MONASH University

Motivation for Starvation and Flexible Adaptation: Investigating the Imbalance Between Reward and Control in an Activity-Based Rat Model of Anorexia Nervosa

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A thesis submitted for the degree of

Doctor of Philosophy

at Monash University

July 2021

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Monash Biomedicine Discovery Institute

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Abstract

Introduction: Anorexia nervosa (AN) is a debilitating psychiatric illness characterized by relentless food restriction and excessive exercise, leading to emaciation. AN has a chronic time course, high relapse rates, and the highest mortality rate of any psychiatric disorder, yet available treatments are largely ineffective, due in part, to an incomplete understanding of the neurobiological drivers that underpin the condition. Functional brain imaging studies have identified an imbalance between diminished reward processing and excessive cognitive control that may underpin the extreme behaviours seen in patients with AN. However, the biological mechanisms underlying these disruptions remain unknown. Understanding these mechanisms is required for a pathway to pharmacological intervention and will likely come from animal-based experimentation. The activity-based anorexia (ABA) paradigm is the most robust and widely used animal model of AN as it recapitulates both voluntary food restriction and voluntary hyperactivity, resulting in rapid and precipitous weight loss. The series of studies described in this thesis utilizes the ABA rat model in combination with a suite of behavioural and neuromodulatory techniques to elucidate the roles of mood, motivation and cognition and their neurobiological underpinnings in the development of pathological weight loss.

Methods: The associations between susceptibility to ABA and hedonic processing and mood-related behaviours were examined using choice paradigms and traditional maze-based assays, while cognitive flexibility was assessed using touchscreen serial reversal learning tasks in both conventional and novel fully-automated testing systems. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) were used in combination with retrogradely transporting Cre recombinase to selectively target and modulate neurons in the medial prefrontal cortex (mPFC) with direct projections to the shell of the nucleus accumbens (AcbSh) in order to highlight a neurobiological link between ABA outcomes and cognitive flexibility.

Results: Susceptibility to body weight loss in the ABA paradigm was not associated with altered hedonic processing and was not predicted by baseline measures of locomotor activity, anxiety-like, or compulsive behaviour. However, in all ABA experiments running in anticipation of food, an index of motivation to eat, was a reliable predictor of resistance to weight loss in ABA, whereas elevated wheel running before food restriction, and development of marked starvation-induced hyperactivity following food restriction onset, was associated with increased susceptibility. Chemogenetic inhibition of the mPFC-AcbSh pathway conferred 100% resistance to ABA and improved cognitive flexibility by reducing perseverative responding following reversal of reward contingencies. These findings suggest that both motivation for feeding and the capacity to adapt to changes in food availability are essential to body weight maintenance under ABA conditions. Moreover, learning rate was increased ~5-fold using the automated home-cage living and touchscreen testing system, the PhenoSys.

Discussion: These results have been synthesized to create a more complete description of the interplay between reward and cognitive control and, as such, may help to inform the development of effective therapeutics for AN. Introduction of a fully automated behavioural testing system to eliminate experimenter influence has proven the utility of this approach for increasing reproducibility and throughput. This represents the new frontier of behavioural neuroscience and will allow cognition and ABA to be examined in the same animals, further increasing our understanding of the causal cognitive factors associated with ABA and ultimately creating better insights into the aetiology of the human condition.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Date: 13th July, 2021

Publications during enrolment

1. Milton LK, Oldfield BJ & Foldi CJ (2018). Evaluating anhedonia in the activity-based anorexia (ABA) rat model. *Physiology and Behavior*, 194, 324-332.

(This manuscript contains data collected as part of Experiment 1; Chapter 2)

2. Milton LK, Mirabella PN, Greaves E, Spanswick DC, van den Buuse M, Oldfield BJ & Foldi CJ (2020). Suppression of cortico-striatal circuit activity improves cognitive flexibility and prevents body weight loss in activity-based anorexia in rats. *Biological Psychiatry*, 10.1016/j.biopsych.2020.06.022

(This manuscript contains data collected as part of Chapter 3 and Experiment 1; Chapter 4)

Acknowledgements

To my extraordinary supervisors, Dr Claire Foldi and Professor Brian Oldfield, words fail me in how to adequately express my gratitude. Claire, you've been my rock. Whenever I started floundering like a turtle stuck on its back (hello ADHD executive dysfunction) you were there to flip me over, point the way and give me a push in the right direction to get me back on track. If I could describe the perfect supervisor (handler) for me, it would be you. Brian, thank you for cultivating such a friendly and supportive environment for my entire post-grad experience, and for the insight and guidance along the way as I found my footing as a research scientist. You both complement each other in the way you've guided and facilitated my post-grad journey and I'll forever be grateful to have won the supervisor jackpot, especially given I know I'm not the easiest student to corral!

A huge thank you to my fellow members of the Oldfield-Foldi (OldFoldi) laboratory conglomerate for their unwavering support over the last 5 years. A particularly big thank you to Dr Paul Mirabella for conducting the electrophysiology work reported in Chapter 4, but predominantly for the ongoing friendship, support and assistance throughout my Honours year and 4 year PhD, you helped keep me sane and were always there whenever and for whatever I needed. Also, a big thank you to Erika Greaves for assistance with wet lab animal procedures throughout, and in particular for assisting Claire with animal processing at the end of the La Trobe touchscreen experiment when I was hospitalized with a serious knee injury, and to Jess Hudson for your help over the years with all the administration tasks that cause my brain to short circuit. A special thank you to Claire's Masters student Alyssa Budin and Honours student Carla Pietrucci for their enthusiasm to learn and patience with me as I tested the waters of mentoring fellow students, thanks for being my human "lab rats".

I would like to thank my Milestone panel members Professor Zane Andrews and Professor Andrew Lawrence for their valuable feedback and suggestions offered at each annual review which helped to improve the overall body of work produced during my PhD.

I would like to thank my external collaborators without whom the touchscreen experiments would not have been possible. Professor Maarten van den Buuse and Dr Emily Jaehne from La Trobe University for providing access to and assistance with running the conventional touchscreen system. Karsten Krepinsky and York Winter from PhenoSys in Berlin for collaboration in designing, building and troubleshooting the PhenoSys automated touchscreen and sorting system and PhenoSoft operating software.

Thank you to Debra Lane for animal care and husbandry in accordance with the specific requirements for my experiments that are especially sensitive to environmental disturbance.

I could not have dreamed of doing a PhD, let alone completing it, without the incredible support, love and encouragement from my family and friends. To my parents Annie and Niall, my sister Morag and my brother Ieuan, thank you from the bottom of my heart for believing in me when I didn't believe in myself, lifting me up (metaphorically and literally) when I was down, and for pushing me when I needed to be pushed. We did it!

To my best friends Phoebe, Freya and Matt, you know more than anyone what I've been through to get where I am now, and I owe you all for helping me get here. You've stuck with me through thick and thin and I can only hope you know how much I appreciate your friendship and unconditional support, I love you guys!

To my netball family, the Monash University Netball Club and the St Justin's Netball Club, especially my Monash Platinum teammates, thank you! Thank you for making the time we spend together, both on and off the netball court, my mental escape from the stress of PhD life! The joy I get from playing netball is largely due to the friendships we've created and your support and encouragement has been invaluable over the last few years, especially through my ACL recovery and rehab.

A considerable thank you to my mental health team over the years, in particular Dr John Stevens and Jacqui Louder, and the inventers of Ritalin (ADHD with a PhD!). Thank you for helping me to navigate life's tumultuous ups and downs to be able to complete a PhD along the way.

Finally, this research was supported by an Australian Government Research Training Program (RTP) Scholarship, Monash University Graduate Research Completion Reward (GRCA), and a Post-Graduate Award granted by the Harrold-Mitchell Foundation.

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CHAPTER ONE

General Introduction and Thesis Overview

1.1 General introduction

Anorexia nervosa (AN) is a debilitating psychiatric illness characterized by persistent food restriction, and, in the majority of cases, excessive physical activity, leading to maintenance of an extremely low body weight (1-4). Patients acutely ill with AN display an endocrine profile indicative of physiological starvation (5, 6), specifically, reduced leptin secretion as a consequence of low body fat (6-10) and increased levels of the “hunger hormone” ghrelin (9, 11-13). In a healthy person, this endocrine milieu drives food consumption and reduction of energy expenditure (5) – yet this does not occur in patients suffering from AN (14), who engage in dieting and exercise behaviour, despite emaciation, often as a means by which to alleviate dysphoric mood (1, 3, 14, 15). Thus, AN represents a stunning paradox whereby there is an appropriate physiological response to the negative energy state but a subsequent failure to translate this signal into the necessary actions to rectify the energy deficit (5, 14), which may potentially be mediated via an interaction between neuroendocrine factors and reward processing (9, 16, 17). As a result, AN has the highest mortality rate of any psychiatric illness (18, 19), with a chronic, unremitting time course that is exacerbated by high relapse rates (20-22) and a lack of adequate therapies (23-25). The overarching question becomes; how is it possible that individuals suffering AN are able to voluntarily restrict food intake to the point of starvation when most of us find it difficult to lose weight and even more difficult to maintain moderate levels of weight loss? At least part of the answer is that neuronal circuits controlling higher-order cognitive and emotional processes and associated behavioural outcomes must override the evolutionary ancient drive for survival (2, 14). Understanding the nature of these interactions is necessary for the development of effective treatment strategies for AN in order to alleviate what is often lifelong suffering in patients.

AN occurs predominantly in female adolescents (26) and is associated with psychological symptoms including an intense fear of weight gain, a drive for thinness, and distorted body image, features that have been historically considered as purely psychosocial in nature, arising from an overemphasis of these “ideals” in social and media contexts (14, 27-29). This has

hampered the development of effective therapies, with pharmacological approaches for patients with AN largely restricted to targeting comorbid psychological symptomology (depressive, anxious and obsessive/compulsive phenotypes; see **Chapter 2**). While these approaches have been helpful in alleviating comorbid symptoms that often worsen the outcomes of AN, medications with anti-depressant, anti-anxiety or anti-compulsive actions have little impact on the core features of the disorder (23-25). Moreover, while some forms of psychotherapy, including cognitive-behaviour therapy (CBT) and family therapy, have shown some short-term improvements for patient outcomes (25), these are often only effective in combination with pharmacotherapeutic approaches and do not prevent relapse (30). It is widely appreciated that the psychosocial aspects of AN are consequential and may explain the initial intent to diet and exercise: however, they offer little explanation as to how and why patients with AN maintain extreme levels of these behaviours in the face of body weight that becomes dangerously low.

Over the last 20 years, a large body of evidence has accumulated to support the notion that AN has biological origins (31), including its genetic aetiology and neurobiological drivers (1, 32-34), that may, in themselves, generate psychological symptoms to emerge (2, 14, 35, 36). The heritability of AN is high (37, 38), with increased (up to 11 times) incidence in female first degree relatives (38-41). Genome wide association studies (GWAS) have shown significant correlations between AN and both psychiatric and metabolic parameters (32, 33). Of these the strongest psychiatric association was with obsessive compulsive disorder (OCD) (32, 42) and the first significant locus associated with AN was with metabolic parameters (43). This has led to a reevaluation of the genesis of the disorder and acceptance of the fact that AN has both psychiatric and metabolic origins, with the latter driving the search for candidate responsible genes. Multiple genetic markers have been identified in patient populations, including those within the dopamine (DA) (44, 45) and serotonin (5-HT) (38, 46-49) systems, which play a role in mood, executive control, reward, appetite and feeding, and physical activity (1, 50-52), as well as the appetitive control system (53-56); see (50, 51, 57-59) for comprehensive reviews. While the genetic studies point towards predisposing/risk factors associated with the aetiology of AN, there has been limited consistency amongst findings across studies, with no candidate marker emerging as a prime candidate (51, 60).

