-3.436$, $P < 0.05$). Similarly, around this time point there was a significant drop in activity level for the food-restricted rat, $t(15) = -2.758$, $P < 0.05$.

As can also be seen in Figure 22 (A), soon after the 4-hour post-injection time point there was a rapid drop in both BAT and body temperature, and activity level in rats that received *ad libitum* food access. This, taken into account with the time point at which the food-restricted temperature and activity levels fell, suggests that food consumption is not required for the formation of temperature increases seen following 2 mg/kg C21 administration. But it is possible that food-consumption assists in, and is required for, the prolonged action of C21 and for temperatures to be sustained at an elevated level for extended periods.

3.3.2 Effect of dopamine D_1 receptor antagonist pre-treatment on thermoregulatory increases elicited by C21 activation of VTA dopamine neurons

The results demonstrate that food consumption is not a contributing factor in the temperature increases caused by C21 administration elicited VTA dopamine neuron activation. It was, therefore, of interest to determine exactly what the regulating factor responsible for the increases seen in the previous study was. Since activation of the dopamine D_1 receptor promotes excitatory responses in the brain, we examined the effects of D_1 antagonist pre-treatment prior to C21 administration.

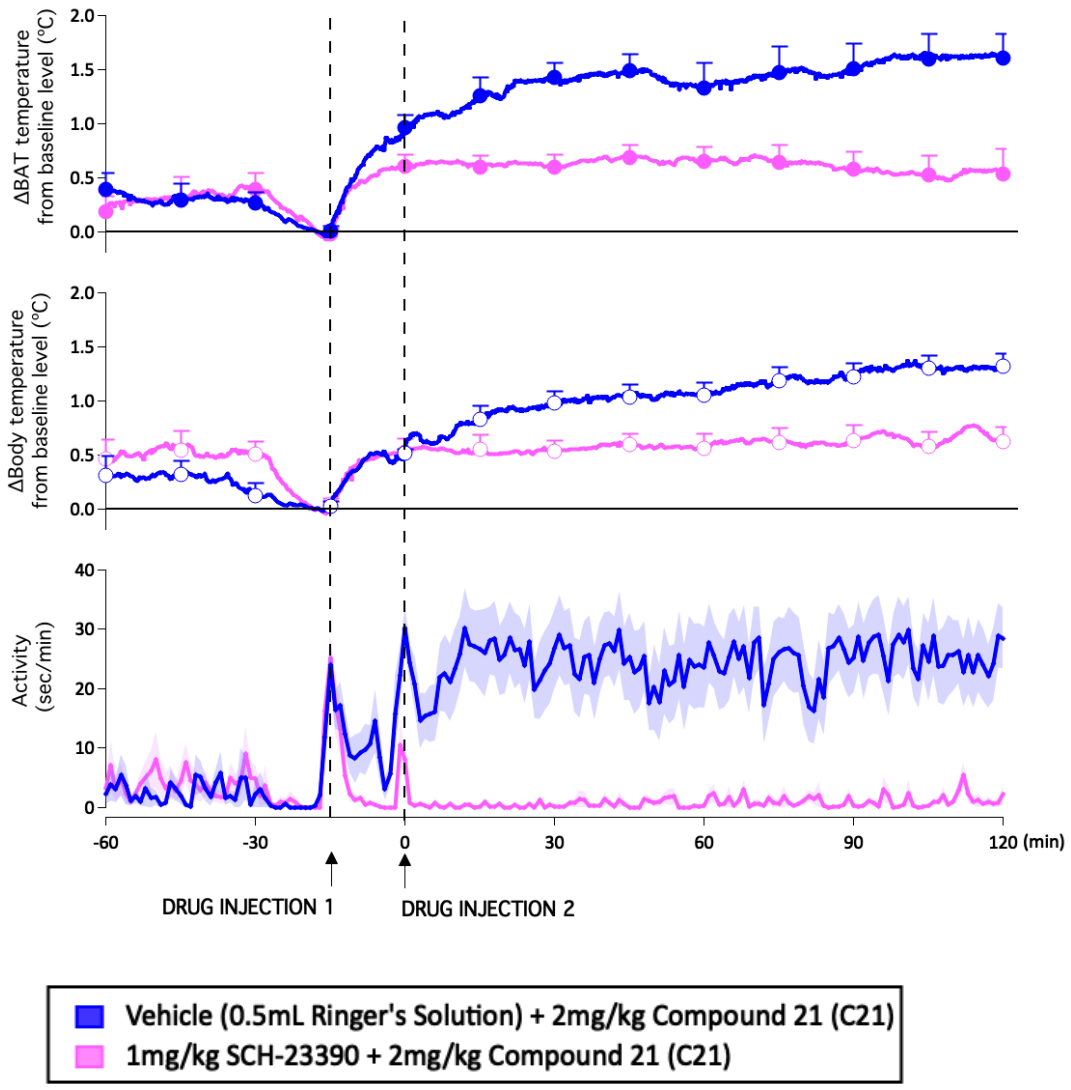


Figure 24: Averaged experimental records (mean ± SEM) following 1 mg/kg SCH-23390 or vehicle pre-treatment administration, followed by 2 mg/kg C21 treatment. BAT and body temperatures, and behavioural activity level are shown. Pre-treatment administration was made 15 minutes (time -15) prior to 2 mg/kg C21 administration (time 0).

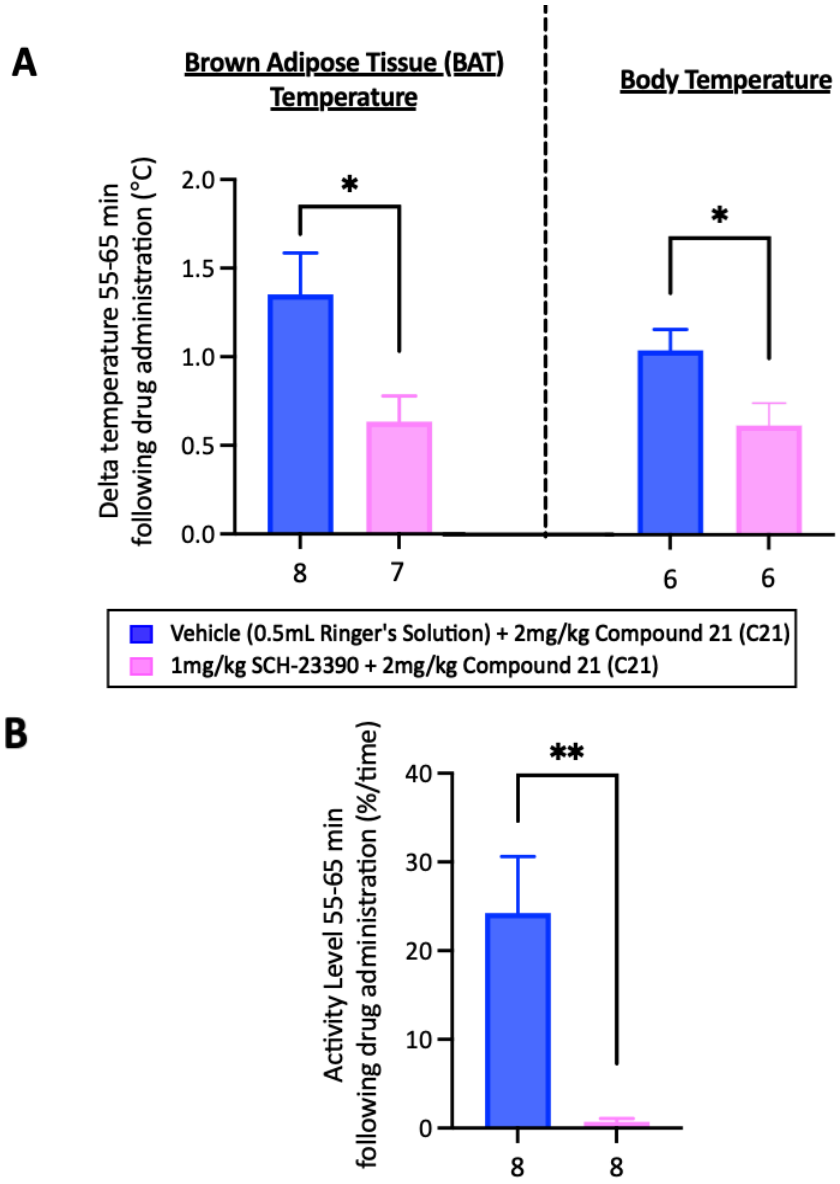


Figure 25: Bar graphs showing group results (mean ± SEM) of vehicle or 1 mg/kg SCH-23390 pre-treatment on C21 administration elicited increases in thermoregulatory variables including (**A)** BAT and body temperature and (**B)** activity level 55-65min post-injection, Asterisk indicates significant different from vehicle pre-treatment * $P < 0.05$, ** $P < 0.01$. Numbers below columns indicate number of animals in each condition.

Indeed, pre-treatment with 1 mg/kg SCH-23390 significantly reduced the temperature increases seen following VTA dopamine neuron activation elicited by C21 administration, as can be seen in Figures 23 & 24. Both BAT ($t(13)=-2.515$, $P < 0.05$) and body temperature ($t(10) = -2.476$ $P < 0.05$) were substantially lower following SCH-23390 treatment prior to C21 administration.

Pre-treatment with SCH-23390 similarly eliminated all increases in activity level associated with C21 administration ($t(7.042) = -3.62$, $P < 0.01$), as shown in Figure 25B, as well as changes in feeding behaviour, demonstrated in Figure 26.

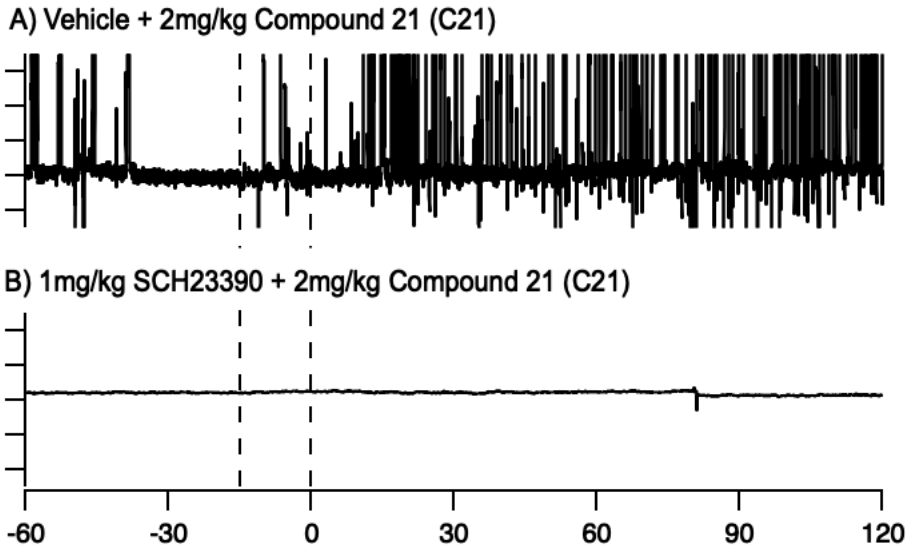


Figure 26: Representative traces from an individual rat showing interactions with the food hopper following vehicle (A), 1 mg/kg SCH-23390 pre-treatment, followed by C21 (2 mg/kg i.p) administration. The drug injections were made at time -15 (pre-treatment) and time 0 (C21). Weight of the food hopper is not indicated, but axes for both treatments are comparable. An artificial 'upper' weight limit was placed for the C21 trace.

However, some elevation of temperature remained with both BAT ($t(6) = -5.00$, $P < 0.05$) and body ($t(5) = -5.157$, $P < 0.05$) being higher than pre-treatment levels. This may be due to activation of other dopamine receptors, such as D_5, or other receptors in general, and should be investigated further. Nevertheless, based on the results of the experiment, it can be concluded that the D_1 receptor is the primary contributor to the increases in temperature observed during the conducted investigation.

3.3.3 Effect of dopamine D_1 receptor antagonist administration on baseline thermoregulatory variables

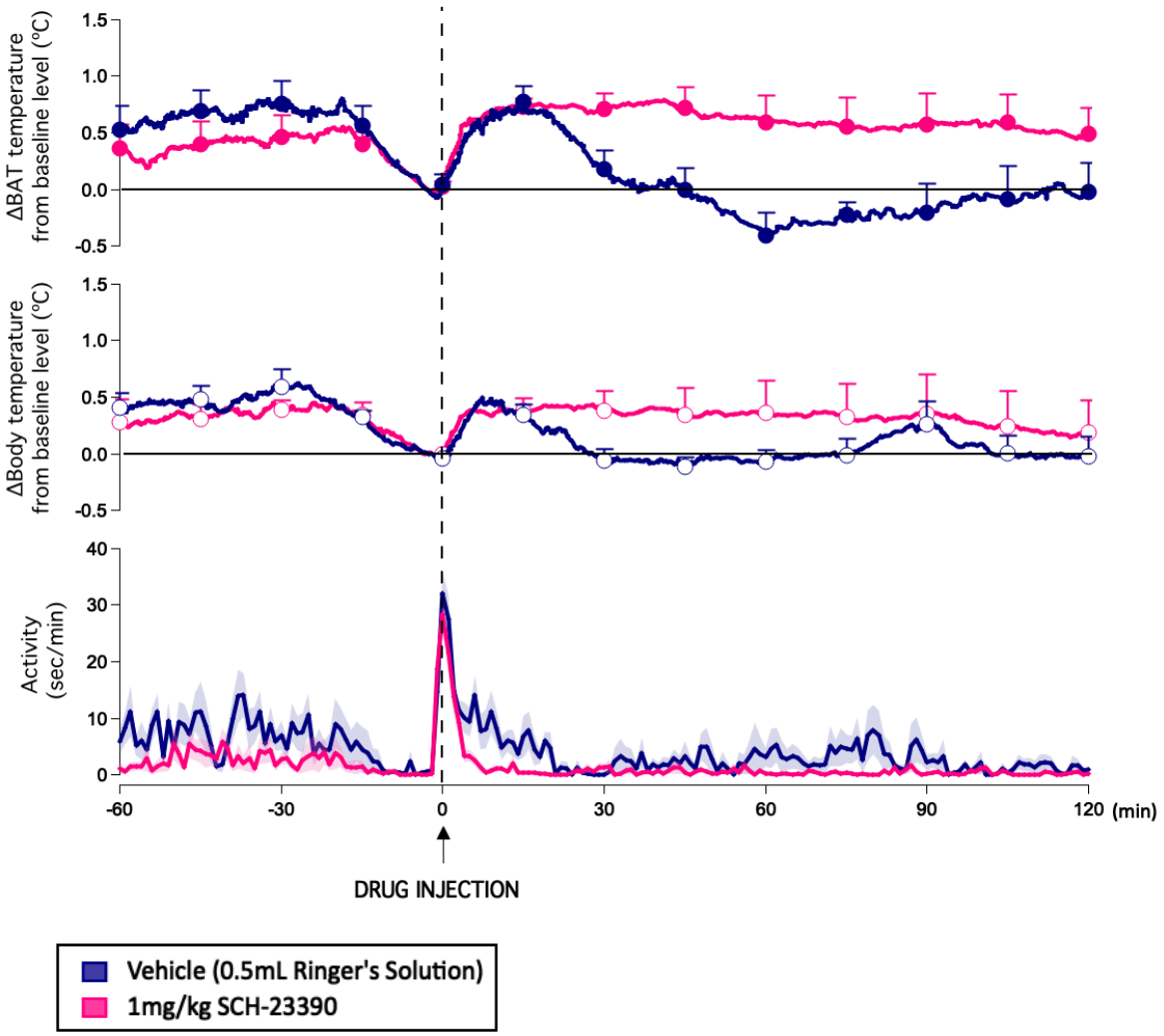


Figure 27: Averaged experimental records (mean ± SEM) following 1 mg/kg SCH-23390 or vehicle administration showing BAT and body temperatures, and behavioural activity level. Drug administration was made at time 0.

The effects of 1 mg/kg SCH-23390 administration on baseline thermoregulatory variables are shown in Figures 27 & 28.

One hour following drug administration there was a significant increase in BAT temperature, compared to vehicle treatment, $t(11) = 3.116$, $P < 0.05$. The elevated temperature was also greater than pre-injection level ($t(6) = -2.534$, $P < 0.05$). However, there was no significant difference seen in regards to body temperature, $t(6.307) = -1.434$, $P > 0.05$. The administration of Ringer's Solution did not affect baseline BAT or body temperatures ($P > 0.05$).