From a neurobiological standpoint, structural and functional neuroimaging studies have shown consistent differences in the brains of patients with AN during acute illness and following weight recovery. These include structural abnormalities in both grey (61-67) and white (62, 64-66)

matter, such as reduced overall grey matter volume (GMV) (62, 64, 65) and global cortical thinning (68, 69). There are also region-specific GMV reductions in areas of clinical importance specific to illness-relevant behaviours including reward processing (61-64, 66), and increased GMV in the prefrontal and orbitofrontal cortex (66, 67), with some of these regional changes correlated to eating disorder psychopathology (64, 66, 68). Global and most region-specific reductions resolve following weight recovery, suggesting they are a consequence of acute starvation, although these changes also likely play a role in the maintenance of AN behaviours (63-65, 68, 69). There are, however, specific morphological abnormalities related to reward processing and cognitive control that remain following weight regain and may indicate brain areas of aetiological relevance (63, 64).

In addition to structural abnormalities, functional imaging studies have found altered brain activity in patients acutely ill with AN as well as following weight recovery (REC-AN). For example, during a behavioural task requiring reward-related decision-making, acutely ill patients with AN have *decreased* activation in reward related regions, associated with decreased sensitivity to and perception of reward (See **Chapter 2**), although this normalizes after weight recovery (70). Acutely ill patients with AN display *increased* activation in reward regions in response to illness-affirming stimuli such as images of underweight women (71, 72) and in prefrontal cognitive control circuitry after illness-relevant aversive stimuli such as images of food (73-75) with the latter still observed in REC-AN patients (73). REC-AN patients display *increased* activation in prefrontal regions, associated with cognitive control (See **Chapter 3**) during reward-related decision making (76, 77), which is insensitive to hunger/satiety (77), *decreased* activation in striatal regions in response to tasting sucrose (78), and lack the normal differentiation in ventral striatal activity in response to positive and negative feedback (79). Finally, both acutely ill and weight recovered patients with AN have perturbed signaling in the insula associated with compromised/disrupted interoceptive awareness of hunger and response to food reward (75, 78, 80-82), such that patients with AN do not appear to be motivated by hunger (77, 83).

While these studies show clear biological determinants of AN and have been important in directing attention to the neurobiological drivers of the condition, it is not possible to determine from brain imaging studies whether these changes are a consequence of current or prior starvation in the acutely ill stage (representing a ‘scar’ when identified in weight recovered

patients) or whether they existed before the onset of AN and play a causal role in AN aetiology (1, 2).

To determine whether these abnormalities in brain activity are causally involved in the onset of AN, it is necessary to use animal models in which neural circuits can be manipulated before and/or during the development of anorectic behaviour (84, 85). While it is impossible to recapitulate the full spectrum of AN symptoms in animal models, such studies have been instrumental in uncovering specific behavioural and neurobiological features associated with pathological weight loss that have led to a more detailed understanding of the processes involved in the human condition (84-87). A number of animal models have served as useful tools to better investigate the biological basis of AN including genetic, stress and environmental models (88, 89). Genetic models include the homozygous anx/anx mouse model that develops the primary symptom of AN, namely, starvation and subsequent emaciation; however, while these mice also demonstrate a range of hypothalamic and neuropeptidergic changes that mimic those seen in patients with AN, animals die prematurely between 3-5 weeks of age (90), making it difficult to extrapolate these findings to the dysfunction seen in humans (87, 89). Stress models have been developed to evaluate the strong association of stress with the onset of AN pathology and centre around the fact that separation or restraint stress often leads to reduced food intake and weight loss in rodents (87-89). However, these types of stressors also have widespread central and peripheral effects that are not typical of those seen in patients with AN (91) and are therefore of limited use in understanding the specific causes of the human condition.

The most robust and widely used animal model of AN is the activity-based anorexia (ABA) rodent model (85, 87, 89). The ABA model was first described 50 years ago (92, 93) and involves pairing unlimited voluntary access to a running wheel with time-restricted access to food (generally 1-2hr/day) (85, 87). Its utility is based in the observation that rats (and mice) exposed to ABA conditions exhibit a *voluntary* reduction in food intake and a paradoxical increase in energy expenditure through *voluntary* and *excessive* exercise, despite rapidly declining body weight (84, 86, 94). Importantly, exposure to *either* time-restricted feeding or access to a running wheel alone does not produce the excessive hyperactivity or precipitous weight loss characteristic of the development of ABA that occurs when the two aspects are combined (89, 94, 95). On the one hand, sedentary rats on the same food restriction schedule initially lose a small amount of body weight (~10%), but readily adapt to the feeding schedule to

increase food intake when it is available in order to maintain body weight (95-99). On the other hand, rats maintained on *ad libitum* feeding with access to running wheels increase food intake to compensate for the energy expended through running (84, 97-99). In this way, the ABA model that combines both components (individually ineffective in promoting profound weight loss) mimics the core features of the human condition and will result in death if left unchecked (84, 85, 92, 100). ABA rats also have elevated ghrelin (97, 101) and reduced leptin (97, 102, 103) in keeping with the endocrine profile of human AN. Following chronic exposure to ABA, rats have significantly decreased cerebral cortex volume (104, 105), which normalizes after weight recovery (105), and prior exposure to ABA is associated with increased anxiety-like behaviour (106-109), findings which both mirror changes seen in human patients with AN. Furthermore, impaired cognitive flexibility is one of the major cognitive deficits that is routinely found in human patients with AN (See **Chapter 4**) (110-112). In this respect, rats at low body weight following ABA also have impairments in cognitive flexibility, which is normalized following weight recovery (113). These observations help cement the utility of the ABA model in reproducing behavioural, physiological/biological and *cognitive* perturbations characteristic of AN (113).

Having established the *bona fides* of the ABA approach, a number of studies have attempted to prevent or rescue the development of ABA in rats, with conflicting results. Interventions targeting the homeostatic control of feeding behavior with manipulations of hormones, including leptin or ghrelin (98, 101, 114, 115) or neuropeptides such as AgRP, NPY or a-MSH (99, 116-118), have variously shown mixed improvements or decrements in the development of the ABA phenotype and/or metabolic outcomes. In regions outside the traditionally accepted hypothalamic/homeostatic control of feeding behavior, the evidence for disrupted DA and 5-HT signaling in patients with AN (1, 2, 50-52) has prompted a focus on such transmitters in ABA. For example, exposure to ABA is associated with a reduction in 5-HT release in the nucleus accumbens (119), whereas increasing 5-HT availability with fluoxetine administration or otherwise increasing the activity of 5-HT receptors again shows variable and often conflicting outcomes in ABA (120-122). This is also the likely case for the involvement of DA signaling on the development of ABA, whereby administration of DA antagonists improves (non-selective D2/3, selective D2) or has no effect on (selective D3) ABA outcomes (123, 124). While it is clear that both 5-HT and DA systems are involved in the ABA phenotype, the variability in outcomes highlights the need for more detailed investigation of the specific circuits and receptor subtypes involved. This more incisive approach is exemplified by the pathway-specific

chemogenetic activation of DA neurons in the ventral tegmental area (VTA) that project to the shell of the nucleus accumbens (AcbSh), which is demonstrated to both prevent and rescue the ABA phenotype (95). What remains to be determined is how pathological weight loss in ABA rats is driven by behavioural aspects of reward, mood and cognition that precede the development of the ABA phenotype and how the neurocircuitry controlling anorectic behaviour in ABA fits with the hypothesis that the human condition arises due to an imbalance between reward processing and cognitive control.

1.2 Thesis Overview

This thesis comprises three experimental chapters aimed at gaining a detailed understanding of the behavioural and neurobiological processes underlying the imbalance of reward and cognitive control in patients with AN. These studies utilize the ABA rat model in combination with standard behavioural test paradigms (**Chapter 2)** and viral-based neuromodulatory approaches (**Chapter 3**) to address the nature of this imbalance. Moreover, the underlying cognitive processes that promote the development of ABA in rats are probed in **Chapter 4** using touchscreen-based operant testing, a paradigm which is, for the first time, also assessed using a novel automated and experimenter-free touchscreen testing system for rats. **Chapter 2** "Evaluating Mood-Related Comorbidities of AN in the ABA Rat Model" addresses the specific components of reward processing that are associated with ABA-induced weight loss, and whether mood-related phenotypes that are commonly comorbid in patients with AN are predictive of susceptibility to ABA in the rat. **Chapter 3** "Corticostriatal Control of Body Weight and Motivated Behaviour" addresses the role of cognitive control in the development of ABA through the incisive use of pathway-specific chemogenetic modulation, and whether any effects on anorectic outcomes are specific to the ABA context. **Chapter 4** "Corticostriatal Regulation and Rapid Assessment of Cognitive Flexibility" builds on the findings of **Chapter 3** to examine *how* the neural pathway involved in susceptibility to ABA is linked to the cognitive phenotype of flexible adaptation, using a task that is then developed and optimized for examination of cognition in the same animals that go on to be exposed to ABA conditions. Taken together, the results from these studies confirm the hypothesis that anorectic behaviour in ABA (and possibly AN) arises from an imbalance between reward processing and cognitive control that specifically relates to the domains of motivation and cognitive flexibility.

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CHAPTER TWO

Evaluating Mood-Related Comorbidities of AN in the ABA Rat Model

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2.1 Introduction

Patients with AN often have comorbid psychiatric diagnoses, the most common of which are depression, anxiety, and obsessive-compulsive disorder (OCD) (1-8). It is important to note in relation to the latter that a secondary diagnosis of OCD requires that the nature of the obsessions and compulsions are not illness relevant, such as obsessions around food and ritualized eating behaviour or compulsive exercise to lose weight (1). As such the classification of patients with AN as suffering OCD is complex and problematic. However, while not always leading to an official comorbid diagnosis, patients with AN routinely exhibit elevated scores on common measures of depressive (9-15), anxious (10, 12-16), and obsessive/compulsive symptoms (17). These issues considered, either a comorbid diagnosis or the expression of symptoms related to other psychopathologies are associated with worsened illness, poorer treatment outcomes, and increased relapse, especially if there is more than one comorbid diagnosis (5, 18-21).

Although not part of the diagnostic criteria for AN, a blunted ability to experience pleasure, referred to as anhedonia (22, 23), and excessive exercise (24) are common features of AN symptomology. Patients with AN have “long been noted to be anhedonic” (25, 26), and excessive exercise is present in up to 80% of the AN population (24, 27, 28). These phenomena in themselves do not necessarily warrant a psychiatric diagnosis, but are behavioural tendencies exhibited by patients with AN, which may, in some cases, arise as symptoms of a comorbid illness. For example, the expression of anhedonia may arise from comorbid major depressive disorder (9, 29) and the propensity to exercise excessively may develop as, or turn into, a mechanism to alleviate dysphoric mood (23, 30-34). That being said, there is evidence that excessive exercise often predates onset of AN symptoms and diagnosis (28, 35), worsens with illness progression (often becoming compulsive (28, 32, 34)) and is associated with poorer treatment outcomes (36, 37) and relapse (38, 39), and endures following AN recovery (although to a lesser extent (37)), indicating that it may be a predisposing and/or facilitating behaviour but is not strictly a symptom of AN. It has also been shown that excessive exercise in childhood is a risk factor for later development of AN (40), with activity

levels generally accelerating in the year before illness onset (35). Patients with AN who exercise excessively exhibit more anhedonic/depressed (11, 23), anxious (31, 33, 41) and obsessive/compulsive (30, 41, 42) symptoms than patients with AN who do not exercise excessively. Moreover, in patients with AN, levels of anxiety (33, 34) and obsessiveness/compulsiveness (43) are associated with increased levels of physical activity and exercise intensity. Eating disorder symptoms are also positively associated with exercise level, such that psychological markers of illness severity increase with levels of exercise/physical activity (11, 31, 33, 34, 36, 43). Taken together, this indicates that excessive exercise in AN has a compounding effect on both eating disorder (ED) and non-ED psychological pathologies. It should be noted that the above conclusions are not universally held, with many studies finding no differences in some of these outcome measures between patients with AN, classified as “excessive” or “non-excessive” exercisers, or between patients with AN and healthy controls (24). This discrepancy may be partly explained by the recent observations based in a systematic review that despite an abundance of research conducted on exercise/physical activity in EDs there is no standard definition, conceptualization, or measure of exercise/physical activity, complicating the understanding of the role exercise plays in the onset, maintenance of and relapse of AN (24). It is also important to note that excessive, and even compulsive, exercise is not restricted to patients with AN, or other ED diagnoses such as bulimia nervosa or eating disorder not otherwise specified (EDNOS), but can exist in non-clinical healthy people (often athletes); it is only when it exists in combination with disordered eating behaviour that it becomes problematic and diagnosed as such (44).

It is difficult to dissect the causal relationship and the direction of this relationship, arising from the interaction between AN and these common comorbid disorders. In fact there is evidence supporting each of the contingencies including support for AN diagnoses preceding the development of comorbid psychiatric disorders (4, 6, 7), coincident onset of AN and a comorbid illness (4, 6, 7), as well as the development of AN following a preexisting diagnosis of depression, anxiety, or OCD (4, 6, 7, 45-47). It is also possible that AN exists as a completely independent disorder with no comorbid diagnosis, although this is very rare. In support of the hypothesis that mood disturbances may increase susceptibility to AN in vulnerable individuals, childhood temperaments and personality traits including picky eating, perfectionism, harm avoidance and rigid patterns of thought and behaviour (17, 48), related to levels of anxiety and compulsiveness (4), have been associated with later development of AN. These traits often become exacerbated in the acute stage of AN and improve with weight recovery but remain

elevated compared to healthy controls (4, 13), indicating that underlying personality traits are partially independent of illness status and may predispose to development of AN (4). Moreover, familial studies provide evidence for a genetic and aetiological overlap between AN and depression, anxiety, and OCD with increased rates of ED diagnoses in first degree relatives of patients with AN (49-52) and increased diagnoses of depression, anxiety, and OCD (and non-pathological symptoms of these conditions) in healthy first degree relatives of patients with AN compared to the general population (50, 53, 54). Additionally, there is genetic overlap between AN and generalized anxiety disorder (55) and major depression (56), and a recent genome wide association study (GWAS) found a genetic association between AN and OCD (57). This evidence suggests the existence of heritable risk factors that cluster together and sometimes result in the development of AN. This is not surprising considering that each of these mood-related pathologies are associated with common neurobiological and behavioural phenotypes with AN, including disruptions to reward processing (58-60).

The neurobiology underpinning reward processing is multifaceted, encompassing aspects of: **1)** the perception of pleasure arising from the receipt of rewards, which is encoded to a large extent by μ opioid, endocannabinoid, and GABA receptors in the ventral striatum (VS), including the nucleus accumbens (Acb); **2)** the valuation of reward arising from the ability to predict and anticipate rewarded outcomes and their relative value, which occurs in subregions of the prefrontal cortex (PFC), particularly the orbitofrontal cortex (OFC); **3)** the motivation for, drive to obtain and calculation of effort required to obtain a reward, which is encoded by regions including the anterior cingulate cortex (ACC) via incorporation of prior reward related outcomes (from ventral striatal regions); and **4)** the process of decision-making, which involves aspects of learning and incorporation of positive and negative feedback that takes place in the anterior ventromedial PFC (vmPFC) and dorsolateral PFC (dlPFC) to promote goal-directed action, sending this information via glutamatergic inputs to the Acb (22, 58, 61, 62). The prediction, anticipation of, and the motivation to obtain reward are encoded by the mesolimbic dopaminergic pathway consisting of dopaminergic (DA) projections from the ventral tegmental area (VTA) to the ventral striatum (22, 63-65). Acb DA signaling is crucial for eliciting *effortful* motivated behaviour in anticipation of reward to generate the goal-directed behaviour planned by the PFC (66-69). Importantly, what may appear to be anhedonia, i.e. a global loss of interest or pleasure in normally pleasurable stimuli, may actually be a manifestation of a deficit or malfunction in any of these reward-related processes rather than an inability to actually experience pleasure (22, 70).

There has been a large body of research directed to reward deficits seen in patients with AN, with abnormalities encompassing brain activity, decision-making and behaviour, all presumably driven to some extent by genetic underpinnings (71). Acutely ill patients with AN display exaggerated subjective sensitivity to punishment (72, 73), which is greater in those with a comorbid anxiety disorder (73), as well as increased neural activity in response to negative feedback in executive control regions (74, 75). They also show increased sensitivity to reward (13, 73), specifically related to aspects of appearance and interpersonal feedback (72), which decreases with weight recovery but remains elevated compared to healthy controls (13). Acutely ill patients with AN exhibit social anhedonia, characterized by a disinterest in social contact and a lack of pleasure in social situations, which is partly recovered with body weight restoration (29). However, tactile anhedonia, in which even the anticipation of touch is unpleasant, persists in patients with AN after body weight recovery (REC-AN) (76). REC-AN patients find high-energy foods aversive (9), prefer low-calorie over high-calorie foods (10), and report increased anxiety, contrary to the pleasure or euphoria felt by healthy individuals (77), in response to the increase in dopamine that accompanies eating (26) or amphetamine administration (78). REC-AN patients also show decreased striatal activity in response to sucrose (15), abnormal striatal activity during tasks requiring reward valuation (79), and increased striatal binding of a competitive DA receptor 2/3 (D2/3) radioligand (25, 80), indicating either upregulated DA receptors or decreased endogenous DA levels (25, 80), with binding in the dorsal caudate positively correlated with harm avoidance (25, 80). Further, ventral striatal activity does not differentiate between positive and negative feedback in REC-AN patients (16), although it does in acutely ill adolescents with AN (74). Additionally, acutely-ill and REC-AN patients have reduced CSF levels of the DA metabolite homovanillic acid (81, 82), which is consistent with the multiple D2 receptor polymorphisms associated with AN (83). Taken together, this evidence implicates perturbed reward processing, particularly disruptions to dopaminergic signaling, in the aetiology of AN. However, it is not possible to determine whether these alterations are a cause or are consequences of AN, which can only be determined from animal models, in which reward processing can be perturbed and anorexic behaviour interrogated in detail.

The activity-based anorexia (ABA) rat model of AN, described in detail in the previous Chapter, pairs unhindered access to running wheels with 90-minutes of food access per day and recapitulates the core features of the human condition including rapid body weight loss and

paradoxical hyperactivity, despite minimal food intake (84-87). In addition, the ABA model is particularly well suited to investigating factors that predispose to pathological body weight loss because ABA exists across a spectrum from Susceptible to Resistant (85). While the majority of rats are susceptible to ABA and display the characteristic body weight free fall, a subpopulation of rats are resistant to weight loss, even when exposed to the same experimental conditions that normally elicit ABA. This natural variability provides an ideal control group that, to date, has not been adequately utilized in the field and that the experiments in this chapter aim to address. Multiple studies have found that rodents previously exposed to ABA display increased anxiety-like behaviour on the elevated plus maze (EPM) and the open field (OF) test (88-91), although others have found no effect (92, 93). These studies all examined anxiety-like behaviour *following* exposure to ABA conditions, therefore, much like human studies, it is unclear whether any differences in anxiety-like behaviour in the ABA rat were a *consequence* of weight loss or whether they play a role in susceptibility to the development of the ABA phenotype. While one study has reported testing on the OF *before* and the EPM *during* the period of ABA exposure, mice had access to running wheels prior to the OF and the EPM was conducted after only 1 day of restricted food access (94) – as such neither provide a reliable uncompromised measure of anxiety-like behaviour before or during ABA. This being said, an interesting finding from this study was that running wheel activity in ABA was correlated with anxiety-like behaviour measured on the EPM, but not on the OF (94).

Examining mood-related behaviours in the rat *before* exposure to the ABA paradigm enables investigation into their causal or predisposing role in the development of AN symptoms that is prohibited by the retrospective or cross sectional nature of human AN studies (4, 6, 7, 23, 46, 95). One of the most well studied and widely used tests to assess anhedonia in rats is the two-bottle preference test, in which healthy rats routinely show strong preference for sweetened water over regular water and anhedonia is inferred by a reduced or abolished preference for the sweet liquid option (22, 58). Anhedonia can be induced in models designed to replicate depressive symptoms such as chronic mild stress (CMS) (96-98) and can be generated via classical conditioning by pairing exposure to the sweet taste with a negative outcome such as lithium chloride (LiCl) induced malaise (99, 100). During CMS, sucrose preference can be reinstated/restored by treatment with antidepressant drugs (96-98).

The most widely used behavioural assay of anxiety-like behaviour is the elevated plus maze (EPM), in which the frequency and duration of exploration on the open, aversive arms of the

maze reflects a level of anxiety that is decreased by anxiogenic agents (e.g. caffeine) and increased by anxiolytic treatments (e.g. benzodiazepines) (101, 102). Measures on the EPM are inherently mediated by the level of baseline activity of individual animals, and, in this way, an assessment of “elevated anxiety” could be misinterpreted from a general increase in locomotor activity that leads the animal to visit the open arms more often. When the EPM is used in combination with the open field (OF) test, this behavioural overlap is controlled for because the OF gives a separate measure of locomotor activity, and in fact this combination provides an “internal control” for anxiety-like behaviour, with the time spent in the centre portion of the OF also sensitive to anxiogenic manipulations (e.g. genetic models of anxiety) and anxiolytic agents (e.g. Benzodiazepines) (103-105). Finally, OCD-like compulsivity can be assessed using the marble burying test (MBT), in which the degree to which rodents will bury novel marbles in an attempt to alleviate the unpleasantness associated with their novelty has been proposed, with some contention, as a test for OCD-like compulsivity. Support for the use of the MBT comes from reports that this behaviour is reduced by administration of selective serotonin reuptake inhibitors (SSRI’s), the most commonly used anti-depressant medication, but is also altered with administration of anxiolytic drugs that have no anti-compulsive activity (106-108). Further, marble burying has been shown to be independent of other anxiety-like traits and to remain constant after multiple tests and is therefore thought to be reflective of repetitive and perseverative (i.e. compulsive) digging behaviour rather than a facet of anxiety (109).