Similarly, there was no significant changes in activity level following vehicle and SCH-23390 injection, for both 0-20 minutes after drug administration $t(7.638)=3.568$, $P > 0.05$, as well as for 55-65 min - $t(6.119)=1.462$, $P > 0.05$.

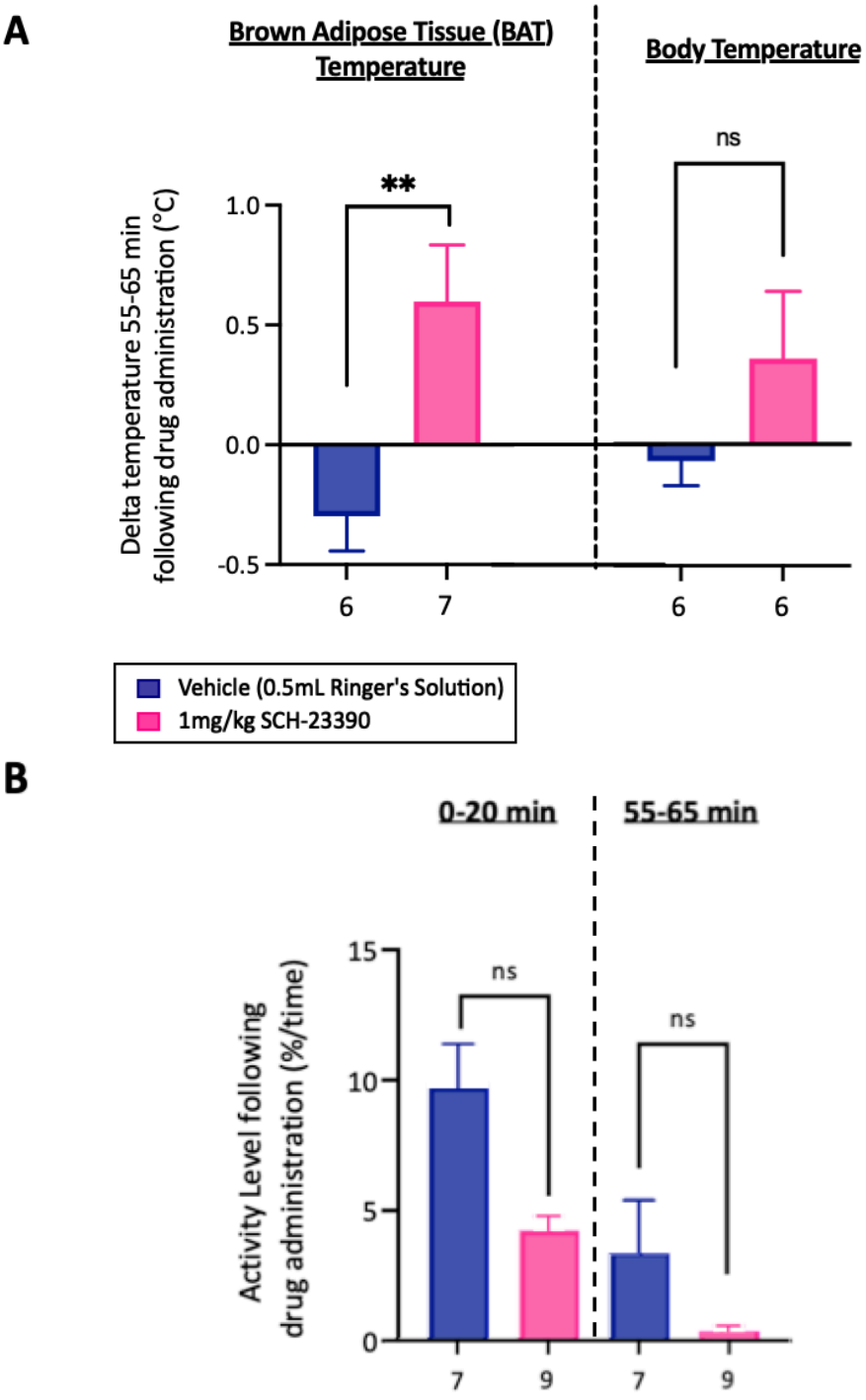


Figure 28: Bar graphs showing group results (mean ± SEM) of vehicle or 1 mg/kg SCH-23390 administration and their effect on thermoregulatory variables including **(A)** BAT and body temperature and **(B)** activity level 0-20min and 55-65min post-injection, Asterisk indicates significant different from vehicle treatment ** $P < 0.01$. ns indicated no significant difference from vehicle treatment. Numbers below columns indicate number of animals in each condition.

In Figure 26, there is an increase in BAT temperature that can be seen immediately following SCH-23390 as well as vehicle injection. For the vehicle injection this temperature increase is likely due to the administration of the injection itself – as no catheters were used for these experiments, it is likely that the manual handling of the rats resulted in stress-induced reaction.

Given that the D_1 receptor is known to be involved in the regulation of feeding behaviour, feeding microstructure was also examined following SCH-23390 and vehicle administration. This is summarised in Table 1 and Table 2.

Table 1: Results of individual vehicle treated rats showing number of meals, average duration of each meal (min:sec), average amount consumed per meal (g) and total amount consumed (g) in a 3 hour period following drug administration

Rat number (arbitrary)	Number of meals	Average duration of meal (min:sec)	Average amount consumed per meal (g)	Total amount consumed (g)
1	5	8:08	0.86	4.73
2	5	1:58	1.20	5.35
3	0	0	0	0
4	0	0	0	0
5	2	15:31	1.83	3.76
6	1	2:06	1.23	1.23

Table 2: Results of individual 1 mg/kg SCH-23390 treated rats showing number of meals, average duration of each meal (min:sec), average amount consumed per meal (g) and total amount consumed (g) in a 3 hour period following drug administration

Rat number (arbitrary)	Number of meals	Average duration of meal (min:sec)	Average amount consumed per meal (g)	Total amount consumed (g)
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0

It should be noted that the Welch Test cannot be performed with 0 values, as such the values for SCH-23390 treated animals were adjusted to 0.0001 for statistical analysis purposes.

The number of meals ($F(1,12)=7.197$, $P < 0.05$), average duration of meal ($F(1,12)=4.938$ $P < 0.05$), average amount consumed per meal ($F(1,12)=11.208$, $P < 0.05$) and total amount consumed ($F(1,12)=9.013$, $P < 0.05$) for the 3 hour period following drug administration were all found to be significantly different from the control group. It should be noted there was large variation in the data for vehicle treated, control, animals and in future a larger sample size should be examined.

3.4 DISCUSSION

3.4.1 Contribution of food consumption to temperature elevation

Surprisingly, the removal of food following VTA dopamine neuron activation did not have an effect on the temperature increase seen with *ad libitum* food access. Indeed, there was only one time point, 4 hours following DREADD agonist administration, at which BAT temperature differed significantly between the food deprived rats and the rats with free food access.

It is possible that this difference was due to poor thermistor probe placement in the BAT tissue of rats; however, this is unlikely to be the case as probe location was confirmed for each animal following the experimental period. The relationship between BAT thermogenesis and food ingestion has previously been studied by Blessing and colleagues (2012), who demonstrated that when *ad libitum* food is available, BAT temperature increases approximately 15 minutes prior to eating as part of the BRAC cycle (11). Heat production continues until the end of the meal. Importantly, their study also showed that the physiological changes associated with eating continue in the absence of food. As such, it is potential that the stimulation of dopaminergic VTA neurons still initiated the desire to eat, and thus, the physiological processes associated with it (temperature increases), even when no food was available. To the best of my knowledge, the present study is the first to suggest this possibility.

At the same time, four hours following dopamine neuron activation, there was also a fall in activity level for the food-restricted rats. It should be noted that based on this study alone, it is difficult to rule out the contribution of increased muscle activity to elevated temperature. There is potential that the temperature increases simply reflect the rats' search for food. As can be seen from the results, the animal was active both with and without food, and the drop in activity level also corresponds to the drop in BAT and body temperature - thus, it is possible that the rat 'gave-up' searching for food. However, if this was the case and muscle activity was the primary contributor, it would be expected that there would be a greater difference in the temperatures of food-provided and food-restricted animals. Likewise, as can be seen from the results, even the rats that received *ad libitum* food experienced a fall in activity soon after their food-restricted counterparts. Based on these observations, it is unlikely the temperature elevation observed was solely a result of increased muscle activity. Thus, whilst the hypothesis was not supported, in that food consumption was not necessary for the development of an elevated temperature following VTA dopamine neuron activation; it does appear, however, that feeding is necessary to maintain the increase for a prolonged period of time.

3.4.2 Contribution of dopamine D_1 receptor to temperature elevation

Pre-treatment with 1 mg/kg SCH-23390, prior to 2 mg/kg C21 administration, significantly decreased the amplitude of temperature increase. This complies with previous reports that observed SCH-23390 pre-treatment block MDMA-induced hyperthermia (314, 315). Whilst commonly associated with the serotonin system, MDMA is also known to increase the release of dopamine (316). Similarly, Beauvais et al (2011) and Broening et al (2005) confirmed SCH-23390 blockade of d-methamphetamine induced hyperthermia in a thermoneutral (22°C and 24°C respectively) environment (317, 318). Therefore, based on the present findings, the hypothesis is supported and it appears the D_1 dopamine receptor is involved in regulating the hyperthermic response seen following VTA dopamine neuron activation.

The current results also showed that blockade of the dopamine D_1 receptor fully abolished any increases in locomotor activity induced by the stimulation of dopamine neurons. This finding is in agreement with other investigators who confirmed that SCH-23390 pre-treatment, at half the dose used here, blocked the development of locomotor sensitization evoked by dopamine targeting psychostimulants amphetamine and cocaine (319-322). Likewise, the erratic feeding behaviour was attenuated in a similar manner. Indeed, SCH-23390 has been observed to attenuate hyperphagia induced by Δ^{9}-tetrahydrocannabinol (THC) (323), which is known to interact with both cannabinoid and dopamine systems. Diazepam induced feeding and sucrose consumption have also been shown to be reduced following SCH-23390 administration (324, 325). Thus, the reduced eating observed in the conducted experiments is unsurprising.

It can be seen that following the initial administration of SCH-23390 or vehicle, there was an initial spike in both BAT and body temperature. This can be attributed to the rats' reaction to the drug injection itself. However, it is interesting that even for D_1 antagonist treated rats, this elevated temperature remained for a minimum of a further 2 hours. In order to test whether this was caused by the SCH-23390 itself, it was separately administered to rats with no following DREADD agonist introduction.

3.4.3 Effect of SCH-23390 on baseline thermoregulatory variables

Interestingly, injection of 1 mg/kg SCH-23390 on its own was found to cause a significant rise in BAT temperature. Faunt & Crocker (1987) did previously demonstrate that SCH-23390 produced a slight, but biphasic, hyperthermic reaction in the rat (174). However, other investigators, such as Salmi and colleagues (1993), produced results that disagree with this outcome and failed to note

any effect on body temperature (326). It should be noted that the present study also failed to report any significant change in body temperature following SCH-23390 treatment. The discrepancy in results could be accounted for by differences in body temperature measurement – whilst this study utilised chronically implanted thermosensors in specific locations, allowing the discrete measurement of BAT and body temperature separately, both Faunt & Crocker and Salmi recorded temperature via a rectally inserted probe.

It was also found in the current study that locomotor activity was unaffected by SCH-23390 treatment. This is in conflict to Hoffman's report (1985), which in fact noted a significant decrease in activity and rearing behaviour following SCH-23390 administration (327). In Hoffman's study, however, this effect was noted to be greatest in the first half-hour following administration. This time point was not examined in the present investigation. The effects of emotional hyperthermia, elicited via injection of the drug itself, can remain for up to 20 minutes post-administration, as such, a longer time period for comparison was initially selected here. However, after examination of the experimental graph the 0-20 minute time frame was also examined to determine if there was a significant decrease in activity shortly after drug administration. This analysis also supported the conclusion that locomotor activity was unaffected. It should further be noted that the rats in Hoffman's study received only a 60-minute pre-exposure to the activity chambers prior to testing. Compared to the 24-hour habituation period used in the present procedure, this is a relatively short habituation period. It is, therefore, possible that the SCH-23390 was decreasing an already hyperactive condition. Nevertheless, SCH-23390 should be further characterized and its behavioural function examined in future studies.

It is also important to note that in the present investigation SCH-23390 was administered systemically. In addition to the dopamine D_1 receptor, SCH-23390 also has considerable affinity for 5-HT 2A/C receptors (328-330), and has also been demonstrated to antagonise PD 128907 (a D_2/D_3 receptor agonist) induced hypothermia (331). Therefore, its action on these receptors cannot be fully ruled out.

3.4.4 Potential D_1 and D_2 dopamine receptor interaction

Although early studies failed to find D_1 receptor mediated thermoregulatory effects; without doubt, it can now be concluded that the D_1 receptor is involved in thermoregulation. Balthazar and associates found that blockade of D_1 receptors impaired dissipation of exercise-induced heat in rats (352). Previously, several groups have also reported that activation of this receptor has

modest hypothermic effects. Salmi (1998) previously reported that the D_1 receptor agonist A68930 induced hypothermia (332). However, these results should be carefully considered, indeed Clifford and colleagues (1999) studied D_1 knock-out mice and found that there were no significant behaviour effects following A68930 administration (333). This, therefore, suggests it may act on other receptors in addition to D_1. Indeed, it should be noted that A68930 also has some affinity for the D_2 receptor (334-336). The majority of studies that have investigated D_1 agonists have found either no change or hyperthermic effects on temperature. In support of the present hypothesis and the results presented here Zarrindast & Tabatabai demonstrated that the administration of the D_1 agonist SKF38393 robustly increases body temperature (337). Nunes (1991) similarly showed that a high, intraperitoneal dose of SKF38393 also reduced hypothermia (338). Interestingly, in the same study, Nunes also suggested that the D_1 and D_2 receptor may potentially function in a cooperative manner (338). This receptor cooperation much be carefully considered.

Waddington (1989, 1995) (339, 340) and White (1997) (341) previously described cooperative interaction between the D_1 and D_2 receptors in the regulation of ventral striatum activity in regards psychomotor behaviour. Indeed, there is additional evidence in the literature which suggests synergistic activation of both the D_1 and D_2 receptor are required for the induction of a variety of responses in rodents (331, 342-349). Particularly regarding thermoregulation, Chaperon (2003) and colleagues have also suggested potential cooperative interaction (331). In the analysis of their results Nunes and associates (1991) suggested that the D_1 receptor may have an indirect modulatory effect on D_2 receptors, which may also vary based on route of drug administration (338). These previous results seem to indicate that the $D_1$and D_2 receptors act together to reduce thermogenesis.

As research techniques have advanced, there has now been identification of a D_1-D_2 receptor heteromer in brain tissue (350, 351). It is likely that the results observed by Chaperon and Nunes were a result of dopamine interactions with this heteromer. One critical aspect of the heteromer function is that it is separated by dopamine or receptor-selective agonists (350). Nunes noted in his study that intracerebroventricularly administered SKF38393 potentiated quinpirole induced hypothermia. Taking the D_1-D_2 heteromer into account, these results can be explained as the initial administration of SKF38393 would separate the heteromer, leaving quinpirole to the act on the individual D_2 receptor, giving a hypothermic result. A similar approach can be taken to account for the results of DREADD agonist administration presented in this thesis. It is possible that once the

DREADD receptors were activated and dopamine release was increased, the heteromer quickly separated into its two components. Following this, during the prolonged action of the DREADD agonists, the dopamine would have been acting on the D_1 and D_2 receptors individually. Given the fact that D_1 receptors are difficult to quantify, it may be the case that they outnumber the D_2 receptors in the specifically affected area, thus resulting in temperature elevation.

As expected, there was also a significant difference between vehicle and D_1 antagonist treated animals in relation to food consumption. SCH-23390 administration has previously been shown to cause hypophagia, at a much smaller dose that what was used here (30 µg/kg) (355).

3.4.5 Conclusion

In summary, based on the findings of this chapter it can be concluded that the dopamine D_1 receptor, is involved in thermoregulation – but not through modification of normal feeding patterns.

Although SCH-23390 administration on its own failed to affect locomotor activity, it substantially reduced activity induced by DREADD agonist administration. Since the hyperthermic effect of DREADD activation was substantially reduced – a response not seen during food depravation, it is possible that the DREADD-driven increase in BAT and body temperature is a result of increased activity. Indeed many studies have shown that strenuous exercise can raise core body temperature by a few degrees (356-359). The results presented here show that the D_1 antagonist, SCH-23390, blocked activity – thus, reducing the DREADD-driven hyperthermic response; whilst at the same time increasing BAT thermogenesis, and effect that appears central and accounts for only a partial and not complete reduction in hyperthermia. Based on this finding it may be the case that D_1 involvement in thermoregulation in indirect and is rather due to it's mediation of activity. This possibility is difficult to assess using the conscious animal experimental model employed in this study. It is suggested that to aid in the further characterisation of SCH-23390, it should be administered to anesthetized animals in order to accurately observe and record potential thermoregulatory effects.

CHAPTER 4: THE ROLE OF THE MESOLIMBIC DOPAMINE SYSTEM IN THERMOREGULATION AND THE AUTONOMIC STRESS RESPONSE

4.1 INTRODUCTION

As mentioned briefly in Section 1.4.1, the dopamine synthesising neurons of the VTA and their rostrally projecting axons to the NAc constitute "the mesolimbic dopamine reward circuit" (151, 360-362). It should be noted that the NAc is made up of a core and shell region with afferent projections from the VTA extensively innervating the shell, as shown in Figure 29. Dopaminergic neurons in the lateral posterior regions of the VTA project to the lateral shell of the NAc, whilst more medial neurons of the VTA project to the medial shell and core (363). The shell contains a large number of dopamine receptors, however, the core has been shown to have more dopamine utilization and greater numbers of dopamine transporters (364). Importantly, the majority of neurons in the NAc are medium spiny neurons bearing the dopamine D_1 and D_2 receptors (309).

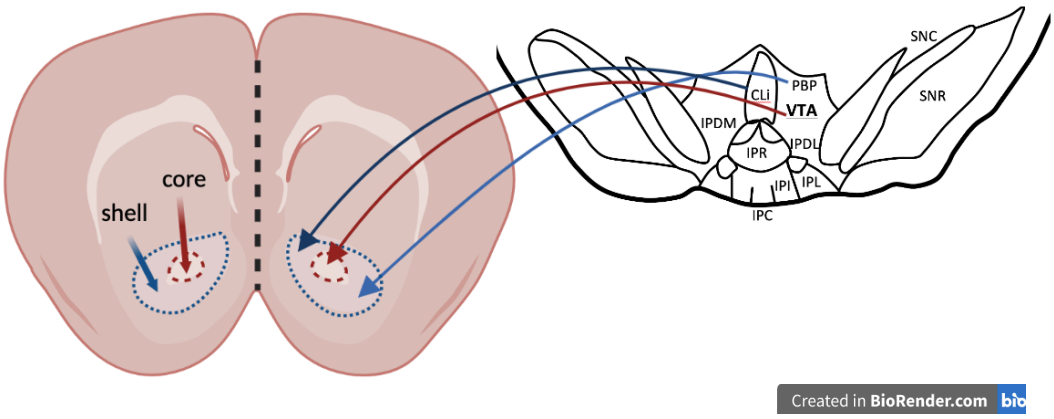


Figure 29: Representation of some afferent projections from VTA to shell and core regions of the nucleus accumbens. Not to scale. Modified from Baik (2020) (363)

The mesolimbic pathway is a key component in CNS regulation of emotional processing. Indeed, fast-scan cyclic voltammetry has shown that negative stimuli inhibit dopamine release in the NAc shell and enhance release in the core (365, 366). Additionally the mesolimbic pathway has also been implicated in voluntary movement, cognition and the reward/reinforcement process that, in particular, responds to natural stimuli and numerous drugs of abuse (104, 105, 367).

Due to its involvement in numerous functions, research into this circuit has substantially increased over recent years; however, several areas of its regulation remain unexplored – in particular, its potential control of thermoregulation and its importance in the emotional hyperthermic response.

One of the major areas of investigation regarding the mesolimbic system has focused on its contribution to locomotion. This has been the result of studies that demonstrated psychostimulant induced hyperactivity was due to activity in the NAc (150-154), as further confirmed by the use of dopamine deficient mice which, under the same circumstances, failed to show similar hyperactivity until dopaminergic signalling was restored in the NAc (368). Boekhoudt and colleagues (2016) further examined the potential involvement of the mesolimbic system in locomotion, using chemogenetics, and confirmed that activation of the pathway resulted in a robust and long-lasting hyperactive phenotype (266). Despite this, other studies that employed optogenetic stimulation of the pathway failed to observe any locomotor increases (156-158). As such, the extent of mesolimbic contribution to locomotor activity remains unclear.

Another area of great interest is the mesolimbic regulation of feeding behaviour. In the same study described in Section 2.4, Boekhoudt and associates also specifically inspected chemogenetic activation of the mesolimbic pathway and the resulting changes in feeding microstructure (275). Similarly to what was observed following exclusive VTA stimulation, mesolimbic activation resulted in smaller meals of short duration that occurred at an increased frequency, with total food intake not being affected. Dramatically, 6-OHDA neurotoxin leisoning of the VTA renders rats indiferent to food, so much so that they neglect to eat (369, 370).

As previously mentioned, both locomotion and feeding behaviour are important modulatory factors of thermoregulation; therefore, given the heavy involvement of the mesolimbic system in these elements, it is possible that the pathway contributes to body temperature regulation.

Further, the results of the previous two chapters, that have demonstrated VTA dopaminergic involvement in thermoregulation, give added support to this theory.

In this chapter, the influence of the mesolimbic system in the process of emotional hyperthermia, and other factors required for normal functioning, were examined using the intruder-stress paradigm and the cold-defence response.

Previous research has already implicated the mesolimbic system in salience processing. Physical stress tests, such as tail pinch, have been shown to increase dopamine content in the NAc (371), and in humans functional MRI studies have indicated high activity in the NAc following noxious thermal stimuli (372, 373). Interestingly, following chronic restraint stress, dopamine D_1 receptor density was also found to be decreased in the NAc (374). Combined studies have shown rapid

increases in VTA dopamine neuron activity following exposure to psychologically stressful stimuli, such as those conducted by Moriya (2018) (375). Baik (2020) and Douma (2020) have suggested that changes in the mesolimbic system are critical for coping with stress, as they allow for adaptation in behavioural responses to environmental stimuli (363, 376). Hence, it is likely that the VTA-NAc connection is also involved in the regulation of psychologically elicited thermoregulatory responses and acts as a link between emotional appraisal and bodily function.

Additionally, almost all animals experience physiological adjustments in response to a wide range of environmental changes, including ambient temperature. Mammals, and other homeothermic animals, maintain their body temperature through regulating heat dissipation and heat generation. Catecholamines are well known to regulate thermogenesis and metabolic rate in mammals, and as such, the cold-defence response was also examined in the present study to confirm mesolimbic influence in thermoregulation (377).

Typically when rats are exposed to cold environments, they are able to use defence responses such as increased thermogenesis in BAT to maintain their core body temperature (53, 378). Specifically in this case, sympathetic nerves activate the β3 – adrenoreceptors in the BAT (77, 379). Adding to the possibility that cold-defence thermogenic responses are also under dopaminergic control is evidence provided by Blouquit (1996), who showed large increases in rat BAT dopamine content following rapid (30 minute) cold exposure (380). Further, it has been demonstrated that chronic cold-exposure profoundly affects VTA dopaminergic neurons, causing a subpopulation of them to cease spontaneously firing action potentials (381).

Since the results of Chapter 2 were inconclusive and it could not be determined whether activation of VTA dopaminergic neurons attenuated emotional hyperthermia, a different approach was utilised for this study. The VTA-NAc pathway was targeted by use of Anti-DAT-Saporin. As such, it was hypothesised that destruction of the VTA-NAc pathway would enhance the psychologically elicited hyperthermic response.

4.2 METHODS

All experiments were performed in male wild-type DAT::Cre Long Evans rats (307 – 560 g) with procedures approved by the Flinders University Animal Welfare Committee.

Surgical procedures were performed under general anaesthesia (2% isoflurane in 100% oxygen, 0.8 L/min) with pre-operative antibiotics (Baytril 0.1 mL s.c) and analgesia (Carprofen 5 mg/kg s.c) being administered. All surgical and post-operative care is fully described in Section 2.2.

Animals were left undisturbed for a minimum of a one-week period prior to any additional surgery or experimental recording in order to allow them to recover from surgical stress.

4.2.1 Chemical Administration and Thermistor Implantation

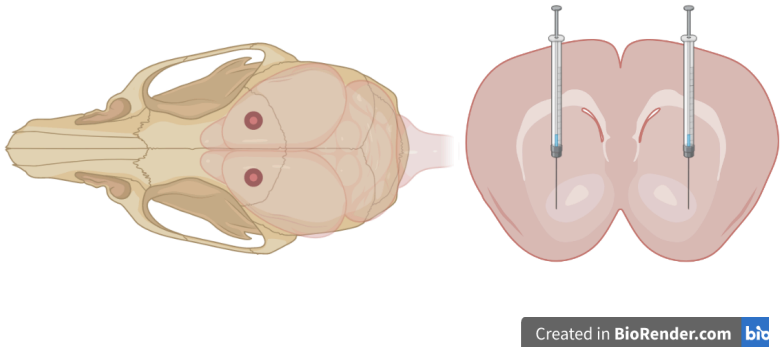


Figure 30: Schematic representation of burr-hole location and Anti-DAT Saporin administration site in the bilateral nucleus accumbens shell. Overhead view of rat skull, red dots indicative of burr-hole location (left). Coronal cross-section of rat brain with injection sites in the location of bilateral nucleus accumbens (right).

In preparatory surgery, rats were mounted onto a stereotaxic frame and the bregma-lambda plane was aligned for a skull-flat configuration. A burr-hole craniotomy was performed to allow for chemical administration and a long-shanked 5 µL glass micropipette was used to administer either 1.0 µl Anti-DAT Saporin (Advanced Targeting Systems, San Diego, CA, USA) or phosphate-buffered saline (PBS) bilaterally into the NAc shell (1.7 mm rostral from bregma, 0.8 mm lateral from midline, 7.0 mm deep from the cortex surface, 500 nl per side) as shown in Figure 30 (382).

The temperature probe implantation procedure has been fully described in Section 2.2.2. In brief, small thermistor based sensors were chronically implanted into the interscapular BAT tissue, and into the mediastinum to facilitate the recording of BAT and body temperature respectively. Thermistor cables were passed subcutaneously and attached to a headpiece screwed to the skull.

4.2.2 Intruder Experimental Design, Measurement of Physiological Parameters and Cold Exposure

Experimental set-up and the 'intruder test' was performed as fully described in Sections 2.2.3 and 2.2.4 respectively. All testing occurred after a minimum of two weeks following Anti-DAT Saporin administration (383).

The 'intruder test' was conducted during the dark cycle, when BAT and body temperature were near baseline value for each individual animal, as judged by the temperature recordings.

One day following the 'intruder test', between 1000 and 1100 hours, the cold exposure trial was conducted. The temperature controlled 'home cage' was located in a modified freezer fitted with an electrical heater, as well as a computer-controlled switch to regulate between the heater and refrigeration system. During the cold exposure experimental period the cage temperature gradually decreased from 26°C to 10°C. The cold state temperature was maintained for three hours, and then gradually returned to pre-cooling level.

4.2.3 Immunohistochemistry

At the conclusion of the experimental period, each animal was deeply anesthetized with pentobarbital (100 mg/kg i.p) and transcardially perfused, as described in Section 2.2.5.

The brain was then removed and post-fixed in the formaldehyde-sucrose fixative overnight. Serial sections (50 μm) were cut from the forebrain using a cryostat (Cryocut 1900, Leica Microsystem Pty Ltf, North Ryde, NSW, Australia). Fluorescent immunohistochemical staining was conducted to visualise TH containing dopamine neurons.

For TH visualisation sections were washed in Immunobuffer three times, with each wash lasting 10 minutes. This was followed by a 30 minute incubation period with 10% NHS (diluted with TPBS). Sections were then placed in a solution of sheep anti-TH antiserum (1:1000 in 10% NHS with TPBS) (MERK Lot#3206348) for three nights. Following this, sections were washed in TPBS three times as described above and incubated with donkey anti-sheep IgG (Alexa 488) (1:500 in TPBS) (Abcam ab150177) overnight. Sections were then mounted onto gelatine-coated microscopic slides, with Triton Water. Once cover-slips were placed on the stained sections, slides were examined using the Olympus AX70 Fluorescence Microscope to confirm loss of dopamine (TH+) neurons.

The number of TH+ cells were then counted for a selection of animals. Three sections were selected per animal and an average was taken. An independent *t*-test was used to determine differences between the vehicle treated and Anti-DAT Saporin treated experimental group.

All of the staining protocol was performed at room temperature.

4.2.4 Data Recording and Analysis

In order to graph and analyse the physiological recordings, the PowerLab Chart files were imported into IgorPro. Statistical analysis was performed using SPSS. Transient artifacts in recordings were removed by interpolating with values at surrounding time points.

Group results are shown as mean ± SEM for each experimental condition and were produced in Prism 9.

To evaluate the effects of intruder introduction, the change in BAT and body temperature from the 5 minute period prior to introduction of the intruder was compared to temperature values at 18-28 minute post-introduction. This longer time period was selected due to interanimal variation, particularly in regard to the onset of the intruder-elicited temperature increases.

The mean value was calculated for behavioural activity as a percentage of the initial 5 minute post-intruder introduction period.

Mean differences in BAT and body temperature increase, as well as the slope of BAT versus body temperature traces and behavioural activity level in the first 5 minutes following intruder introduction, were compared using an unpaired *t*-test between groups. $P < 0.05$ was considered significant.

For evaluation of cold exposure, the response magnitude over time was calculated as the area under the curve above baseline. Similarly, an independent *t*-test was performed to determine any significant differences between Anti-DAT Saporin treated and control groups.

Ultradian rhythms for control and Anti-DAT Saporin treated animals were examined by investigating the 12-hour light and dark period at least 24 hours following experimental recording set-up. The following parameters were examined for BAT and body temperature: average magnitude of temperature increase in an episode, episode onset temperature, episode peak temperature, interval between onsets, interval between peaks and total number of events in light or dark conditions.

Food microstructure was also examined as previously described in Section 2.2.8.

4.3 RESULTS

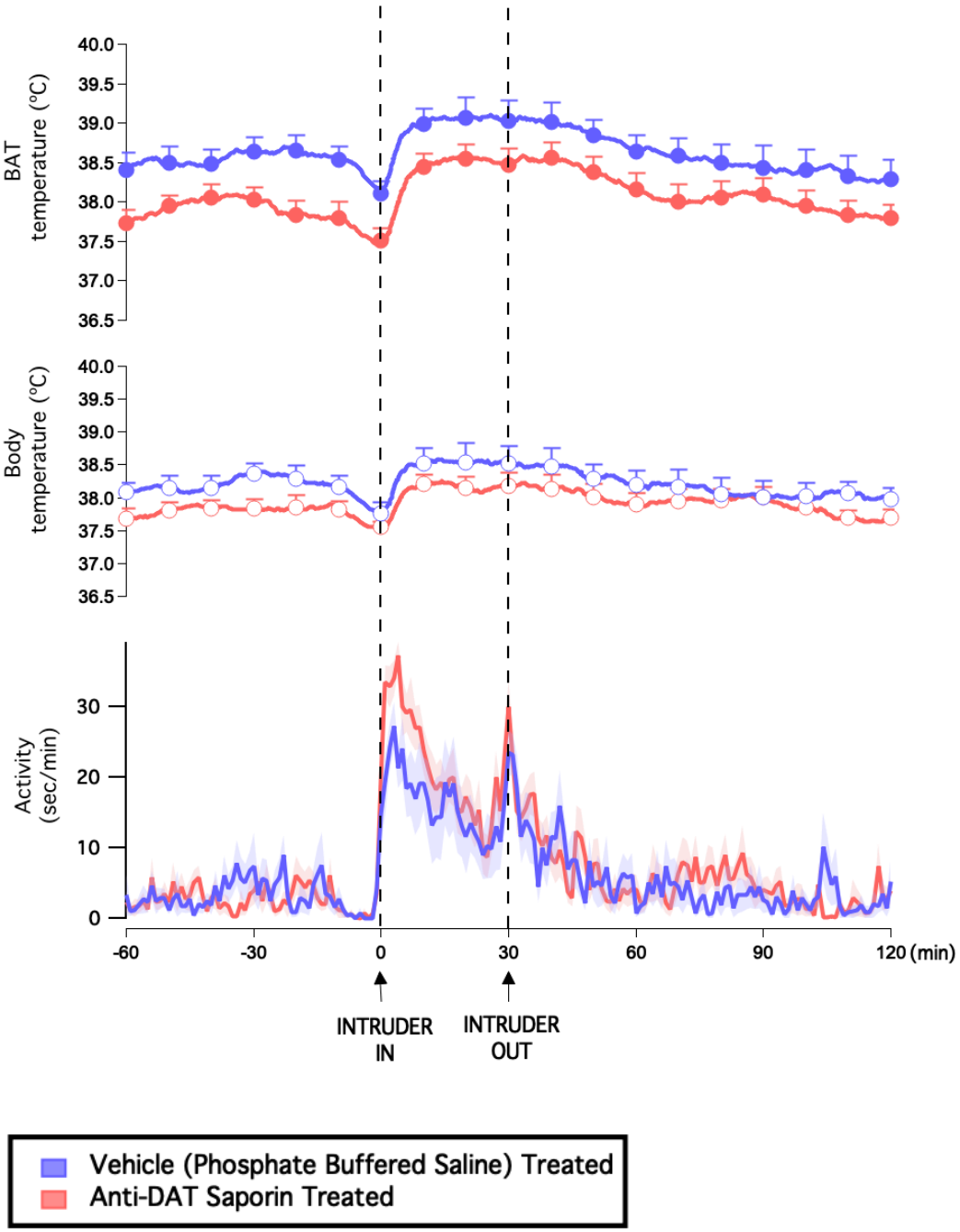


Figure 31: Group data (mean ± SEM) showing pre- and post-intruder introduction periods. Averaged experimental records from 'resident rat' showing BAT and body temperatures, and behavioural activity. Intruder was introduced at time 0 and removed 30 minutes later.