This Chapter examines whether depressive, anxiety-like and compulsive behaviours, as defined by a bank of the tests and behavioral assays outlined above, contribute to the development of ABA in adolescent female rats. The overarching aim therefore is to uncover a behavioural predictor of susceptibility to pathological weight loss in the ABA model. This objective is in keeping with broader efforts to define predictive markers of future susceptibility to ABA in rodents and AN in humans that encompass not only behavioural but biological traits. While the experiments described in this Chapter are restricted to the former, it should be recognized that despite our intention to incorporate the most appropriate behavioral tests that may highlight a predisposition, this list is not exhaustive and that other predictive tests may be uncovered in the future.

2.2 Methods

2.2.1 Experiment 1: evaluating anhedonia in ABA

2.2.1.1 Animals

Female Sprague-Dawley rats (Animal Resources Centre, Western Australia) with initial body weights of 140-180 g were used in these studies. All experimental procedures were approved by the Monash Animal Resource Platform Ethics Committee (MARP-2015-046). A singly housed male rat was present in all experimental rooms to synchronise the estrous cycles of the female rats via the Whitten effect (110).

2.2.1.2 Baseline preference for sweetened water

To determine baseline preference for sweetened water over a range of concentrations, rats (*n*=27) were individually housed in standard wire top cages in a temperature (27°C) and humidity (21%) controlled room with a 12h light-dark cycle (lights on 0700h). Two-bottle choice tests were performed during the light phase to ensure that fluid consumption during the test was not influenced by feeding behaviour that largely occurs during the dark phase in order to minimize confounds and ceiling effects. Rats had *ad libitum* access to food and water for the duration of the experiment.

2.2.1.2.1 Determining optimal concentrations of nutritive and non-nutritive sweeteners

Two-bottle choice tests were conducted twice daily (1100-1230h and 1430-1600h), in which rats (*n*=12) had access to two bottles, one containing tap water and the second containing varying concentrations of nutritive or non-nutritive sweetener diluted in tap water (Sucrose 0.5-6%; Saccharin 0.0025-0.10%). The side (left/right) of the sweetened water was counterbalanced within and between rats across sweeteners and concentrations to eliminate any side preferences. Each rat received each concentration of each sweetener once with tests considered reliable and included in analyses if ≥0.25 ml of the sweetened water was consumed. Preference was calculated as: sweetened water intake / (total water intake) x 100.

2.2.1.2.2 Determining preference for nutritive versus non-nutritive sweeteners

Three-bottle choice tests to determine sweetener preference, and whether this was influenced by the nutritive value of the sweetener, were also conducted in a naive cohort of rats (*n*=15) between 1100h-1230h on three non-consecutive days. The three bottles contained tap water, 1.5% sucrose or 0.02% saccharin diluted in tap water, with their location (left/middle/right) counterbalanced across rats and between tests. The concentration of each sweetener was determined from the results of the above experiment, and selected as the lowest concentration that generated individual preference scores from ~50-100 while ensuring that group means were similar and variation was sufficient to reveal any effects of treatment (i.e. no ceiling or floor effect). Preference for each solution was calculated as a percentage of total fluid consumed, e.g. 1.5% sucrose intake / (total water intake) x 100.

2.2.1.3. The activity-based anorexia (ABA) paradigm

Rats were individually housed in transparent activity wheel and living chambers (Lafayette Instruments, IN, USA; model 80859) in a temperature (22-24°C) and humidity (30-50%) controlled room under a reversed 12h light/dark cycle (lights off at 1400h). The ABA paradigm utilized in these experiments involved three phases. Initially, animals were acclimated to the running wheel cages and testing room for 4 days, during which they had *ad libitum* access to food and water and unrestricted access to the running wheels. There was then a 7-day habituation period under the same conditions to determine baseline running wheel activity. Following habituation, the ABA phase was initiated with food access restricted to 90 min/day at the onset of the dark phase while rats retained unhindered access to the running wheels and water. ABA persisted until rats reached ≤80% of their baseline body weight (taken at the end of habituation before food restriction starts), on the second day of spontaneous weight gain or for a maximum of 10 days, whichever occurred first, at which point *ad libitum* access to food was reinstated (wheels remained unlocked and voluntarily accessible). During habituation, ABA, and recovery rats and food baskets were weighed daily from 1330-1400h, with food baskets in ABA also weighed upon removal at 1530h.

2.2.1.4 Assessment of running wheel activity

Each running wheel was connected to an Activity Wheel Counter (Lafayette Instruments, IN, USA) mounted on each cage which was connected by USB interface to a computer running the Activity Wheel Software (Lafayette instruments). Running wheel data were recorded in 10-minute interval bins for the duration of each experiment, whereby one count constitutes one wheel revolution. Daily running wheel activity (RWA) on day n comprised total wheel revolutions over 24h from 1400h on day n to 1400h on day n+1. RWA in the hour before feeding 1300-1400h constitutes the window of food anticipatory activity (FAA), which is used as an index of motivation to eat (as detailed in **section 2.4 Discussion**). To control for individual differences in overall RWA, FAA was calculated as a proportion of total daily RWA (i.e. RWA between 1300-1400h on day X / total RWA on day X).

2.2.1.5 Preference for sweetened water in the ABA paradigm

2.2.1.5.1 The effects of individual components of the ABA paradigm on saccharin preference

In order to assess the independent effects of the different components of the ABA paradigm on preference for sweetened water, a series of two-bottle choice tests were conducted using 0.02% saccharin. The non-nutritive sweetener saccharin was used in this instance in order to limit additional caloric intake given we were investigating the effects of food (caloric) restriction. Rats (*n*=12) were individually housed as described above with *ad libitum* access to water, and intermittent access to running wheels and standard laboratory rodent chow (Barastoc Feeds, AU) as described below. For the first 9 days of the experiment ("novelty" block) running wheels were locked and rats were not handled nor disturbed except for the administration of saccharin choice tests. From day 9 onward, rats were handled and weighed daily between 1330-1400h, with food baskets and water bottles also weighed during this time. Half of these animals (*n*=6) were then subject to 8-day blocks of food restriction first, followed by access to running wheels (restrict-run), while the other half (*n*=6) were given access to running wheels first, followed by food restriction (run-restrict), with 2 days of *ad libitum* feeding and locked running wheels between experimental blocks. During food restriction access to food was for 90 min/day between 1400-1530h, as occurs in ABA. Two-bottle saccharin choice tests were administered between 1400-1530h **a)** during exposure to the novel environment alone (day 5 and 7), **b)** following handling alone (day 9 and 12), **c)** throughout periods of running wheel access and 90

min/day food restriction for both groups (days 14, 17, 19, 22, and days 24, 27, 29, 32), and **d)** over the following 3+ weeks after cessation of food restriction and running wheel access (days 34, 41, 48, 55). The location (left/right) of saccharin was counterbalanced between and within animals across tests.

2.2.1.5.2 The effect of running wheel access on sucrose preference

Following the results of the above experiment (see **Results section 2.3.2**), it was necessary to also examine the effects of running wheel access on preference for sucrose-sweetened water. A separate, naïve cohort of rats (*n*=14) were individually housed as described above, with *ad libitum* access to food and water. Two-bottle sucrose choice tests were conducted prior to wheel access (wheels locked) (1.25%), and following 3 (1.25%) and 5 (1.5%) days of voluntary access to unlocked running wheels. Additionally, a separate naïve cohort of rats (*n*=8) were given sucrose (1.5%) choice tests only after 3 (group 3d; *n*=4) or 7 (group 7d; *n*=4) days of voluntary access to unlocked running wheels.

2.2.1.5.3 The effect of ABA induced body weight loss on preferences for sweetened water

Finally, two-bottle choice tests were conducted before, during and after ABA was induced. Naïve rats were exposed to either sucrose (1.5%; *n*=11) or saccharin (0.02%; *n*=12). Rats, food baskets and water bottles were weighed daily between 1300-1400h. Two-bottle choice tests were conducted between 1400-1530h, coinciding with the ABA food availability window. Baseline preference was tested after 7 days of running wheel access (habituation day -4). After 11 days of running wheel habituation, ABA (90 min/day food restriction) commenced at 1400h on day 12 (ABA 1). ABA was maintained until body weight loss reached ≤80% of baseline body weight (measured on day ABA 1 immediately prior to commencement of ABA) or until the second day a rat spontaneously put on weight, or for a maximum of 10 days, whichever occurred first. Choice tests were conducted at the cessation of ABA for Susceptible rats (i.e. those that reached ≤80% of baseline body weight) or on the first day of spontaneous weight gain for Resistant rats (i.e. those that were able to maintain body weight >80% of baseline) and for all rats on the day that their body weight reached ≥100% of baseline after return to *ad libitum* feeding (weight restoration/ABA recovery).

2.2.2 Experiment 2: Behavioural predictors of pathological weight loss in ABA

2.2.2.1 Animals and housing

To determine whether baseline levels of anxiety-like and compulsive behaviours and general locomotion are related to ABA susceptibility, these behaviours were assessed in a naïve cohort of female Sprague-Dawley rats with initial body weights 120-160g (*n*=44; Monash Animal Research Platform, Victoria) before exposure to behavioural testing and the ABA paradigm. Tests were conducted on separate and consecutive days in the following order: elevated plus maze (EPM), open field test (OF), marble burying test (MBT). All experimental procedures were approved by the Monash Animal Resource Platform Ethics Committee (#15171). A singly housed male rat was present in the housing and ABA room to synchronise the estrous cycles of the female rats via the Whitten effect (110).

2.2.2.1.1 Experimental groups

In order to examine how these behaviours were involved in the individual components of the ABA paradigm, four experimental groups were utilized, with animal allocation to groups based on body weight. Rats were ranked on body weight and allocated into groups such that all groups had the same mean body weight rank. Animals either underwent exposure to the ABA paradigm (ABA; *n*=20; 20 rats were allocated to ABA to allow for the divergence into ABA Susceptible and ABA Resistant groups each with adequate n), had *ad libitum* access to food paired with access to a running wheel (RW; *n*=8), had time-limited food restriction with no wheel access (FR; *n*=8), or *ad libitum* food access and no running wheel (control; *n*=8; **Figure 2.1**). ABA and RW only were individually housed in activity living chambers with *ad libitum* voluntary access to a running wheel, FR only and control were singly housed in standard wire topped cages. Following 7 days of habituation, with *ad libitum* access to food for all animals, food access was restricted to 90-minute per day at the onset of the dark phase (1400h-1530h) for the ABA and FR only groups, whilst RW only and control remained on *ad libitum* feeding. Food restriction lasted for a maximum of 10 days, with Susceptible ABA rats returned to *ad libitum* feeding when their body weight dropped to ≤80% of baseline (taken after 7 days of habituation). Body weight and food intake were recorded daily between 1330h-1400h over the 10-day experimental period, with 90-minute food intake recorded upon food removal at 1530h.

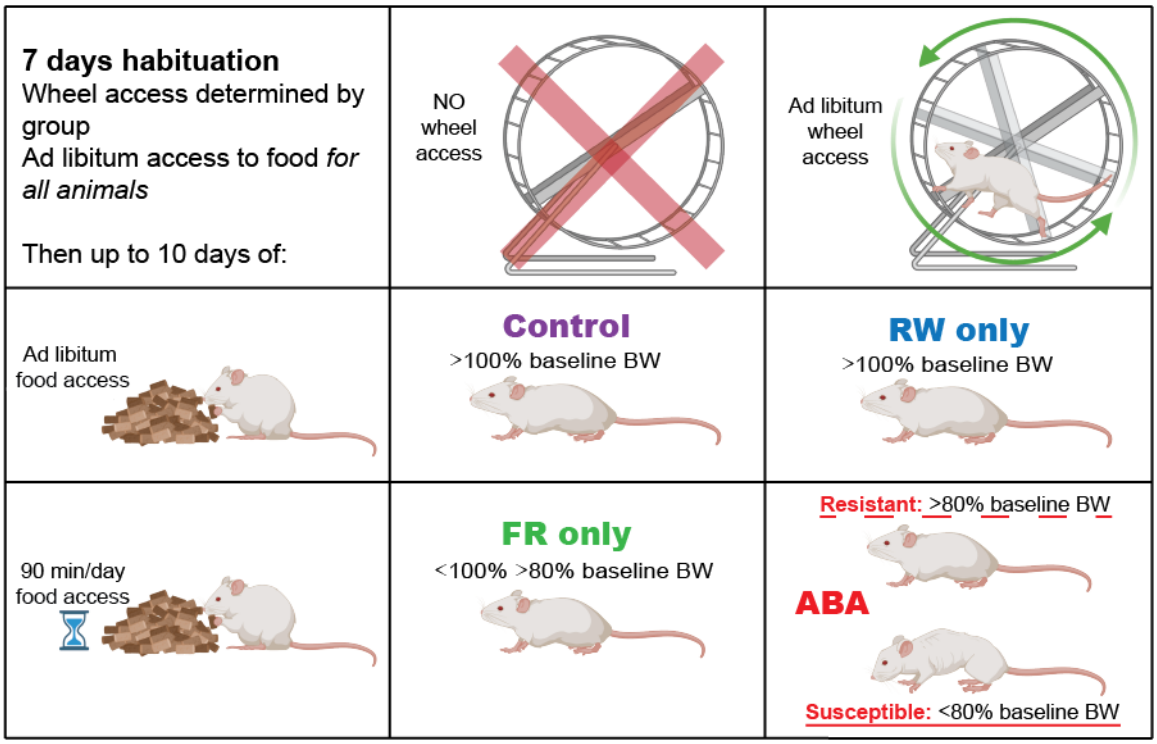

Figure 2.1 Experimental groups compared in Experiment 2

2.2.2.2 Running wheel activity

Running wheel activity was collected as per **section 2.2.4**. In addition, mean hourly RWA over the 24 1-hour blocks of the day was calculated for habituation, ABA, and the change from habituation to ABA (ABA-Habituation) as both absolute wheel revolutions, and as a percentage of total daily wheel revolutions.

2.2.2.3 Anxiety-related behaviour and general locomotion

Anxiety-like behaviour was primarily measured using the elevated plus maze (EPM) which consisted of an elevated 4-arm platform in a plus shape (70 cm long x 10 cm wide) where two of the arms along one axis have walls (40 cm high; closed arms) and the other two arms along the perpendicular axis are open (aversive arms) (101-103). At the beginning of each 10-minute trial, rats were placed in the centre (10x10 cm) of the EPM facing an open arm. The amount of time spent in the closed arms relative to the open arms gives a measure of anxiety-like behaviour, where a greater amount of time spent in the closed arms is associated with greater anxiety-like behaviour and administration of anxiolytic drugs increases the proportion of time spent in the open arms (101, 102). Additionally, measures of exploration were obtained by

recording both the frequency of head dips over the central zone or open arms of the EPM and rearing behaviour.

The open field (OF) test was used to examine general locomotor activity and consists simply of a deep open topped box (60x60x55 cm deep). In each 10 min trial, the total distance travelled reflected general locomotor activity (104) and the proportion of time spent in the central portion (central square of a 3x3 grid; 20x20 cm) was used as a secondary measure of anxiety-related behaviour (103, 105). General locomotor levels were a measure of inherent interest but also served as a control for EPM performance which is sensitive to variation in general locomotion as well as anxiety-like behaviour (102). Considering that administration of some anti-anxiety drugs increases the proportion of time spent exploring the open centre area of the OF, it's been suggested that this test is only sensitive to "normal" anxiety and not "pathological" anxiety (104, 105). A measure of exploratory behaviour was obtained by recording the frequency of rearing.

Both tests were recorded with an overhead camera connected to a computer and analyzed with TopScan Lite tracking software (V 2.0; CleverSys VA, USA).

2.2.2.4 Compulsive behaviour

The marble burying test (MBT) was used to assess compulsive digging behaviour. Rats were placed in an open field box (60x60x55 cm deep) with a 5cm deep layer of sawdust bedding on which 9 marbles (blue glass; 8mm diameter) were placed in an even grid pattern. The "classical" measure of compulsiveness in the MBT is number of marbles buried at the end of the test duration (in this case 10 min), where a greater number indicates increased compulsive tendencies (106, 107, 109). However, this measure does not account for the likelihood that rats will bury and unbury marbles throughout the test, and produces an unwanted confounding ceiling effect (111), therefore the number of bouts of marble burying and unburying as well as the latency to first dig in the sawdust and total digging time was also recorded. Each test was recorded with an overhead camera and all behaviours were manually scored.

2.2.3 Statistical analyses

Except otherwise noted all statistical analysis was performed in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Significance for all tests was set at $p<0.05$. One-way RM

ANOVA followed by Tukey's post-hoc multiple comparisons was used to analyze mean three-bottle preference. One-way RM ANOVA followed by Dunnett's post-hoc multiple comparisons with baseline as the reference time point were used to independently analyze cohort mean saccharin preference following food restriction or running wheel access. Linear regression was used to analyze the relationship between ABA test day body weight % and sucrose/saccharin preference, FAA and subsequent 90-min food intake, FAA and subsequent 24h body weight % loss, daily food intake and daily body weight % loss, daily RWA and daily food intake, daily RWA and daily body weight % loss, and OF total distance and Habituation mean daily RWA. An SPSS mixed model ANOVA custom syntax (previously described: 112) which enables analysis of data with decreasing group size over time was used to independently longitudinally analyze daily RWA in habituation, FAA in ABA, and 90-min ABA food intake in Experiment 1, and daily RWA in ABA in both Experiment 1 and 2. Independent samples t-tests were used to analyze Experiment 1 ABA mean daily food intake and change in RWA and FAA from habituation to ABA, and mean daily body weight % loss and food intake for the two *ad libitum* fed groups in Experiment 2, and Experiment 2 ABA Susceptible vs. ABA Resistant parameters of the EPM, OF and MBT. Two-way RM ANOVA followed by post-hoc multiple comparisons with a Bonferroni correction were used to analyze mean daily RWA and FAA in Experiment 1, whereas Tukey's post-hoc multiple comparisons were utilized in Experiment 2 analyses of daily RWA in habituation, mean hourly RWA (both absolute and percentage of daily RWA) in habituation, ABA, and change from habituation to ABA, and EPM duration in and number of entries into zones. In experiment 2, one-way ANOVA with Tukey's post-hoc multiple comparisons were used to analyze mean daily body weight % loss and food intake for the three food-restricted groups, and change in mean daily RWA and FAA for the three groups with wheel access.

2.2.4 Exclusions

In Experiment 1 the body weight loss trajectories of three rats were abnormal and inconsistent with the similarities in trajectories for the rest of the cohort; one displayed an initial 7-day plateau followed by a drastic drop, while two others showed a steady but slow drop to 80% on ABA day 10. In Experiment 2, one ABA rat also showed an abnormal body weight loss trajectory displaying a steady but slow drop to just above 80% on day 9, followed by a tiny increase in weight on day 10, and then a drop below 80% on day 11. Data collected from these four animals were therefore removed from all analyses.

2.3 Results

2.3.1 Baseline preference for sweetened water

To determine whether preference for sweetened water over regular water was evident across a range of concentrations, a battery of two- and three-bottle choice tests were conducted. Two-bottle choice tests revealed that rats consistently preferred both sucrose- (pink **Figure 2.2 A**) and saccharin-sweetened (orange **Figure 2.2 B**) water to tap water (blue), even at very low concentrations. Moreover, three-bottle choice tests using 1.5% sucrose and 0.02% saccharin (**Figure 2.2 C**) confirmed preference for both these solutions over tap water (sucrose $p<.0001$; saccharin $p=.0156$) and demonstrated a clear preference for sucrose over saccharin ($p<.0001$).

2.3.2 Effect of running wheel access on preference for saccharin (non-caloric) sweetened water

To independently examine the various aspects of the ABA paradigm on preference for sweetened water, a longitudinal series of two-bottle choice tests were conducted following the introduction of each separate feature. Preference for saccharin was not altered by single housing (Day 0-9 **Figure 2.2 D** and **E**), handling (Day 9-14 **Figure 2.2 D** and **E**), or time-limited food access alone (light grey shaded blocks **Figure 2.2 D** and **E;** right **Figure 2.2 F**, $p=.7608$). However, access to running wheels induced a rapid aversion to saccharin in all animals (dark grey shaded blocks **Figure 2.2 D** and **E;** left **Figure 2.2 F,** $p<.0001$), evident after 3, 5 and 8 days of running wheel access (all ps$<.0001$). Aversion to saccharin was not due to acute competition between wheel running and saccharin availability as it persisted when the wheels were locked for the final choice test (Day 22 **Figure 2.2 D** and Day 33 **Figure 2.2 E**). Strikingly, saccharin aversion was maintained, or lessened to a lack of preference, for a further 3+ weeks following locking of running wheels (final white blocks **Figure 2.2 D** and **E**).

2.3.3 Effect of running wheel access on preference for sucrose (caloric) sweetened water

To determine whether the specific properties of saccharin underpinned this running wheel-associated aversion, a naïve cohort of rats were singly housed in activity chambers with locked running wheels and displayed initial preference for 1.25% sucrose-sweetened water (**Figure 2.2 G**), which was consistent with expectations from baseline tests conducted under standard

housing conditions. However, most rats also developed an aversion to sucrose after 3 days of access to running wheels (experimental day 5 **Figure 2.2 G**) and *all* rats avoided sucrose after 5 days of running wheel access (experimental day 7), despite the slightly sweeter sucrose solution (1.5%). In contrast, when access to running wheels was introduced 7 days *prior* to initial exposure to 1.5% sucrose, they displayed reliable baseline preference (7d RWA, **Figure 2.2 H**), which was not evident when access to running wheels preceded initial sucrose exposure by only 3 days (3d RWA).