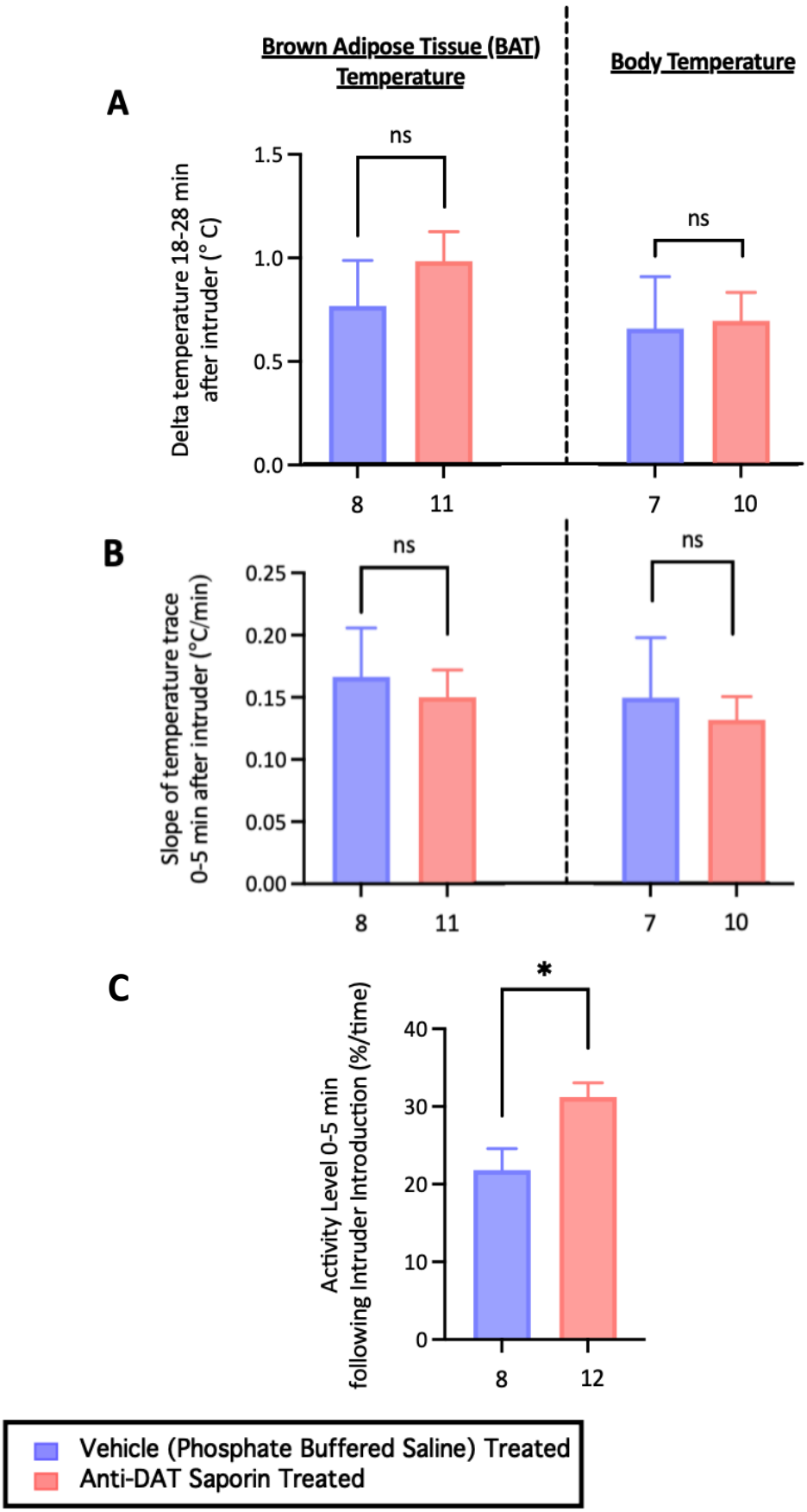


Figure 32: Bar graphs showing group results (mean ± SEM) of the effects of vehicle or Anti-DAT Saporin administration into the NAc on intruder-elicited thermoregulatory responses **A** *ns* no significant changes in BAT and body temperature recorded in the 'resident rat' 18-28min after introduction of the intruder rat **B** *ns* slope of 0-5min BAT and body temperature signal is not significantly different than following vehicle administration **C** Behavioural activity during 0-5min after introduction of the intruder rat (percent total time) *ns* behaviour following intruder introduction was not different compared to vehicle treatment. Numbers below the columns indicate number of rats in each experimental condition.

Table 3: Individual results for vehicle treated rats showing activity level for the 30 minute intruder period, in 1 minute intervals. The top three highest activity values (excluding intruder introduction and removal at times 0 and 30) have been highlighted in yellow (highest peak value), orange (second highest peak value) and purple (third highest peak value)

Vehicle (PBS)		Activity Level (arbitrary units)							
		Rat no: 1	2	3	4	5	6	7	8
Time value post-intruder introduction (minute)	0 **(intruder IN)**	29.10	8.97	5.85	21.38	7.57	28.30	3.57	12.38
	1	31.17	16.80	21.30	30.62	12.05	32.73	1.98	7.40
	2	29.17	27.60	27.07	31.17	14.35	37.05	15.97	12.55
	3	29.83	19.45	41.47	30.22	9.65	31.47	28.67	27.53
	4	20.95	10.98	28.88	19.15	16.82	33.78	13.58	26.20
	5	39.70	0.00	29.57	22.33	13.30	34.85	27.85	24.65
	6	6.32	3.80	14.60	32.85	17.17	36.17	17.63	18.65
	7	31.30	1.03	1.63	17.10	19.78	36.08	17.40	29.17
	8	21.23	0.00	4.07	6.13	9.35	33.80	38.20	22.48
	9	33.00	2.30	3.80	20.60	1.92	37.38	43.97	9.72
	10	20.20	33.45	4.63	10.43	11.50	27.22	31.70	13.80
	11	26.35	3.15	0.00	4.13	6.70	37.97	19.48	36.97
	12	25.90	0.00	2.88	0.00	5.97	38.00	3.38	29.57
	13	8.57	0.00	0.47	0.00	10.10	37.60	19.48	38.37
	14	5.70	0.00	0.00	0.00	4.93	31.15	38.13	34.58
	15	32.65	0.00	0.00	0.00	24.03	32.33	42.67	22.63
	16	32.75	0.00	0.00	0.00	9.63	28.45	36.05	30.75
	17	10.10	0.00	21.28	0.00	2.75	33.40	46.08	39.52
	18	5.82	0.00	35.73	0.00	0.85	32.22	10.23	37.02
	19	8.97	0.00	7.63	0.75	14.22	39.75	16.48	17.28
	20	7.32	0.00	0.70	0.00	4.88	24.53	40.80	14.25
	21	7.13	0.00	0.43	16.57	10.30	32.20	26.15	15.47
	22	12.68	0.00	0.00	13.30	7.45	25.72	23.28	13.30
	23	9.10	0.00	4.47	33.45	3.47	16.45	7.27	13.33
	24	20.28	0.00	1.22	1.55	1.95	24.70	12.05	9.75
	25	20.92	0.00	0.00	10.28	1.35	36.85	1.25	12.00
	26	18.70	0.00	0.00	20.42	0.55	30.55	5.05	3.85
	27	7.40	0.00	0.00	0.00	7.13	43.22	38.20	8.10
	28	15.55	0.00	0.00	0.00	4.90	28.85	16.25	22.03
	29	36.35	3.27	19.57	9.75	13.68	32.13	3.57	14.67
	30 **(intruder OUT)**	31.05	15.53	23.30	14.05	16.28	18.53	40.60	28.82

	Peak value
	Second highest peak value
	Third highest peak value

Table 4: Individual results for Anti-DAT Saporin treated rats showing activity level for the 30 minute intruder period, in 1 minute intervals. The top three highest activity values (excluding intruder introduction and removal at times 0 and 30) have been highlighted in yellow (highest peak value), orange (second highest peak value) and purple (third highest peak value)

Anti-DAT Saporin		Activity Level (arbitrary units)											
		Rat no: 1	2	3	4	5	6	7	8	9	10	11	12
Time value post-intruder introduction (minute)	0 (intruder IN)	36.38	20.38	9.05	16.48	9.52	7.90	11.05	16.25	18.47	36.27	36.12	16.53
	1	31.10	35.30	44.92	29.30	16.85	28.97	26.23	30.50	42.33	39.12	40.67	37.50
	2	31.28	36.80	32.95	24.78	21.90	35.17	41.80	42.75	41.00	25.50	47.52	12.95
	3	39.03	42.38	21.55	19.53	25.55	39.03	45.33	34.15	34.20	28.53	39.05	40.55
	4	40.55	34.77	23.25	31.75	36.87	38.10	44.88	39.42	48.75	33.27	41.07	34.35
	5	49.58	41.07	14.22	24.98	12.20	41.22	39.55	32.52	36.05	7.13	42.67	18.23
	6	35.47	35.45	9.20	31.53	29.53	30.07	36.38	20.75	27.65	17.08	35.13	41.42
	7	32.10	47.52	3.90	24.75	35.92	36.45	45.00	40.45	34.80	1.50	42.05	10.43
	8	34.45	31.10	13.20	9.70	31.42	27.70	37.20	39.63	27.75	8.37	36.83	26.72
	9	25.40	23.60	35.28	28.85	35.50	31.85	37.95	30.10	39.65	1.65	19.33	16.65
	10	25.17	28.37	4.88	25.33	34.75	40.10	33.35	5.42	14.37	25.28	18.73	29.08
	11	23.15	29.12	2.65	8.73	21.32	33.15	31.95	12.40	28.88	14.45	39.30	12.35
	12	14.78	34.53	1.80	18.82	8.55	25.05	47.10	14.67	30.22	0.00	26.25	11.53
	13	13.65	24.40	5.07	22.00	15.05	22.50	40.20	37.02	5.97	7.97	22.70	2.65
	14	13.50	34.62	3.72	9.38	17.23	37.15	29.12	19.33	16.15	13.35	33.60	4.52
	15	7.85	25.28	6.52	19.00	20.12	17.23	17.08	37.27	31.32	4.13	18.92	7.63
	16	13.53	26.90	19.42	22.48	4.18	15.50	33.87	12.58	33.38	0.50	22.90	30.90
	17	25.20	21.58	2.25	13.75	11.25	28.10	37.77	1.30	22.07	0.30	36.90	39.95
	18	3.27	20.92	5.65	15.53	33.87	30.38	30.32	0.00	16.30	0.52	21.53	8.77
	19	0.00	17.83	4.10	5.95	27.53	29.72	23.30	0.00	18.53	32.40	20.20	1.63
	20	7.37	25.63	9.20	32.35	12.08	28.35	26.95	0.00	9.75	7.90	32.85	14.75
	21	16.30	11.93	0.00	24.40	17.12	33.52	21.40	0.00	0.00	19.85	40.45	2.43
	22	7.90	4.88	0.00	17.00	14.47	25.48	16.00	0.00	12.97	2.18	24.05	32.67
	23	7.45	26.90	0.00	12.90	8.15	40.62	40.40	1.82	23.83	1.28	17.25	3.80
	24	7.67	3.45	0.00	2.25	4.47	28.37	29.57	1.57	13.40	0.00	14.88	5.97
	25	8.90	2.65	0.00	5.32	9.52	14.63	29.78	2.13	4.07	0.00	28.07	0.00
	26	12.12	3.35	0.00	19.60	28.65	5.55	26.70	6.92	13.55	0.00	35.00	0.00
	27	11.78	0.00	0.00	16.45	23.23	24.25	21.45	42.17	13.78	25.48	37.85	23.17
	28	25.15	0.18	0.00	25.85	0.95	40.75	6.47	16.85	22.30	15.15	8.67	20.75
	29	37.00	27.55	7.18	15.75	3.27	47.22	18.82	9.15	18.40	31.72	25.15	32.73
	30 (intruder OUT)	38.13	40.65	14.88	29.53	27.67	44.17	20.05	36.25	33.08	0.50	39.15	36.15

	Peak value
	Second highest peak value
	Third highest peak value

Effect of vehicle or Anti-DAT Saporin treatment on intruder-elicited variables

The effects of introducing the intruder rat following vehicle or Anti-DAT Saporin pre-treatment are shown in Figures 31 & 32. Similar to what has previously been observed, following vehicle pre-treatment once the intruder rat was introduced there was a rapid increase in both BAT and body temperature. This response was maintained after pre-treatment with Anti-DAT Saporin, and resulted in temperature increases that were not significantly different to the vehicle treatment. It is interesting to note, as clearly seen in Figure 30, that for Anti-DAT Saporin treated animals baseline BAT temperature was significantly lower than for vehicle treated animals: $t(16.4)=3.42$ $P < 0.05$. Any differences in baseline body temperature were not significant. Therefore, it is also of interest that the intruder-elicited increase in BAT temperature, for Anti-DAT Saporin treated animals, was substantially greater than the increase for body temperature.

Typically, once the intruder animal is introduced to the 'resident rats' home cage, the rat becomes very active, investigating the intruder and often climbing on top of the cage. This robust response was also seen in this experiment for both vehicle and Anti-DAT Saporin treated animals. However, it is important to note that the Anti-DAT Saporin pre-treated animals displayed significantly higher levels of activity compared to their vehicle treated counterparts ($t(18)=2.95$, $P < 0.05$).

Examination of individual trace values, shown in Tables 3 & 4, showed that Anti-DAT Saporin treated animals typically reached peak activity levels within the first 5-10 minutes of intruder introduction. In contrast, vehicle treated animals tended to display peak activity towards the middle of the intrusion period. Further, the top three activity peaks for control animals were typically scattered throughout the entire intrusion time. Analysis of the average value of the first 10 minutes, following intruder introduction, of each animal confirmed a significant difference between control and Anti-DAT Saporin treated groups: $t(18)=3.036$, $P < 0.01$.