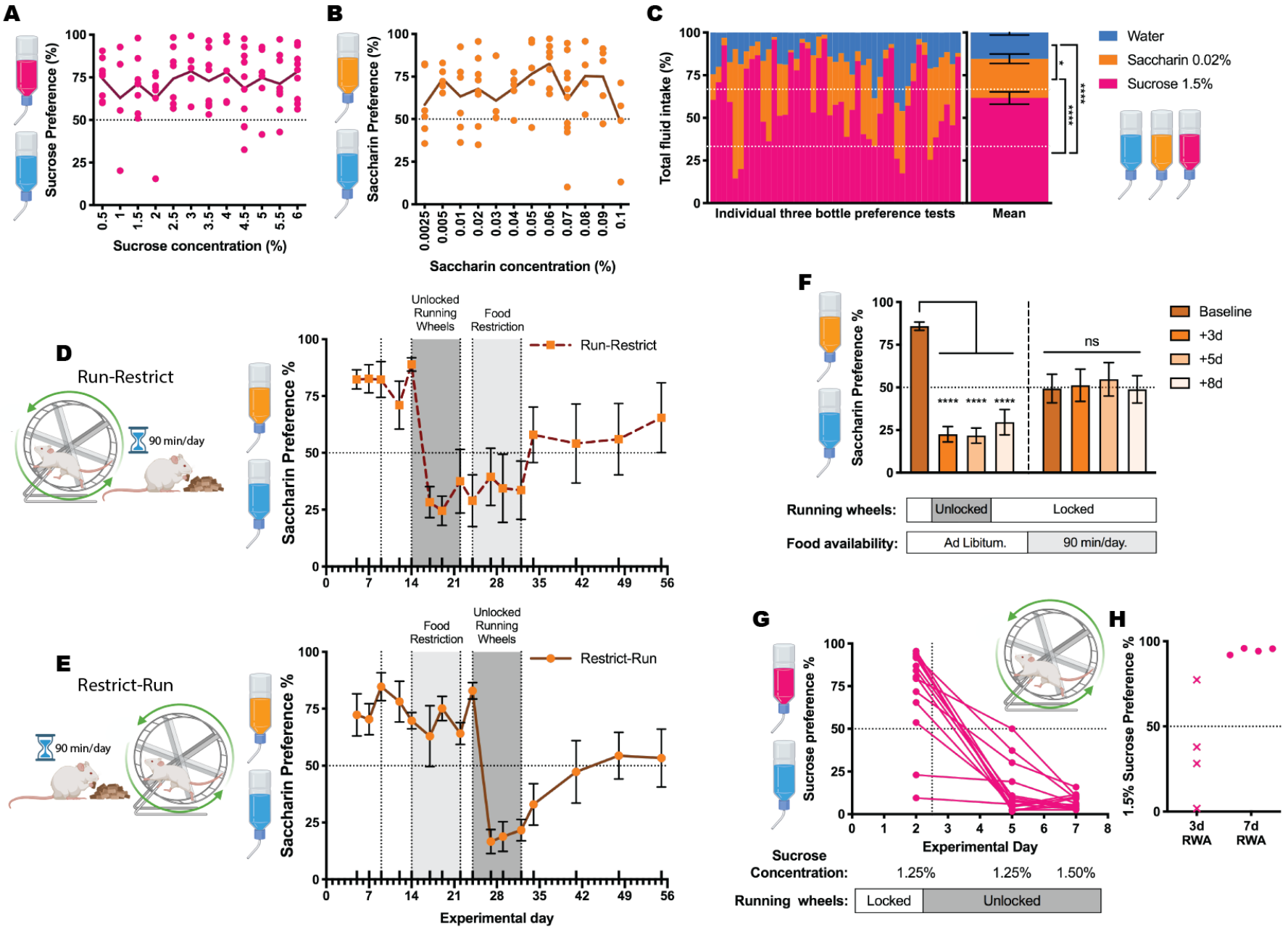
A
Sucrose Preference (%)
Sucrose concentration (%)
B
Saccharin Preference (%)
Saccharin concentration (%)
C
Total fluid intake (%)
Individual three bottle preference tests
Mean
Water
Saccharin 0.02%
Sucrose 1.5%
D
Run-Restrict
90 min/day
Unlocked Running Wheels
Food Restriction
Run-Restrict
Saccharin Preference %
E
Restrict-Run
90 min/day
Food Restriction
Unlocked Running Wheels
Restrict-Run
Saccharin Preference %
Experimental day
F
Saccharin Preference %
Baseline
+3d
+5d
+8d
ns
Running wheels:
Unlocked
Locked
Food availability:
Ad Libitum.
90 min/day.
G
Sucrose preference %
Experimental Day
Sucrose Concentration:
1.25%
1.25%
1.50%
Running wheels:
Locked
Unlocked
H
1.5% Sucrose Preference %
3d RWA
7d RWA

Figure 2.2 Evaluating sucrose and saccharin preference in response to the individual parameters of the ABA model. Dose-response plots for 90 min two-bottle choice tests using increasing concentrations of sucrose **(A)** or saccharin **(B)** with ≥0.25ml of sweetener consumed; dots are individual tests from 12 rats, line is group mean. **C)** Individual triple replicate tests for 15 rats (left) and group mean ± SEM (right) preference scores for 90 min three-bottle choice tests of water (blue), 0.02% saccharin (orange) and 1.5% sucrose (pink); One-way repeated measures ANOVA, $F(1.204, 52.99)=53.80$, $p<.0001$, post-hoc Tukey's multiple comparisons revealed sucrose was preferred over both saccharin and water (both ps<.0001) while saccharin was still preferred over water ($p=.0156$). **D and E)** Two-bottle saccharin (0.02%) choice tests for two groups of rats ($n=6$ each) testing the effects of novelty (first unshaded block), handling (second unshaded block), food restriction (light grey block), and unlocked running wheels (dark grey block); see **Methods section 2.2.1.5.1** for details. Rats had *ad libitum* access to food and locked running wheels except where indicated by grey blocks. Tests were conducted between 1400-1530h on days indicated by upward ticks. **F)** Cohort mean saccharin preference scores at baseline (dark orange; corresponding to left most test in grey blocks in **D and E**), and after 3, 5 and 8 days (medium, light and very light orange respectively) of access to unlocked running wheels (left half; corresponding to tests in dark grey blocks in **D and E**) or 90 min/day food restriction (right half; corresponding to tests in light grey blocks in **D and E**). Data for each block were collapsed across both groups and analyzed independently from the other block; data are cohort mean ± SEM. One-way RM ANOVA revealed a significant effect of running wheel access on saccharin preference $F(1.833, 20.17)=78.29$, $p<.0001$, with Dunnett's post hoc multiple comparisons showing saccharin preference was significantly decreased from baseline after 3, 5 and 8 days of access to running wheels (all $ps<.0001$). One-way RM ANOVA revealed no main effect of food restriction on saccharin preference, $F(2.205, 24.25)=0.3045$, $p=.7608$. **G)** Two-bottle sucrose (1.25%) choice tests for a naïve cohort of rats ($n=14$) showed normal preference for sucrose at baseline (day 2, locked wheels), that was abolished or became an aversion to sucrose after 3 days of access to an unlocked running wheel (day 5; wheels unlocked at 1530h after test on day 2), and was maintained even when the sucrose concentration was increased (day 7; 1.5%). **H)** Seven days of running wheel access (7d RWA) *prior* to initial sucrose (1.5%) exposure resulted in reliable preference that was not evident in rats given only 3 days of running wheel access (3d RWA) prior to initial sucrose exposure. *$p<.05$, ****$p<.0001$.

2.3.4 Involvement of anhedonia in susceptibility to ABA

Considering that baseline sucrose preference was reliably shown when initial exposure followed 7 days of wheel access, an extended period of habituation to the running wheel was employed before exposure to ABA conditions (**Figure 2.3 A**) and resulted in a 70/30 split in Susceptible and Resistant phenotypes (**Figure 2.3 B**). This produced expected baseline 1.5% sucrose preference scores (**Figure 2.3 C**), although large variability remained for 0.02% saccharin preference (**Figure 2.3 D**). Following ABA exposure, a reduction in sucrose or saccharin preference only occurred in a subset of rats (middle columns **Figure 2.3 C** and **D**), and there was no relationship between preference and body weight on the day of the ABA choice test (day reaching removal criterion for Susceptible rats and the first day of spontaneous weight gain for Resistant rats; **Figure 2.3 E,** r=.1935, R^2=.03744, p=.3763). Sucrose preference was largely unchanged across phases of *ad libitum* and restricted feeding, and associated body weight loss and regain, with most rats maintaining a strong preference regardless of ABA susceptibility and body weight change (**Figure 2.3 C**). Only one rat exhibited the hypothesized transient “anhedonia”, indicated by the “V” shaped curve starting with high baseline sucrose preference, a lack of preference following the development of ABA, and a high sucrose preference rebound following body weight recovery (**Figure 2.3 C**). Three rats tested with saccharin show an exaggerated form of this V shape which is skewed towards saccharin aversion at lowest body weight (Susceptible rats) or first day of spontaneous weight gain (Resistant rats), while the saccharin preference scores for the remaining rats were relatively steady across the experimental phases with rats maintaining their initial level of saccharin preference (or lack thereof or aversion) regardless of ABA susceptibility (**Figure 2.3 D**).

What became apparent from this experiment was that there was variation in susceptibility to body weight loss under ABA conditions (**Figure 2.3 B**). While the majority (n=16, 70%) of rats were susceptible to ABA-induced body weight loss dropping to ≤80% of baseline within 6 days of ABA, a proportion (n=7, 30%) were resistant to ABA and able to maintain their body weight above 80% of baseline before exhibiting spontaneous weight gain (**Figure 2.3 F**). Unsupervised clustering analysis of body weight loss curves (**Figure 2.3 F**) unveiled two characteristically different trajectories; rats that were susceptible to ABA-induced body weight loss (solid black lines) generated steep downward curves with continuous and accelerating weight loss over time, whereas rats that were resistant to ABA (dashed green lines) produced curves with an initial shallow descent tapering into an extended plateau before subsequently ascending with

spontaneous weight gain. The distinct difference between and clustering within these two characteristic trajectories highlights that body weight loss within the ABA paradigm exists along a continuum between susceptibility and resistance becoming increasingly distinct with diverging trajectories as the paradigm endures (i.e. day 4-5, **Figure 2.3 F**).

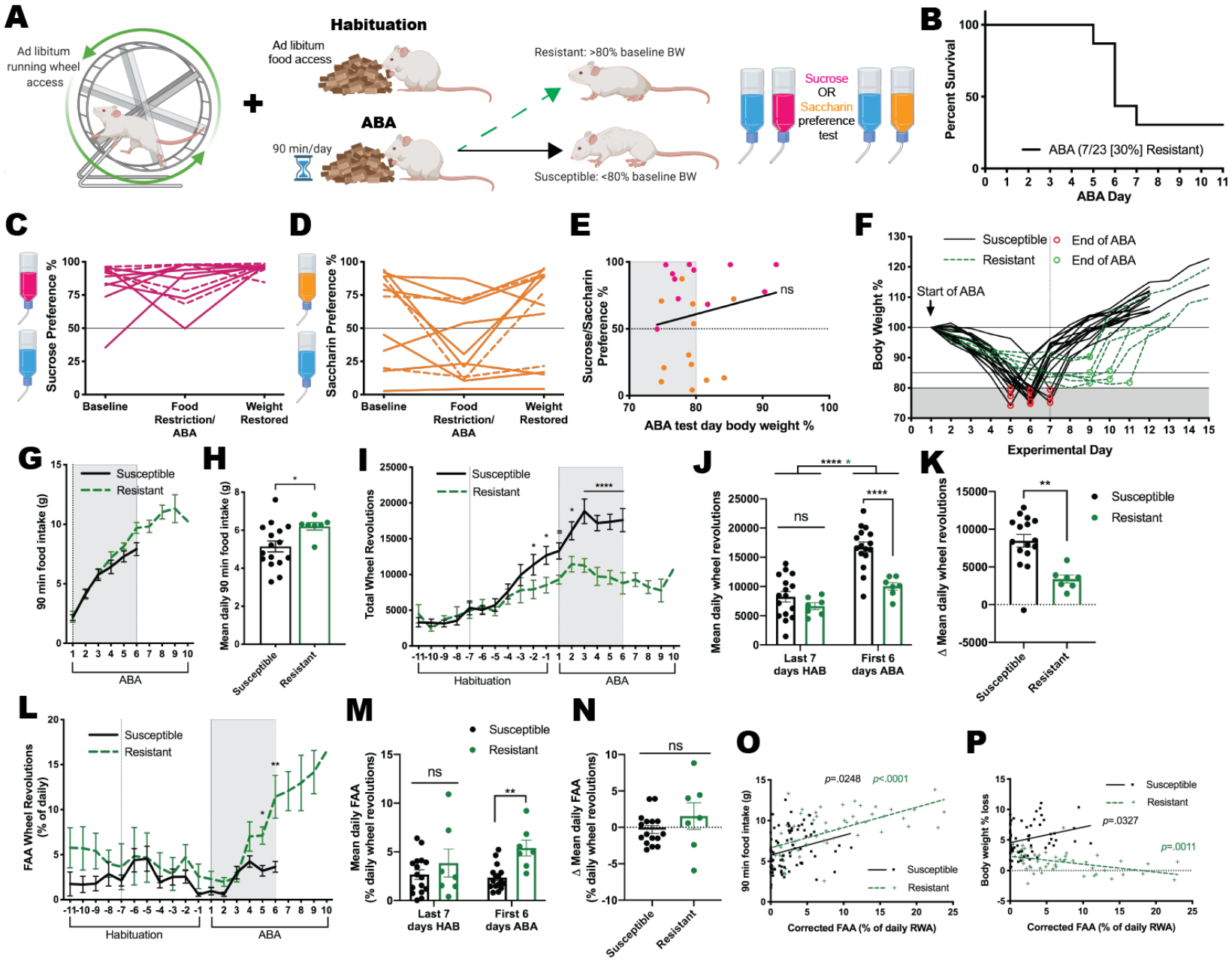
A
Ad libitum running wheel access
Habituation
Ad libitum food access
ABA
90 min/day
Resistant: >80% baseline BW
Susceptible: <80% baseline BW
Sucrose OR Saccharin preference test
B
Percent Survival
ABA (7/23 [30%] Resistant)
ABA Day
C
Sucrose Preference %
Baseline
Food Restriction/ ABA
Weight Restored
D
Saccharin Preference %
E
Sucrose/Saccharin Preference %
ns
ABA test day body weight %
F
Susceptible
Resistant
End of ABA
Start of ABA
Body Weight %
Experimental Day
G
90 min food intake (g)
ABA
H
Mean daily 90 min food intake (g)
I
Total Wheel Revolutions
Habituation
J
Mean daily wheel revolutions
Last 7 days HAB
First 6 days ABA
K
Δ Mean daily wheel revolutions
L
FAA Wheel Revolutions (% of daily)
M
Mean daily FAA (% daily wheel revolutions)
N
Δ Mean daily FAA (% daily wheel revolutions)
O
90 min food intake (g)
p=.0248
p<.0001
Corrected FAA (% of daily RWA)
P
Body weight % loss
p=.0327
p=.0011

Figure 2.3 Anhedonia and other outcome measures in ABA. **A)** Schematic of the experimental design. **B)** Survival plot for *n*=23 female Sprague-Dawley rats exposed to ABA; *n*=16 Susceptible, *n*=7 Resistant. Each rat received three two-bottle 1.5% sucrose (**C**, *n*=11) or 0.02% saccharin (**D**, *n*=12) choice tests given **1)** at baseline (after 7 days RWA, 4 days prior to ABA), **2)** during restricted food access when body weight dropped below 80% baseline (Susceptible; solid black line) or on the first day a rat spontaneously put on weight (Resistant; dashed green line), and **3)** the day a rat first exceeded 100% of baseline body weight. **E)** Body weight % does not predict sucrose (pink) or saccharin (orange) preference on the test day within the food restriction phase. Linear regression with line of best fit and one data point per animal, Y=1.345*X - 46.67, *r*=.1935, R^2=.03744, *p*=.3763. **F)** Body weight percentage loss during ABA for Susceptible (solid black line) and resistant (dashed green line) rats. *Ad libitum* access to food was returned when rats reached <80% baseline body weight (susceptible; red circle), or after the second day they spontaneously put on weight or after 10 days of ABA, whichever occurred first (resistant; green circle). **G, I** and **L)** Group mean ± SEM. Two-way mixed model ANOVA followed by post-hoc multiple comparisons with a Bonferroni correction. **H, J, K, M** and **N)** Group mean (bars) ± SEM with individual animals (circles). **H, K** and **N)** Independent samples t-test. **J** and **M)** Two-way RM ANOVA followed by post-hoc multiple comparisons with a Bonferroni correction. **G)** Daily 90 min food intake during ABA. Time *F*(9, 105.743)=45.025, *p*<.0001; Outcome *F*(1, 22.672)=1.706, *p*=.205; Interaction *F*(5, 105.651)=0.863, *p*=.508. **H)** Mean daily food intake over the first 6 days of ABA (the maximum length of ABA for Susceptible rats; light grey shading in **G**), *t*(21)=2.311, *p*=.0311. **I)** Total daily RWA (wheel revolutions). Habituation: Time *F*(10, 196.129)=20.300, *p*<.0001; Outcome *F*(1, 21.123)=0.444, *p*=.512; Interaction *F*(10, 196.129)=2.069, *p*=.029; Susceptible rats ran significantly more than Resistant rats on habituation days -2 *p*=.40 and -1 *p*=.014. ABA: Time *F*(9, 104.65)=2.537, *p*=.011; Outcome *F*(1, 21.250)=23.438, *p*<.0001; Interaction *F*(5, 104.666)=1.637, *p*=.157; Susceptible rats ran significantly more than Resistant rats on ABA day 1 *p*=.05, day 2 *p*=.023 and days 3-6 all *p*s<.0001. **J)** Mean daily RWA (wheel revolutions) over the last 7 days of habituation and the first 6 days of ABA. Phase *F*(1, 21)=77.37, *p*<.0001 (Susceptible *p*<.0001, Resistant *p*=.0133); Outcome *F*(1, 21)=10.86, *p*=.0034; Interaction *F*(1, 21)=14.22, *p*=.0011. No significant difference between groups at habituation (*p*=.5328) but significantly greater mean daily ABA RWA for Susceptible rats (*p*<.0001). **K)** Change in mean daily RWA from habituation to ABA, *t*(21)=3.771, *p*=.0011. **L)** Food anticipatory activity (FAA) expressed as wheel revolutions in the hour before feeding as a percentage of total daily RWA. ABA: Time *F*(9, 111.462)=18.154, *p*<.0001; Outcome *F*(1, 31.035)=7.315, *p*=.011; Interaction *F*(5, 111.194)=1.813, *p*=.116; Susceptible rats ran significantly more than Resistant rats on ABA day 5 *p*=.043 and day 6 *p*=.003. **M)** Mean daily FAA over the last 7 days of habituation and the first 6 days of ABA. Phase *F*(1, 21)=0.7541, *p*=.3950; Outcome *F*(1, 21)=9.868, *p*=.0049; Interaction *F*(1, 21)=1.687, *p*=.2081. No significant difference between groups at habituation (*p*=.4721) but significantly greater mean daily FAA during ABA for Resistant rats (*p*=.0070). **N)** Change in mean daily FAA from habituation to ABA, *t*(21)=1.299, *p*=.2081. FAA versus subsequent 90 min food intake **(O)** and subsequent 24h body weight % loss **(P)** during ABA; linear regression with line of best fit and all individual data points. **O)** Susceptible Y=0.2418*X + 5.748, *r*=.25, *p*=.0248, R^2=.06289; Resistant, Y=0.2502*X + 6.63, *r*=.59, *p*<.0001, R^2=.3493. **P)** Susceptible Y=0.2541*X + 4.552, *r*=.27, *p*=.0327, R^2=.07152; Resistant, Y=-0.1307*X + 2.36, *r*=.44, *p*=.0011, R^2=.1909. = *p*=0.05, **p*<0.05, ***p*<0.01, ****p*<0.0001.

2.3.5 Other determinants of susceptibility to ABA

The variability in susceptibility to ABA-induced body weight loss prompted post-hoc analyses of variables that may predict susceptibility or resistance to ABA. While it appeared that Resistant rats eat more than Susceptible rats from day 4, there was no difference between groups in food intake over time (p=.205, **Figure 2.3 G**); however, this analysis was limited due to the fewer number of data points contributed by Susceptible rats. Analysis of mean daily food intake over the first 6 days of ABA (maximum ABA duration for Susceptible rats) circumvented this limitation and indeed revealed that Resistant rats ate significantly more per day, on average, than Susceptible rats (p=.0311, **Figure 2.3 H**). As expected, daily RWA increased over the habituation phase following acclimatization to the wheels (p<.0001, **Figure 2.3 I**). Interestingly, a significant interaction differentiated rats that would go on to be susceptible or resistant to ABA (p=.029), with rats that went on to be susceptible running significantly more on the last two days of the habituation period than those that went on to be resistant (day -2 p=.40, day -1 p=.014, **Figure 2.3 I**).

While all rats increased RWA over time during ABA (p=.011), the magnitude of starvation-induced hyperactivity was markedly greater in Susceptible compared to Resistant rats (p<.0001) and this was evident from day 1 of food restriction (day 1 p=.05, day 2 p=.023, day 3-6 ps<.0001, **Figure 2.3 I**). Mean daily RWA (**Figure 2.3 J**) significantly increased from the last 7 days of habituation (period following initiation of daily handling) to the first 6 days of ABA (maximum duration for Susceptible rats) for the entire cohort (p<.0001), and independently for both Susceptible (p<.0001) and Resistant (p=.0133) rats. Whilst both groups exhibited similar mean daily RWA in habituation (p=.5328, **Figure 2.3 J**), starvation-induced hyperactivity in ABA was significantly greater for Susceptible versus Resistant rats (p<.0001), reflected by a significantly greater magnitude of increase in mean daily RWA from habituation to ABA for Susceptible rats (p=.0011, **Figure 2.3 K**).

Similar to daily RWA, all rats increased food anticipatory activity (FAA) as ABA progressed (p<.0001, **Figure 2.3 L**). In contrast to daily RWA, Resistant rats had significantly greater FAA than Susceptible rats (p=.011), particularly evident on days 5 (p=.043) and 6 (p=.003) (**Figure 2.3 L**). Again, in contrast to mean daily RWA, there was no overall change in mean daily FAA from habituation to ABA (p=.3950, **Figure 2.3 M**). There was no difference in mean daily “FAA” between groups during habituation (p=.4721), but groups were significantly different during

ABA, this time with Resistant rats having significantly greater mean daily FAA than Susceptible rats (p=.0070; **Figure 2.3 M**), although there was no significant difference between groups in the magnitude of change in mean daily FAA from habituation to ABA (p=.2081, **Figure 2.3 N**). Of note, Susceptible and Resistant rats had significantly different *overall* mean daily RWA and FAA but in opposing directions, whereby Susceptible rats had significantly greater mean daily RWA (p=.0034, **Figure 2.3 J**) whereas Resistant rats had significantly greater mean daily FAA (p=.0049, **Figure 2.3 M**).