4.3.2 Effect of Anti-DAT Saporin treatment on cold defence response

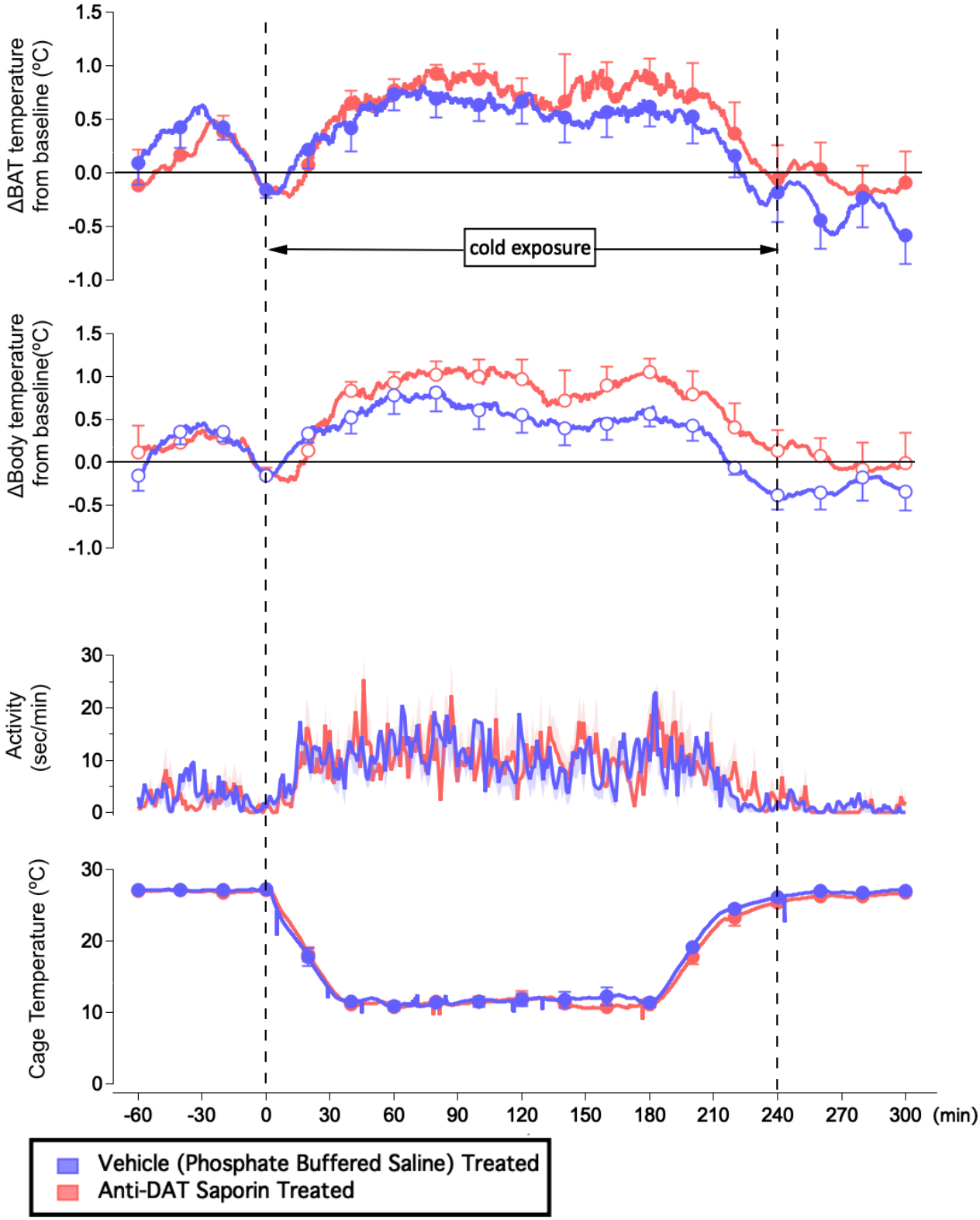


Figure 33: Averaged group data (mean ± SEM) showing effect of cold exposure on physiological parameters from vehicle and Anti-DAT Saporin treated rats. **A**: Relative changes from pre-cooling level were shown in BAT temperature (n=7 For vehicle, n=6 for Anti-DAT Saporin) and body temperature from pre-cooling level. Absolute value was shown in behavioural locomotor activity and cage temperature. Two vertical dashed lines on the graph indicated the onset and the end time of cold exposure, respectively.

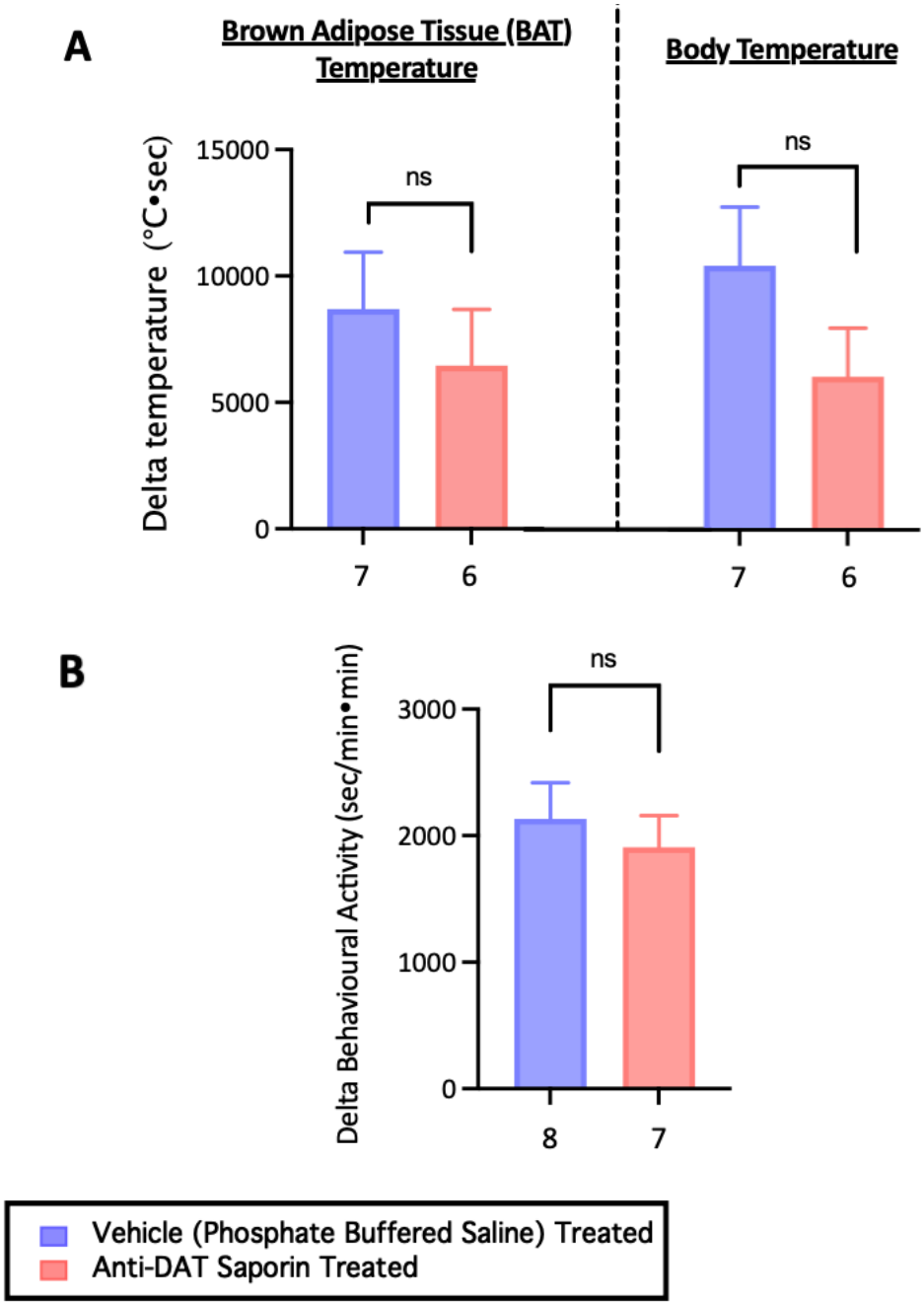


Figure 34: Bar graphs showing group results (mean ± SEM) of the effects of bilateral vehicle or Anti-DAT Saporin administration into the NAc on cold-defence thermoregulatory responses **A** BAT and body temperature changes during the 240min cold exposure were expressed as the area under the curve. *ns* no significant change compared to vehicle treatment. **B** Change in behavioural activity over the 240min cold exposure period expressed as the area under the curve. *ns* no significant change compared to vehicle treatment. Numbers below columns indicate number of animals in each experimental condition.

Autonomic physiological and behavioural responses to cold exposure in vehicle and Anti-DAT Saporin treated rats

Rats were exposed to caged-environmental cooling. After cooling began, the ambient cage temperature gradually decreased – reaching a final temperature of 10°C within 30 minutes, as

seen in Figure 33. The temperature was maintained at this level for a further 180 minutes, after which it returned to pre-cooling level within 40 minutes following the end of the cold state.

In both vehicle and Anti-DAT Saporin treated animals once cooling started both BAT and body temperature began to increase immediately. For vehicle treated animals BAT temperature increased by 1.0 ± 0.2°C, and body temperature by 0.7 ± 0.2°C. This is similar to what has previously been reported (175). The Anti-DAT Saporin treated animals showed a comparable increase in BAT temperature, and a slightly lower elevation of body temperature (0.5 ± 0.2°C), although this change was not found to be significant, as seen in Figure 34.

As expected, vehicle treated rats also showed an increase in behavioural activity during the cold-exposure period. This likely assists in maintaining body temperature. The same response was also observed in the Anti-DAT Saporin treated animals.

4.3.3 Effect of Anti-DAT Saporin treatment on normal thermoregulatory parameters

Other thermoregulatory factors were also examined following destruction of the mesolimbic pathway to investigate whether this pathway was involved in any aspect of thermoregulation; including parameters of ultradian rhythm events and feeding behaviour.

Effect of Anti-DAT Saporin treatment on regulatory thermogenic factors

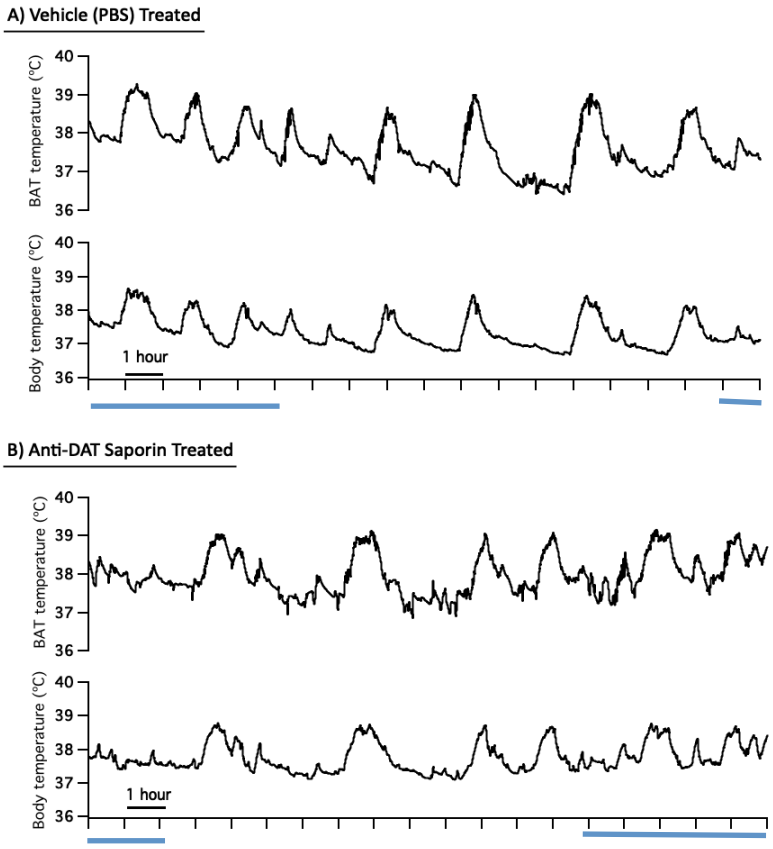


Figure 35: Representative trace of typical ultradian rhythms for BAT and body temperature in A) vehicle treated and B) Anti-DAT Saporin treated rats. Blue lines below graphs indicate 'Night' period.

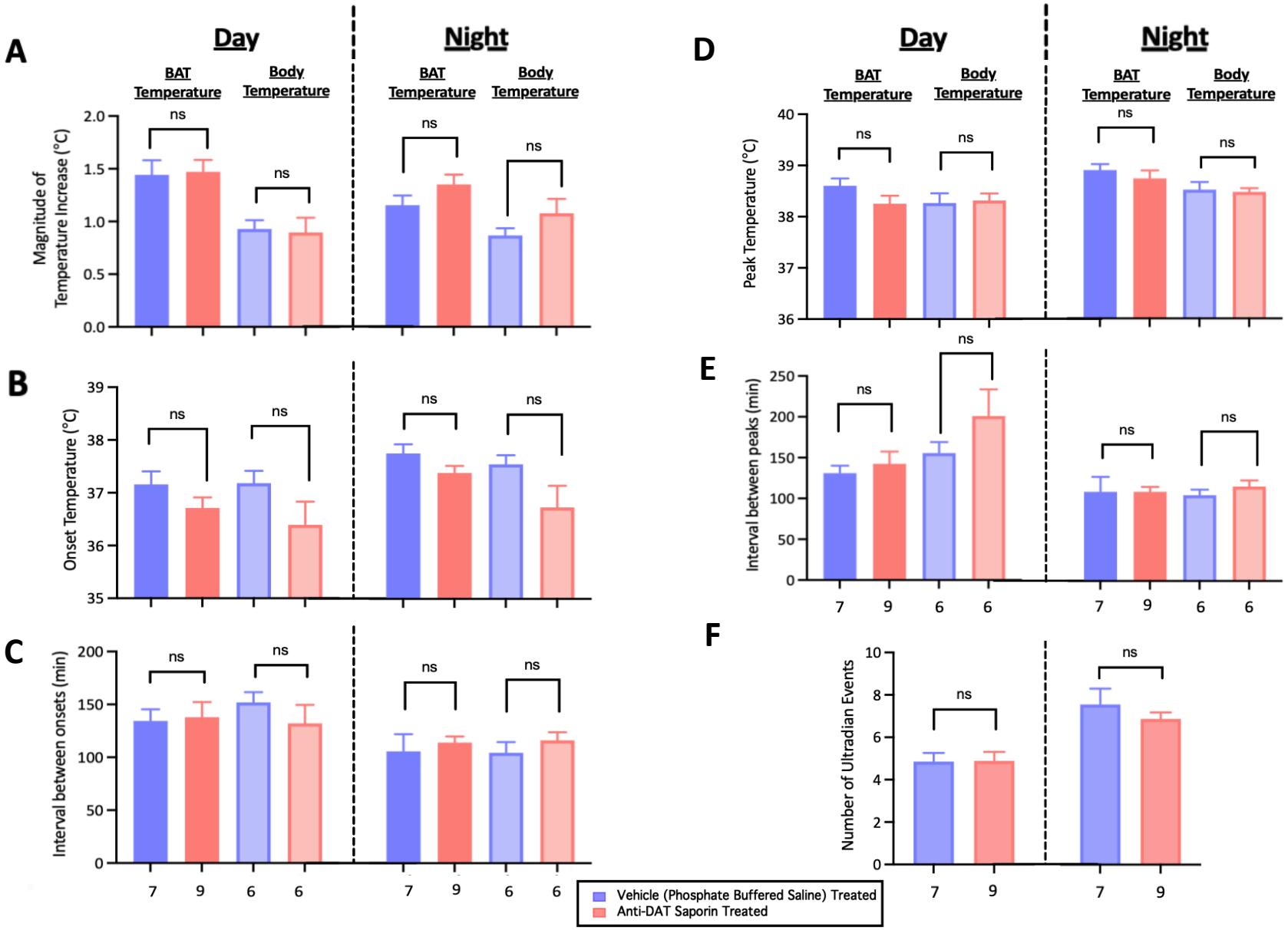


Figure 36: Averaged group data (mean ± SEM) showing effect of vehicle and Anti-DAT Saporin treatment on ultradian rhythm parameters: magnitude of temperature increase, episode onset temperature, interval between onsets, peak temperature reached, interval between peaks and total number of events. *ns* no significant difference between vehicle and Anti-DAT Saporin treated animals. Numbers below columns indicate number of animals.

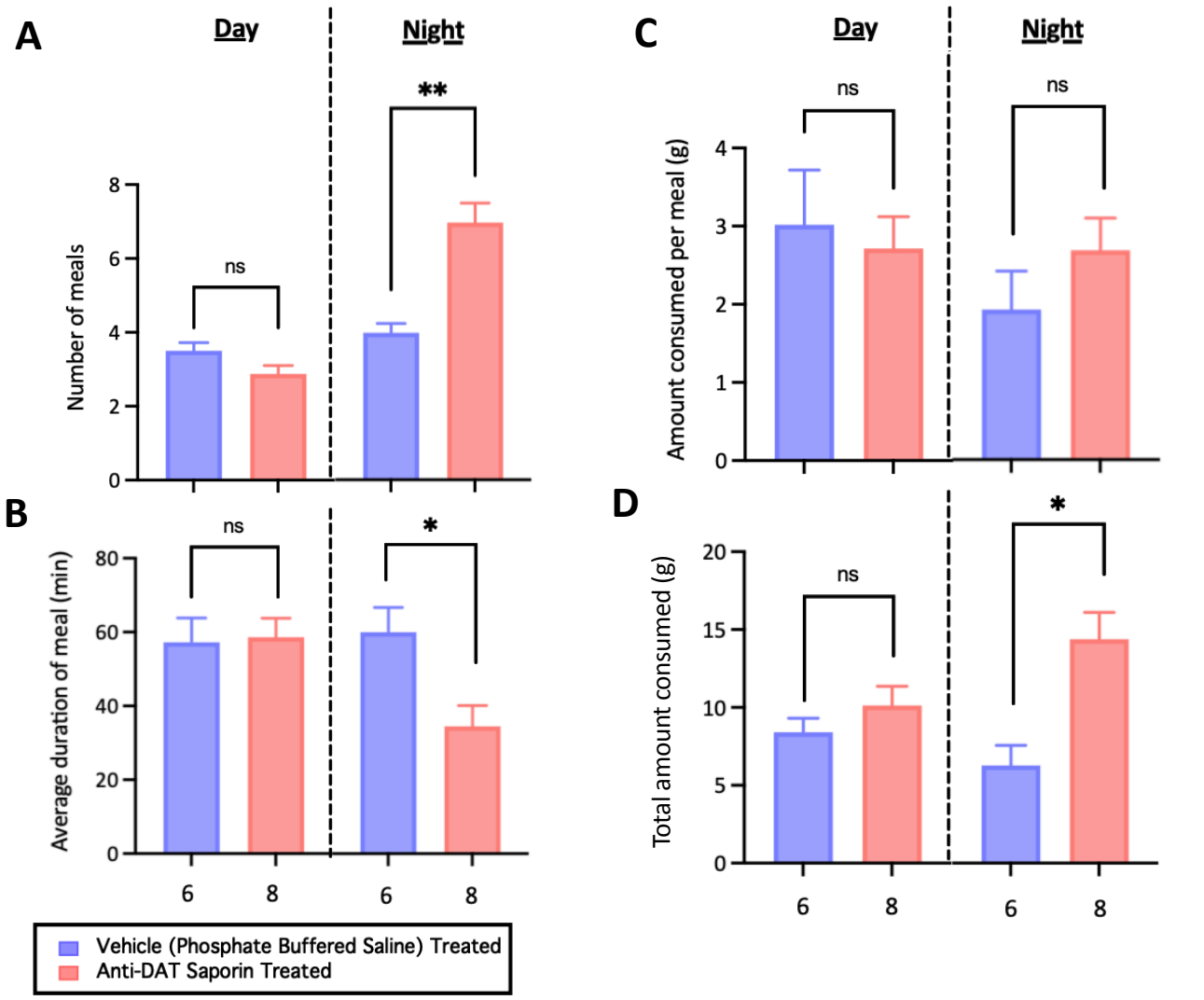


Figure 37: Bar graph showing group results (mean ± SEM) of the effect of vehicle or Anti-DAT Saporin treatment on feeding behaviour. **A** total number of meals **B** Average duration of each meal (min) **C** Average amount consumed per meal **D** Total amount consumed. Numbers below columns indicate number of rats in each experimental condition. Asterisks indicate significant difference between Anti-DAT Saporin and vehicle treated animals ** P <0.001, * P < 0.05, *ns* indicates no significant difference.

Figures 35 & 36 show the analysis of typical ultradian cycles for both light and dark periods in vehicle and Anti-DAT Saporin treated rats. In comparison to vehicle, the Anti-DAT Saporin treated animals showed no significant difference in the ultradian factors examined.

As expected however, there were differences within treatment groups regarding several factors. For Anti-DAT Saporin treated rats there was a significant difference between ultradian rhythm onset temperature for BAT in light and dark conditions ($t(8)=4.153$, $P < 0.05$). Additionally there were significant differences in regards to peak BAT temperatures reached during the day and night

($t(8)=4.965$, $P < 0.05$), interval between BAT peaks ($t(8)=2.452$, $P < 0.05$) and the magnitude of BAT and body temperature increases at night ($t(5)=3.844$, $P < 0.05$).

Similar differences were reflected in the vehicle treated rats with differences in BAT onset temperature in light and dark periods ($t(6)=4.786$, $P < 0.05$), body onset temperature in light and dark periods ($t(5)=5.129$, $P < 0.05$), BAT peak temperatures reached ($t(6)=3.550$, $P < 0.05$) and body peak temperatures reached ($t(5)=3.273$, $P < 0.05$)

As seen in Figure 37, this study also found that Anti-DAT Saporin treated rats had statistically significant increases in the number of meals and the total amount consumed during night hour (number of meals: $t(9.9)=5.05$, $P < 0.001$; total amount consumer: $t(11)=3.67$, $P < 0.05$), however there was also a significant decrease in the average duration of each meal during the night, compared to vehicle treated animals ($t(12)=1.91$, $P < 0.05$).

4.3.4 Confirmation of dopamine neuron destruction

In order to confirm destruction of the mesolimbic pathway, as judged by destruction of dopamine neurons in the VTA, the brains of experimental animals were stained for the TH marker. Representative images of the immunohistochemical staining following vehicle and Anti-DAT Saporin treatment is shown in Figure 38.

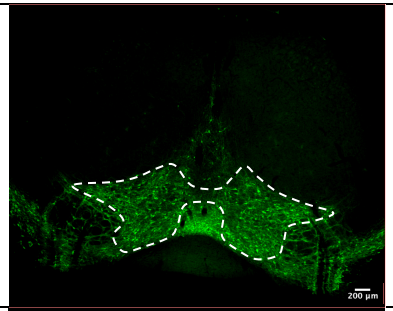
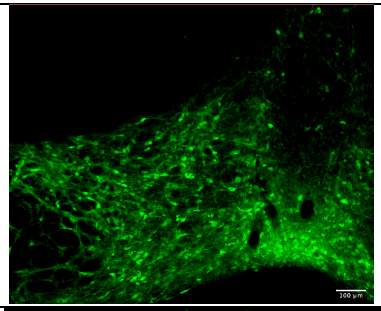
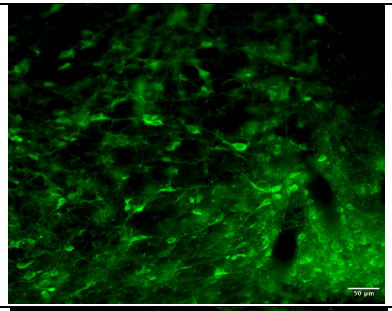
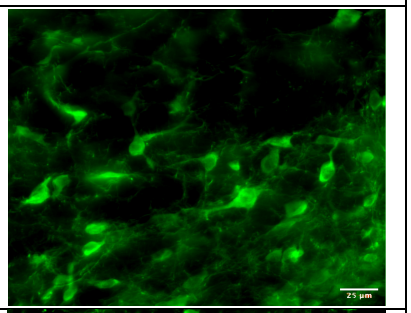
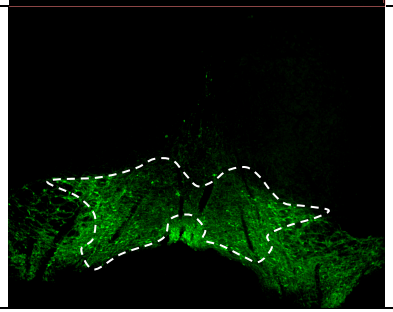
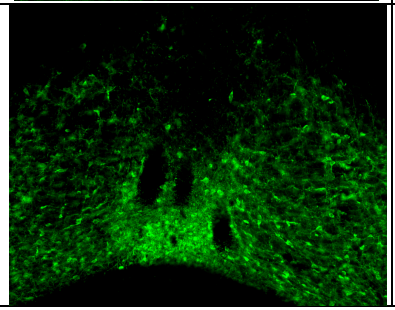
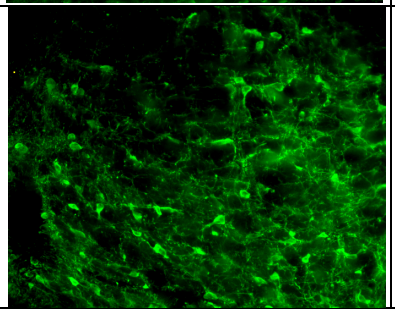
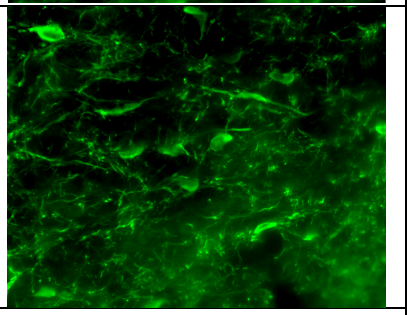
Tyrosine Hydroxylase (TH) staining	4x	10x	20x	40x
Vehicle (PBS) Treated				
Anti DAT-Saporin Treated				

Figure 38: Representative immunohistochemical demonstration of dopamine (green - TH) neuron presence in coronal sections through the midbrain of a vehicle and Anti DAT-Saporin treated animal. Subregion from which cell counts were taken is outlined in white, first panel. Scale bar is located in the bottom right corner of each 'Vehicle (PBS) Treated' image; 4x = 200µm, 10x = 100µm, 20x = 50µm, 40x = 25µm

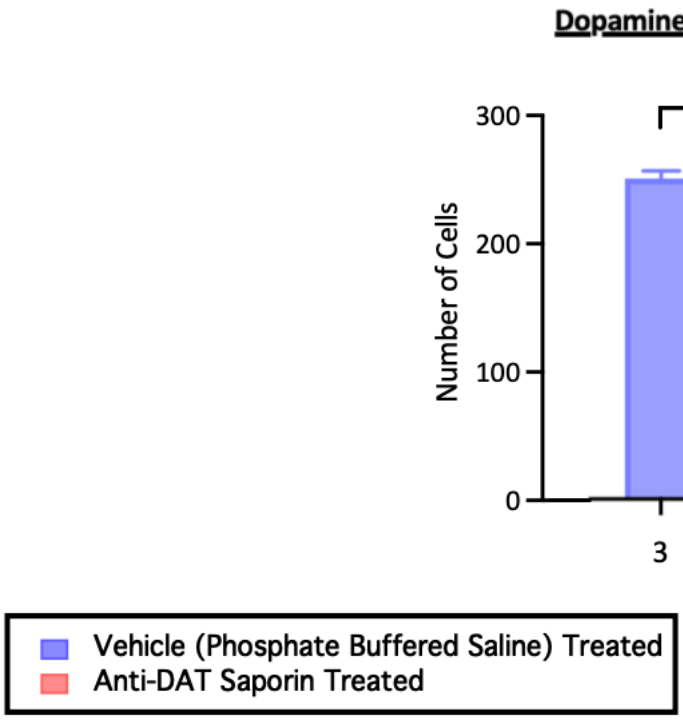


Figure 39: Summary of TH+ cells in the VTA following bilateral vehicle or Anti-DAT Saporin injection into the NAc. Asterisks indicate significant difference from vehicle treatment, ** $P < 0.001$. Numbers below columns indicate number of animals in each experimental condition

Following staining, unfortunately the majority of brain sections from experimental animals were damaged. Because of this, only a small number of animals could be used for cell counts. However, although not all sections were counted, dopamine destruction was visually confirmed for all experimental animals. Even if cells were not fully destroyed, significant morphological differences were noted where Anti-DAT Saporin treated cells showed small and irregularly shaped cell-bodies.

Despite the limited sample size, it was confirmed that administration of Anti-DAT Saporin significantly decreased the number of dopamine (TH+) neurons in the VTA, as seen in Figure 39 ($t(5)=8.603$, $P < 0.001$).

Typically, in order to support the claim of lesioning there must be an assessment of total neuron loss. In the future it would be ideal to not only confirm the loss of TH+ neurons but also whether non-dopamine cell types were affected. An attempt was made to perform NeuN staining, however, as shown in the Appendix, the results indicated that none of the surviving TH cells stained for NeuN. Further, the NeuN staining that was seen was identified as cytoplasmic rather than staining the nucleus. As such, these results were not evaluated as part of the main thesis. Cannon and Greenamyre (2009) (384) previously examined NeuN staining of dopamine cells in the

substantia nigra. Although NeuN is one of the most commonly used general immunohistochemical markers for neurons and is reportedly expressed in most mammalian neurons, the study found that expression was very variable and dopamine cells with no, or very faint, staining were identified. Interestingly, NeuN expression was demonstrated to be higher in non-dopamine cells of the ventral midbrain. Based on these findings, it was suggested that NeuN is not a useful quantitative marker for dopamine cells; a conclusion supported by the results of the present study.

4.4 DISCUSSION

4.4.1 The robust nature of emotional hyperthermia

For the experiments involving the intruder stress paradigm, the vehicle treated rats showed rapid increases in BAT and body temperature following intruder introduction. This finding is consistent with the expected result, with previous studies finding similar increases in temperature. This, therefore, indicates that the results currently observed in control animals are likely to be accurate. Ootsuka has demonstrated increases of up to 1.4°C for BAT temperature (9). Although this is higher than what was observed here, there are several factors that could account for this discrepancy. Most importantly, comparisons between the two studies must be drawn carefully as Ootsuka's study used Sprague-Dawley rats, while the current study used Long-Evans rats. The difference in strain may potentially explain the difference in temperature increase magnitude; however, it nevertheless demonstrated the robustness of the emotional hyperthermic response.

4.4.2 Impact of Anti-DAT Saporin on locomotor activity

It is interesting that the only factor of the intruder response that was significantly affected was locomotor activity following intruder introduction. In fact, Anti-DAT Saporin treated animals were approximately 10% more active than the vehicle treated counterparts. Clinical studies in humans have previously given evidence for the role of the mesolimbic dopaminergic pathway in the regulation of locomotor activity (385, 386). Importantly, psychostimulant-induced hyperactivity in rodents has also been linked to actions in the NAc (216, 261, 368, 387-390). Although these studies examined long term locomotion in an open field, which is different to the long-term 'home cage' locomotion examined here, in 2013 Wang et al demonstrated that chemogenetic activation of midbrain dopamine neurons in mice included hyperactivity in a home cage as well as an open field (262).

Indeed, Heusner (2003) demonstrated that dopamine deficient mice, which showed no hyperactivity following psychostimulant administration, had a hyperactive phenotype appear when dopaminergic signalling in the nucleus accumbens was restored (368). This is in opposition to the presented results. Similarly, Boekhoudt et al (266) and Boender (391) respectively showed that enhanced dopamine activity in the VTA-NAc pathway facilitated locomotor hyperactivity.

Importantly, through closer examination of individually recorded traces it was observed that control animals seemed to react fairly slowly to the intruder, and their peak activity levels were reached only half-way through the intruder period, after which it was sustained for the remainder

of the intrusion time. In contrast, animals treated with Anti-DAT Saporin displayed an immediate, large but short-acting burst of activity when the intruder was introduced. This may be related to impaired attention to the intruder stimulus itself and enhanced responsivity (392). It was observed in Chapter 2 that enhanced VTA dopamine neuron activity resulted in deficits in sustained attention regarding food consumption. This is also potentially the case here, and could indicate that there is a fine balance that needs to be maintained in order to enable appropriate levels of attention retention to tasks and novel objects.

Therefore, despite the discrepancies with previous research, the present data reinforces the idea that the mesolimbic dopamine system is a good target for new and novel therapies to treat psychiatric disorder hyperactivity, as seen in attention deficit hyperactivity disorder (ADHD), which has been linked to low dopamine activity in the VTA (393), and schizophrenia (394).

4.4.3 Could the mesocortical pathway be responsible for temperature regulation instead?

Nevertheless, the obtained results indicate that following treatment with Anti-DAT Saporin, the typical response of emotional hyperthermia remained following intruder-stress. This is interesting as DAT knockdown in the NAc has been shown to improve anxiety related behaviours in mice (395). Thus, it may mean there is involvement of other compensatory systems, or that the VTA-NAc pathway is unlikely the main regulating factor. Indeed, the response may be regulated by the mesocortical pathway between the VTA and PFC. It is critical to note that the mesolimbic and mesocortical dopaminergic pathways are separate and distinct - with different neurons projecting to their respective areas, resulting in differing function and areas of regulation (363, 396).

Although compared to expression by projection in the NAc, the PFC, medial PFC (mPFC) in particular, expresses fewer dopamine reuptake transporters and an overall lower dopamine level concentration (147, 397-399); it appears to be a promising area for the study of thermoregulation. Further, in relation to the previous findings described in Chapter 2, the activity of D_1 neurons of the PFC has been shown to increase following food intake (400, 401). This finding has been confirmed via optogenetic photostimulation of D_1 neurons, which also increases feeding (298). Further, the same physical stressors which were demonstrated to increase dopamine levels in the NAc, such as tail pinch and noxious thermal stimuli, also increase extracellular dopamine in the PFC (371, 402, 403).

4.4.4 Factors influencing temperature during cold exposure

For the cold-defence experiments, both rats that were treated with vehicle and Anti-DAT Saporin displayed similar responses. As expected, in vehicle treated rats the drop in ambient temperature resulted in increased BAT thermogenesis, and a resultant maintenance of body temperature. This is once again comparable to what has previously been observed in Sprague-Dawley rats (53). The response in Anti-DAT Saporin treated rats was not statistically different; however, there does seem to be a small delay in the initiation of BAT thermogenesis and a slight difference in relation to the rate of temperature increase, and return to baseline following cold exposure. For these results it is vital to consider probe placement. Similar to what has been observed in a previous study by Mohammed (2016) (53), BAT temperature initially decreased during cold exposure. This likely occurs because the BAT thermistor is positioned near the vein of Sulzer. Thus, since the interscapular BAT is in close proximity to the skin, temperature readings are likely to be influenced by skin temperature and the ambient environment. The fur above the interscapular BAT was also shaved to enable the temperature probe implantation, and thus could have further affected the cold defence response (53). It should be noted that in an ideal scenario, body temperature should remain at a constant level (175, 404), however, there was an increase seen in the current results. This can once again be accounted for by probe placement. As discussed by Mohammed, heated blood from the interscapular BAT flows only a short distance to the heart, therefore mediastinum temperature may partially be influenced by BAT temperature (53, 405).

4.4.5 Acute or chronic stress – does it make a difference?

Another factor that is critical to consider is the difference between acute and chronic stress. Based on previous research it appears that dopamine release can either be enhanced or inhibited based on the intensity and duration of a stressor (363). It is possible that the curve of dopamine release in response to stress exposure is an inverted 'U' – with mild and moderate stress that is short-lasting having activating effects on dopamine release, whereas it is inhibited by chronic and intense stress (406, 407). Following acute restraint or acute foot shock, for 10-240 minutes, an increase of approximately 125-150% in dopamine level was found in the NAc of rodents, and an even larger increase (approximately 139-250%) in the mPFC (408). This, in turn, supports the idea that sensitivity to stress is highest in the mesocortical dopamine system or it is possible that a lower threshold of stimulation is required for PFC projecting dopamine neurons (381, 409). Following chronic stress, in the form of inescapable cold, Finlay et al (1995) showed NAc and PFC dopamine levels were unchanged, or were indeed lower than in controls (410). Even stressors that

are not physical, such as simply watching other rats receive severe foot shocks, elicits significant extracellular dopamine increases in the NAc shell (411); and presentation of a predator odour produces elevation of dopamine levels in the PFC (375, 412). As only an acute stress paradigm was examined in this study, it is possible that if the mesolimbic system was involved in regulating the physiological response to either environmental or psychological stress, the effects of pathway destruction may have been more apparent if chronic stress was investigated. Indeed, chronic stress results in morphological changes to VTA dopamine neurons – including decreased soma size (413) and can even lead to cell loss (414). Interestingly though, studies have found long-lasting neuroadaptive changes in VTA dopamine neurons following even single exposures to stress (415, 416), meaning acute stress may alter the responsivity of neurons to future stimulation.

It is also possible that only specific subsets of VTA neurons are affected by stress. It was previously demonstrated through electrophysiological studies that aversive stimuli inhibited VTA dopamine neuron firing; however, recently a subset of VTA dopamine neurons has been identified where the opposite is true, and their firing rate is increased (408, 417).

4.4.6 Mesolimbic regulation of ultradian rhythm and feeding behaviour

Examination of ultradian rhythms found that there were no significant changes between Anti-DAT Saporin and vehicle treated animals. This speaks to the robustness of this event, and likely indicates there are many other mechanisms that are responsible for its correct regulation. However, it is important to note, as seen clearly in Figure 30, Anti-DAT Saporin treated animals had a lower resting BAT temperature. This gives some evidence that the mesolimbic dopamine pathway is, in part, involved in some aspects of thermoregulation.

The examination of feeding structure showed that the destruction of the VTA-NAc pathway resulted in a substantial increase in the number of meals, a decrease in the duration of meal and a total increase in the amount consumed during the dark period. It is not surprising that these changes were seen at night, since this is when rodents are commonly most active (11, 418-420). What is surprising, however, is that these results seem to closely resemble the findings of Chapter 2, in which VTA dopamine activity was enhanced. Since in this set of experiments the mesolimbic dopamine pathway was destroyed, it was expected that this would result in the opposite effects being seen. Previous chemogenetic activation of the mesolimbic pathway has resulted in smaller and shorter meals, with increased feeding frequency (275). Elevated dopamine signalling in the NAc was also demonstrated to reduce meal size. Based on the results, shorter meals would

typically suggest that animals were satiated more quickly (421), however, the unaffected meal size and increase in total amount of food consumed indicated an opposite effect on satiety. Based on these findings new insights have been found into how dopamine neuronal activity may be controlled in the regulation of food intake, and may be implicated in dopamine's role in overeating. In support of this, it has been well documented that weight gain in antipsychotic drug treatment may be attributed to dopamine's effect on meal size (422-424). Grinker, however, suggested that dopamine signalling may affect food intake differently in obese patients, compared to those who are thin (425).

4.4.7 Efficacy of Anti-DAT Saporin

Following immunohistochemical analysis, although a significant reduction in dopamine neurons was observed, many un-lesioned cells remained. Thus, since the majority were not destroyed, it is highly likely that the action of Anti-DAT Saporin was not substantial enough to elicit a significant change in response and behaviour. This result reflects what was previously observed by Wiley and colleagues (2003) (224). Instead of the cell counts used here, their study assessed Anti-DAT-SAP action through densitometric analysis of autoradiograms – showing the binding of [^{3}H]mazindol to DAT in the striatum and SNc. It was demonstrated that only high doses (>20 µg) of toxin showed profound, yet incomplete, loss of binding in the SNc. Further, based on the examination of multiple midbrain sections the group also concluded significant, but lesser degree, loss of dopaminergic neurons in the VTA. It should, nevertheless, be considered that Wiley's study also utilised a higher injection volume (10 µL), and different injection location (intraventricular), to what was used in the present study.

Saurer and associates (2005) (426), conversely, selected a 0.5 µg/side dose, and administered 1.0 µL of Anti-DAT Saporin bilaterally into the NAc - a method very similar to what was used here. Their study found that Anti-DAT Saporin exposure blocked morphine's suppressive effect on Natural Killer cell activity. Interestingly though, whilst their results were significant, no quantification of Anti-DAT Saporin cell destruction was reported in the study. This lack of efficacy quantification makes the results of the present study difficult to confirm as only a very limited comparison can be made.

Therefore, whilst Anti-DAT Saporin may be a promising research tool, it is not a flawless method, and does not result in the complete destruction of all dopamine cells in a target region. Importantly, several research groups have discussed that DAT is expressed differently in the core

and shell of the NAC (427), and interestingly, on only about half of VTA dopaminergic neurons (428-430). Taking this into account, the results of the current study's immunohistochemical analysis are unsurprising. As such, it is suggested that in future investigations of the mesolimbic system several adjustments to the research method should be made: a different administration site can be trialled - such as the core region of the NAc, a sufficiently high dose of Anti-DAT Saporin should be used to ensure maximal destruction of dopaminergic neurons, and more appropriate toxins should also be examined to enable the targeting of a greater number of cells in the VTA. A dual viral-vector chemogenetic approach - using a retrograde transport type Cre vector in a region (e.g. NAc) that has connectivity to another brain region (e.g. VTA) which has a Cre-dependent vector administered, can also potentially be employed to target and modulate the function of neuronal pathways. However, if this method is to be used, it must be considered that the activity of collateral projections will also be affected, as explained by Campbell and Marchant (2018) (431). Collateral projections should also be considered when using Anti-DAT Saporin, and whilst this goes beyond the scope of the current project, it is a critical factor to examine in the future.

4.4.8 Conclusion

In conclusion, the hypothesis that the mesolimbic pathway was responsible for mediation of thermoregulation and emotional hyperthermia could not be supported. Instead, it is suggested that it is likely the mesocortical pathway that warrants further investigation with regards to these thermoregulatory alterations.

CHAPTER 5: SUMMARY AND GENERAL DISCUSSION

In this thesis, I have aimed to further uncover the role of the dopamine system in the process of thermoregulation and its contribution to the regulation of the autonomic stress response. Using newly developed techniques, such as DREADD technology and targeted toxins in rats, VTA dopaminergic neurons and the VTA-NAc mesolimbic pathway was targeted in order to explore the results of dopamine signalling enhancement, or reduction, on such factors as BAT temperature, body temperature, locomotor activity and feeding behaviour.

This chapter briefly summarizes the findings and presents an overall review of chemogenetic dopamine neuronal activation in the VTA, and destruction of dopaminergic neurons in the mesolimbic pathway; and how these findings add to an understanding of how the dopamine system contributes to thermoregulation and the control of thermoregulatory factors. The limitations of the studies are also discussed, and some possible future directions for research are presented - focusing on highlighting the potentials of DREADD technology in the creation of novel therapies for dopamine signalling related disorders.

Particularly in recent years, new technologies that directly control neural activity in freely moving animals, such as chemogenetics and optogenetics, have rapidly grown in popularity (432, 433). These techniques allow for the investigation of the whole animal as well as the integrated behavioural functions of selective neural circuits and populations. Thus, the findings of prior studies that have used techniques such as lesions, electrical stimulation and pharmacological manipulation can be confirmed. By confirming these previous findings with new techniques that regulate variables in differing ways, these novel studies - including the ones presented here, can contribute to the field of translational neuroscience. In addition, selectively controlling the activity of specific populations of neurons leads to major discoveries, which would not have been previously been possible through the use of conventional techniques.

5.1. Summary of results

In Chapter 2, the important discovery was made that the chemogenetic activation of midbrain VTA dopamine neurons profoundly affects thermoregulation and thermoregulatory variables in rats. Enhancement of VTA dopamine neurons induced locomotor hyperactivity and disrupted normal feeding behaviours – leading to significant and long-lasting increases in both BAT and body

temperature. These results are critical to the understanding of thermoregulatory pathways and present vital information that adds to the elucidation of VTA function.

To further investigate these findings, in Chapter 3, it was investigated whether the altered feeding behaviour following DREADD activation contributed to the changes in typical thermoregulation. It was also examined whether or not the excitatory D_1 receptors similarly influenced the increase of baseline BAT and body temperature. Based on the results obtained it was concluded that changes in feeding behaviour were not the primary cause of temperature elevation following VTA dopamine neuron activation. Additionally, the D_1 receptor was found to be a major regulator of body temperature.

In conclusion, Chapter 4 explored the contribution of the mesolimbic pathway to the process of emotional hyperthermia and the cold-defence response. It was demonstrated that disruption of the mesolimbic dopamine system resulted in the altered feeding patterns and modest changes to locomotor activity, but failed to significantly affect the robust cold-defence and intruder-stress response.

These results have implication for the understanding of the neural substrates and pathways that are involved in thermoregulation, and also provide a starting basis in the novel production of targeted treatments for temperature regulation disorders, and associated dysregulated factors including locomotor impairments and altered food consumption – as is seen in many psychiatric disorders such as ADHD, or obesity.

5.2 Pharmacological and chemogenetic manipulation – why do they produce different results?

There were some discrepancies between the present results and what has been previously reported. These differences can mainly be accounted for by differences in pharmacological and chemogenetic manipulation. Pharmacological manipulations tend to regulate post-synaptic activity, by either blocking reuptake or stimulating pre-synaptic neurotransmitter release. Conversely, endogenous activity is regulated by chemogenetic techniques. It is these differences that can potentially account for the differing effects and the duration of action.

A significant benefit of utilising chemogenetics is that it has anatomically restricted effects in comparison to systemic administration of drugs that alter neurotransmitter function throughout the brain. Despite this, it would be ideal to target neuronal populations based on both cell-type

and projection. Since the VTA projects to both the NAc and PFC, it would be highly beneficial to be able to regulate the activation of these two pathways separately. Using a cannula to infuse DREADD agonists directly over the targeted area is possible; but this would be suitable for only short-term studies (434-436). Implantation of a cannula is also invasive and has been known to cause damage to surrounding tissues. Potential clogging of equipment would also have to be considered.

New technologies are constantly emerging, being refined and developed. One such technique is a combination of Cre and Flp recombinase that allows for cell-type and projection specificity (437).

In the future, the current experimental paradigms should be more thoroughly tested with smaller doses of CNO and C21. Previous studies have demonstrated maximal results with doses lower than what was used here, such as 1 mg/kg. However, in a limited earlier pilot study conducted prior to the present investigation, no significant results were found following the use of 1 mg/kg (data not shown).

5.3 Can DREADDs be used to treat humans?

Interestingly, the use of DREADDs in humans for the treatment of various disorders is not a drastically new idea. Adeno-associated viruses have already been suggested for use in gene therapy and it is indeed a promising system in that no animal studies have yet reported any effects of DREADD expression without activation (432, 438). However, prior to any application in humans there must be further testing done in relation to safety, the steps of which have already begun for the treatment of eating disorders (439-441) and epilepsy (442, 443). The major issue with its implementation, however, has been the conversion of CNO to clozapine. The development of C21 may avoid these issues, and indeed Chen (444) has shown that it is suitable for use in humans. Further, the current study has demonstrated that activation of dopamine neurons using CNO and C21 produces comparable results. Despite this, it was found that enhancement of dopaminergic activity with C21 affected several physiological aspects, and created other confounding factors that were not related to off-target effects. The most significant of these was locomotor activity. Therefore, in future, and especially if this technology is to be applied in humans, functions that are not dependent on activity should be examined following neuronal activation – for example, functional brain responses assessed using neuro-imaging techniques.

5.4 Refinement of viral vector and Anti-DAT Saporin administration

In relation to the current studies, bilateral injections of 500 nl were found to produce DREADD expression in a relatively large area, with some transduction of the SNc. Although the influence of the SNc was able to be ruled out in the majority of results, based on previous research, smaller injection volumes should be investigated to reduce any activation of off-target cells. This will likely be an effective method, as researchers such as Burnett and Krashes (206) have shown that infusion of smaller virus volumes does indeed result in more restricted anatomical specificity.

Similarly in relation to the administration of the Saporin toxin, large variation was observed in the extent of the area that was affected. The investigation of smaller injection volumes could also help with anatomical specificity and would produce more definitive and specific results.

The current experiments also did not account for the weight of the animals used. Indeed, the coordinates used to establish an appropriate injection site for DREADD and Anti-DAT Saporin administration were obtained from *The Rat Brain in Stereotaxic Coordinates*, which is based on 290 g Wistar rats (382). The rats used in this thesis were both a different strain, Long-Evans, and were generally of a greater weight. As such, due to cranial differences, the actual sites of injection may not have been ideal for the targeted brain regions. *The Rat Brain in Stereotaxic Coordinates* does include a table with basic adjustments; however, these are stated to only be suitable for hooded rats between 250-350g. Indeed the current project utilised some rats as large as 560g. In 2018, Yang's research group suggested the use of several equations to deduce relative coordinates and ensure craniometric parameters are measured as accurately as possible (445). Future investigations should employ these equations to ensure the correct brain regions are targeted. Further, as there will always be slight variations when using a brain atlas, it is advisable to verify proposed coordinated in test animals first.

Lastly, the study largely focused on the activation of VTA dopamine neurons. In future research it is vital to also investigate the effect of inhibition through use of the hM4Di receptor, or the kappa-opioid receptors (KORD) (263, 446). Since KORD utilises a different ligand, this could also allow for testing of inhibition and excitation in the same experimental animal.

5.5 Translation of results across species, ages and sexes

Even if all of the previous factors were considered, the challenge of translating results across species will remain. It cannot be disputed that the brain and body are incredibly complex, and

healthy function requires the activity and coordination of many complicated systems including dopaminergic, GABAergic and glutamatergic to name just a few. In intact animals, these systems are all under the control of several inhibitory and excitatory inputs working together (447). Therefore, the manipulation of a single pathway, or neurotransmitter system, in a rat model for a limited period of time is unlikely to completely translate to, or be fully reproducible in humans. The exact effects of DREADD activation will remain unknown in relation to neuron location downstream in neuronal pathways. To fully characterise the effects of DREADD technology, there are aspects that should be tested either directly with electrophysiology or calcium imaging (448, 449).

Specifically in relation to the VTA, the anatomy of rats and humans must be taken into consideration. So, whilst both rats and humans have their midbrain dopaminergic neurons divided into the VTA and SNc, they are slightly varied (145, 450). Compared to humans, the rat VTA is located more medially in the midbrain, with projections going primarily to the NAc and PFC; in primates, including humans, the VTA is relatively small – suggesting it may serve a different function, adding to difficulties in result translation (125, 450). Indeed, an inverted U-shape curve between dopamine levels and cognitive function has been suggested by Cools and D'Esposito (451), which supports the ideas presented here, proposing that a fine balance of dopamine is required to maintain healthy function. This also further highlights the complexity of dopamine's action.

Additionally, an important factor to consider when interpreting and translating results is the issue of senescence – that is, the physiological and progressive event that leads to impairment of normal function of the organism. The nervous system is one of the most significantly affected systems during the aging process, and a noteworthy study conducted by Norrara and colleagues (2018), found that there were substantial differences in the number of dopaminergic neurons in the VTA of young and aged groups of rodents (452). Whilst the present experiments utilised 'young' rats that were around 3 months old, the age of animals is not identified in many other investigations. As such, care should be taken when drawing firm conclusions from any previous finding regarding physiological responses to stress. Indeed, it is possible that the body's physiological response is changed throughout the aging process due to loss of VTA dopamine neurons. Although not directly related to aging, in future the level of viral vector expression should also be studied, and its stability tested. This was not directly addressed in this thesis, and since physiological testing occurred over a period of time, up to several weeks, it is potential that viral

vector expression may have decreased during this time period - thus affecting results obtained late in the testing period.

It is also critical to note that the investigations done in this thesis utilised male rats. Indeed it has been shown that VTA circuits show sexual dimporphism that is programmed by sex hormones during perinatal life (376). As a result, females tend to show overall amplified VTA dopamine turnover and release (453). This is caused by estradiol, which has been found to stimulate dopamine function – with estrogen receptors being expressed on both dopaminergic neurons of the VTA and those projecting to the NAc. Interestingly, androgen receptors are found on dopaminergic neurons projecting to the mPFC (454). This may highlight the different strategies that males and females adopt to deal with 'stress'. Whilst males favour the 'fight or flight' response and often attempt to gain control; females tend to rely on a passive strategy termed 'tend and befriend' (376, 455). As such, social defeat is demonstrated to increases dopamine turnover in the ventral striatum, whilst social support involves the mesolimbic dopamine system (456). This may also account for the results found in this thesis, and it is vital to further investigate the role of the VTA and mesolimbic pathway in females, because generalizations between the two sexes cannot be made.

5.6 Avenue for further examination

Dopaminergic neurons of the VTA also express serotonin (5-HT) receptors and 5-HT neurons are located closed to its poorly defined borders. Indeed, the 5-HT system has previously been suggested to participate in thermoregulation (83, 457), and regulation of BAT thermogenesis (458). Ishiwata (2017) has even suggested that 5-HT, and not dopamine, is what modulates heat regulation through action in the VTA (459). Interestingly, behavioural studies in rats have also demonstrated sustained hyperlocomotion following 2-mehtyl 5-HT administration in the VTA (460), which is consistent with the observations made Chapter 2 of this thesis. Chemogenetic activation of the 5-HT_{2C} expressing neurons in the VTA was also found to modulate food-motivated behaviour (461). Further, it has been demonstrated that BAT serotonin in mobilized during both acute and chronic cold exposure (462). This evidence suggests that the 5-HT system should similarly be further explored in relation to its role in temperature regulation.

5.7 Conclusion

This investigation demonstrated that both targeted toxins and DREADD technology are powerful tools for the investigation of brain circuitry, and the effect of neural activation or reduction on resultant physiological conditions. Selective activation of VTA dopaminergic neurons produced significant effects in various thermoregulatory aspects including locomotor activity and feeding behaviour. It was determined that the resulting temperature increases were contributed to, but not solely caused by this disrupted feeding behaviour; and further demonstrated that this was largely regulated by the dopamine D_1 receptor. Further investigation of the mesolimbic dopamine system indicated that whilst it did have a significant impact in the regulation of normal feeding behaviours, it is unlikely to be the primary system involved in thermoregulation and maintenance of emotional hyperthermia. Together these findings improve the fundamental understanding of the dopamine system and its regulation of bodily functions. By providing further evidence of the VTA as a brain region involved in thermoregulation, new therapies aimed at easing the burden of thermoregulatory disorders such a Parkinson's and psychogenic fever can be investigated and refined. Although BAT thermogenesis has previously been shown to be regulated by the VTA, based on the data presented in this thesis it cannot be conclusively confirmed whether VTA dopamine-induced thermogenesis is a direct effect or results as a consequence of changes in activity and motivated behaviours. In order to decisively conclude the action of dopamine neurons in the VTA, and their involvement in thermoregulation, further investigations must be performed on non-conscious animals to establish BAT nerve activity and remove confounding factors such as locomotor activity.

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APPENDIX

Thermistor Probe Production

This method was used to create probes for the measurement of BAT and body temperature. Two strands of Insulated wire (New England Wire Teach, Lisbon, NH USA Part #N12-50F-254-O) was twisted together and cut to a length of 7cm for the BAT temperature probe and 12cm for the body temperature probe. A thermistor temperature probe (surface mount thermistor, NCP15XH103J03RC, Murata, SMD, 10K) was then soldered on the end of double wire, with one wire attached to each side of the thermistor.

A thin layer of nail polish was applied to cover the thermistor. Once the nail polish had dried, one end of the wire was connected to a 9V battery, and the opposite end with the thermistor was submerged in Ringer's Solution to ensure no gaps in the coating. If the presence of air bubbles emerging from the thermistor was noted, more nail polish was applied and the process was repeated. The thermistor was then covered with a layer of silicone gel (ACC Silicones Ltd, Somerset, UK) and allowed to dry for 24hours.

The wires from the thermistor probe were soldered onto a 8-pin head socket piece (6-7467, ROHS, Switzerland). Each wire was connected to a pin, meaning each thermistor was connected to a total of 2 pins. The head socket piece was further modified to include a nut in the centre to facilitate the attachment of a 'gooseneck cable'. UV-gel was applied over the soldered pins to secure the wire. The thermistor probes were then calibrated to ensure accurate temperature readings. This was achieved connecting the probes to LabChart recording software and then immersing in a water bath of 40°C, as taken from a mercury thermometer. Once the temperature readings were stable, a calibration point was recorded and the voltage reading for the temperature was taken. Ice was them slowly added until water bath temperature had reached 35°C and 30°C respectively, at which two more calibration points were taken. Calibration temperatures were chosen based on the physiological range for their operation.

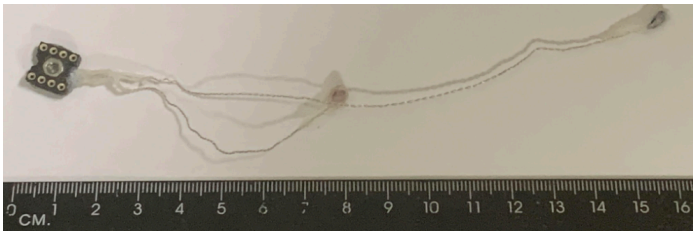
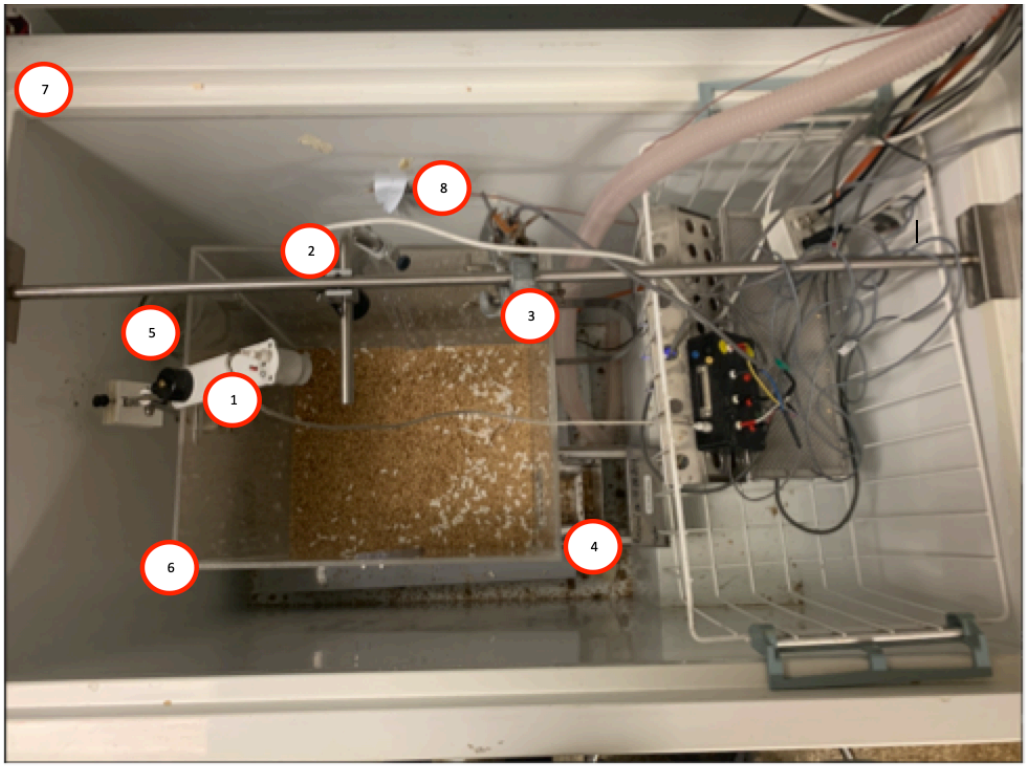


Figure 40: Photo of head socket with BAT (black) and body (red) probes attached

Experimental Set-up:



Home Cage Set-up:

1. Counter-balanced Swivel device
2. Camera
3. Infrared Motion Sensor
4. Food Hopper
5. Drinking Water
6. 'Home Cage'
7. Modified Freezer Unit
8. Temperature Sensor

Recording Set-up:

1. Recording Chamber
2. Computer
3. 'Day/Night' Cycle Timer
4. Temperature Controller
5. Bridge Amplified
6. PowerLab
7. Thermistor Amplifier

Figure 41: Home Cage set-up and recording device connections.

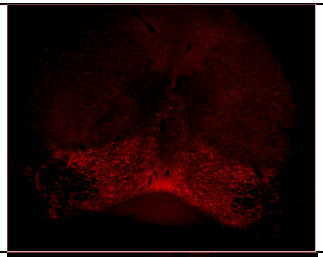
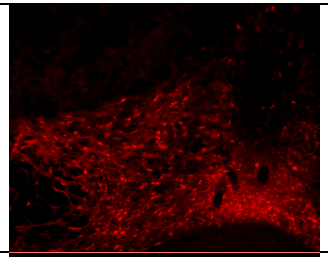
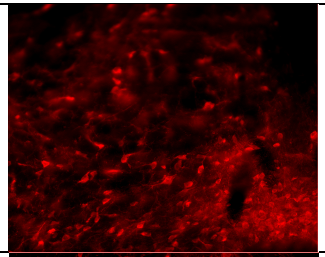
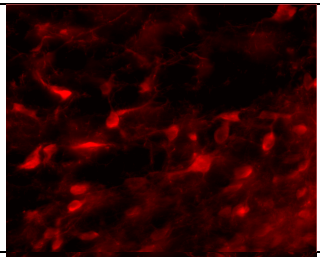
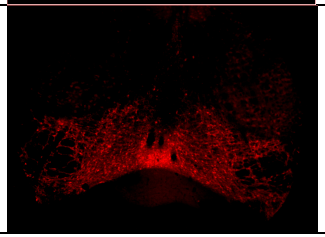
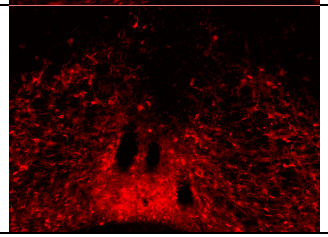
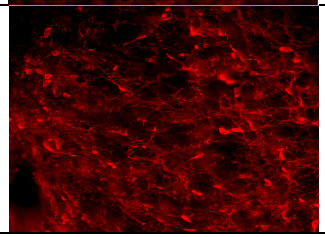
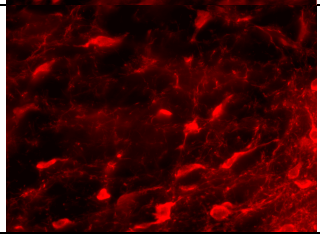
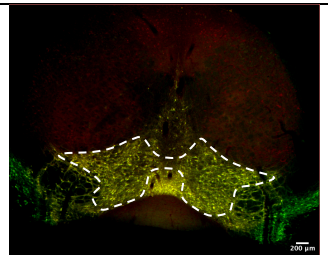
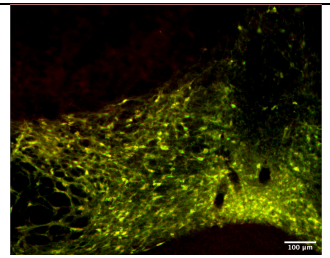
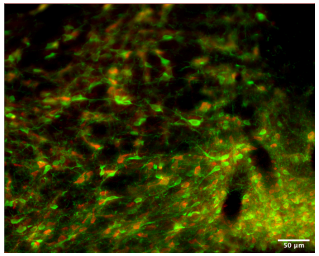
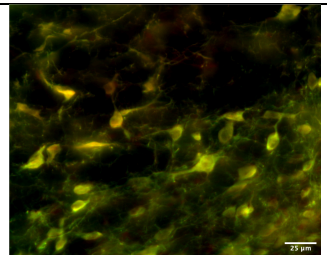
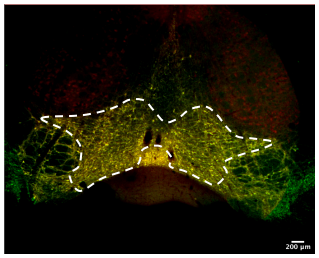
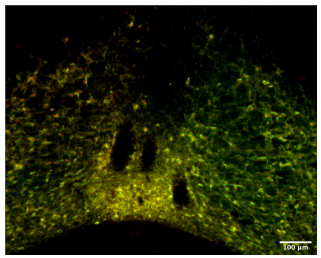
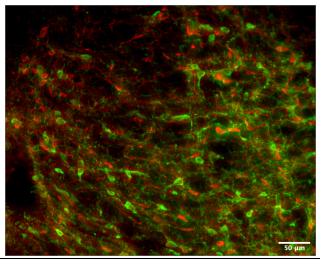
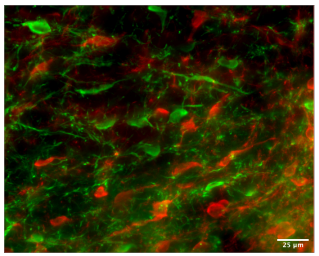
NeuN staining	4x	10x	20x	40x
Vehicle (PBS) Treated				
Anti DAT-Saporin Treated				
Overlay with TH staining:				
Vehicle (PBS) Treated				
Anti DAT-Saporin Treated				

Figure 42: Representative immunohistochemical demonstration of general neuron (red - NeuN) presence in coronal sections through the midbrain of a vehicle and Anti DAT-Saporin treated animal. Overlay images with dopamine (green – TH) staining are also shown with white outline indicating VTA region. Scale bar is located in the bottom right corner of each overlay image; 4x = 200µm, 10x = 100µm, 20x = 50µm, 40x = 25µm