While FAA was positively correlated with subsequent 90-minute food intake for both groups, the relationship was only weak for Susceptible rats (r=.2508, p=.0248), whereas it was strong for Resistant rats (r=.5910, p<.0001), accounting for only 6% (R^2=.06289) versus 35% (R^2=.3493) of the variation in 90-minute food intake respectively (**Figure 2.3 O**). Strikingly, the relationship between FAA and body weight loss in the following 24h was in opposite directions depending on ABA outcome, with a weak *positive* correlation for Susceptible rats (r=.2674, p=.0327) but a moderate *negative* correlation for Resistant rats (r=.4369, p=.0011), accounting for 7% (R^2=.07152) and 19% (R^2=.1909) of the variation in body weight loss respectively (**Figure 2.3 P**).

2.3.6 Impact of Individual and combined components of the ABA paradigm on body weight and food intake

Because we demonstrated that preference for sucrose and saccharin was abolished, or became an aversion with running wheel access, it was necessary to examine the effects of each component of the ABA model (i.e. food restriction [FR] and running wheel [RW] access) separately. Further, based on the identification of Susceptible and Resistant ABA profiles, it is plausible that a preexisting behavioural phenotype could be identified that predicts ABA susceptibility or resistance. We therefore conducted behavioural tests of anxiety-like behaviour (EPM), general locomotion (OF), and compulsive behaviour (MBT) in a cohort of animals which were then split into four groups exposed to individual (FR or RW), combined (ABA), or none (control) of the ABA paradigm components (**Figure 2.4 A**).

There was a near even split between ABA Susceptibility and Resistance with 9/19 (47%) rats being Resistant to ABA induced body weight loss (**Figure 2.4 B**). Rats that were Susceptible to ABA displayed the traditional body weight free fall, while Resistant rats showed body weight

loss trajectories that plateaued, similar to rats that were food restricted without access to running wheels (FR), although the plateau of body weight maintenance was at a lower body weight than FR rats (**Figure 2.4 C** and **D**). This resulted in a significant difference in mean daily body weight % loss (p<.0001), with Susceptible rats losing significantly more body weight % on average per day than both Resistant and FR rats (both ps<.0001), while the latter two groups did not differ in weight loss (p=.1473; **Figure 2.4 E**). This pattern was mirrored for food intake, with Susceptible rats eating less over time than rats that were resistant to ABA or food restricted without wheel access (**Figure 2.4 H** and **I**), such that there was a significant difference in mean daily food intake (p<.0001) with Susceptible rats eating significantly less per day, on average, than both other groups (both ps<.0001) – although the food intake of Resistant rats was similar to FR rats (p=.9982; **Figure 2.4 J**). Meanwhile, when *ad libitum* feeding was maintained, access to a running wheel did not alter body weight over the experimental period (**Figure 2.4 C** and **F**) with no significant difference in mean body weight % change between control rats and those that had access to a running wheel (p=.1446; **Figure 2.4 G**). This was accomplished through an increase in food intake over time (**Figure 2.4 H** and **K**) presumably to compensate for increased energy expenditure from wheel running, such that they ate significantly more per day, on average, than the control rats (p=.0003; **Figure 2.4 L**)

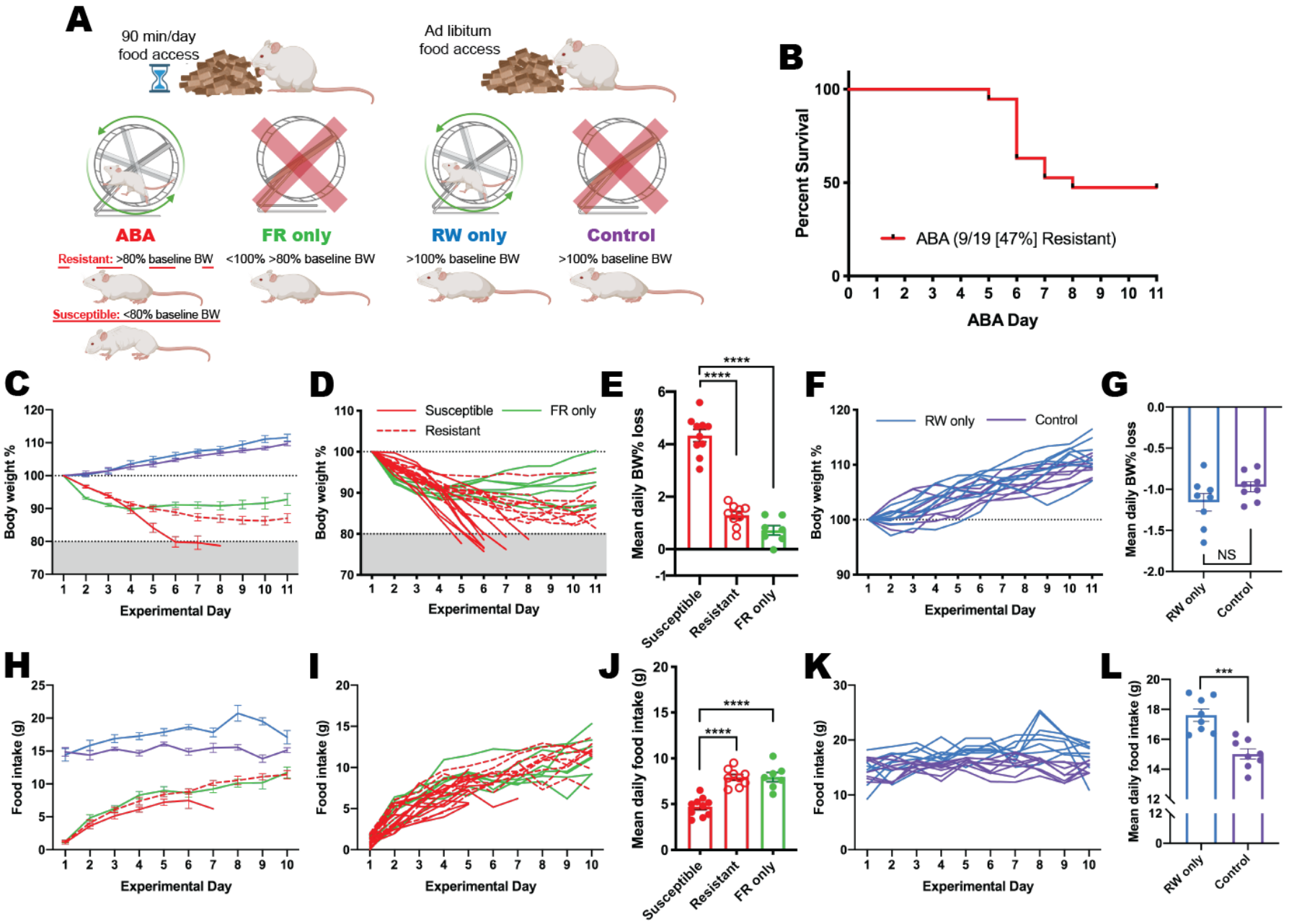
A
90 min/day
food access
Ad libitum
food access
ABA
FR only
RW only
Control
Resistant: >80% baseline BW
<100% >80% baseline BW
>100% baseline BW
>100% baseline BW
Susceptible: <80% baseline BW
B
Percent Survival
ABA (9/19 [47%] Resistant)
ABA Day
C
Body weight %
Experimental Day
D
Susceptible
Resistant
FR only
Body weight %
Experimental Day
E
Mean daily BW% loss
****
****
Susceptible
Resistant
FR only
F
RW only
Control
Body weight %
Experimental Day
G
Mean daily BW% loss
NS
RW only
Control
H
Food intake (g)
Experimental Day
I
Food intake (g)
Experimental Day
J
Mean daily food intake (g)
****
****
Susceptible
Resistant
FR only
K
Food intake (g)
Experimental Day
L
Mean daily food intake (g)
***
RW only
Control

Figure 2.4 Body weight and food intake following exposure to individual or combined components of the ABA paradigm. A) Schematic showing the four experimental groups. **B)** ABA survival plot. 9/19 (47%) rats were resistant to ABA. Group mean ± SEM **(C)** and individual **(D** and **F)** daily body weight % over the experimental period. **E** and **G)** Mean daily body weight % loss. Group mean ± SEM **(H)** and individual **(I** and **K)** daily food intake over the experimental period. **J** and **L)** Mean daily food intake. **E)** One-way ANOVA: $F(2, 23)=101.3$, $p<.0001$. **J)** One-way ANOVA: $F(2, 23)=27.29$, $p<.0001$. Tukey's post-hoc multiple comparisons revealed that Susceptible rats lost significantly more body weight % **(E)** and ate significantly more **(J)** on average per day than both Resistant and FR only groups (all ps<.0001). **G)** Independent samples t-test, $t(14)=1.545$, $p=.1446$. **L)** Independent samples t-test, $t(14)=4.846$, $p=.0003$. **E, G, J** and **L)** Individual animals (circles) and group mean (bars) ± SEM. NS = not significant $p>.05$, ***$p<.001$, ****$p<.0001$.

2.3.7 Overall RWA differentiates Susceptible and Resistant rats

In order to determine the effect of food restriction on RWA we compared animals that were exposed to ABA conditions to a group that had access to the running wheel but maintained on *ad libitum* food intake (**Figure 2.5 A**). As in Experiment 1, daily RWA was markedly different for Susceptible (solid red; **Figure 2.5 B**) and Resistant rats (dashed red; **Figure 2.5 C**), while this latter group was relatively similar to the RW only group (blue; **Figure 2.5 D**) at the level of overall daily running activity. The comparison between Susceptible and Resistant groups was of course of primary interest, therefore the longitudinal analysis of daily RWA was restricted to these groups, although the RW only group is also plotted for visual comparison (**Figure 2.5 D**; also the case for mean hourly RWA **Figure 2.5 H-M**). During 7 days of habituation (*ad libitum* access to food; **Figure 2.5 E**) there was a significant increase in RWA over time (p<.0001), which was different depending on whether rats went on to be susceptible or resistant to ABA (p<.0001), with Susceptible rats displaying overall significantly greater RWA in habituation than Resistant rats (p=.0101). This was largely driven by a sharp increase in daily RWA over the last two days of habituation (days 6 and 7; **Figure 2.5 E).** On these days, rats that went on to be susceptible to ABA ran substantially more than rats that went on to be resistant (both ps<.0001). The effect of the onset of food restriction on daily RWA is striking, with Susceptible rats rapidly developing severe starvation-induced hyperactivity, while Resistant rats show a much-blunted increase in daily RWA that is more in line with that observed in the RW group (**Figure 2.5 E**). Indeed, the daily increase in RWA over time was of a significantly greater magnitude for Susceptible compared to Resistant rats, and that the rate of increase over time was dependent upon whether rats were Susceptible or Resistant (all ps<.0001; **Figure 2.5 E**). Moreover, all animals ran a similar amount on average per day during the habituation phase and increased mean daily RWA at the initiation of ABA (all ps<.0001); however, RWA during the ABA phase (“starvation-induced hyperactivity”) was substantially greater for Susceptible rats compared to both Resistant and RW rats (both ps<.0001), with these latter two groups being similar (p=.9777; **Figure 2.5 F**), a feature that is more simply reflected in the RWA change for individuals from habituation to ABA (p=.0003; Susceptible > Resistant p=.0022, Susceptible > RW p=.0008; **Figure 2.5 G).**

2.3.8 RWA across the light cycle differentiates ABA susceptible and ABA resistant rats

During habituation, the majority of RWA for all rats occurred in the dark phase (grey shaded area **Figure 2.5 H** and **K**), particularly in the latter half, for both absolute (**H**) and proportional (**K**) RWA measures (both *p*s<.0001). Under conditions of ABA, there was a dramatic change in the profile of RWA across the day that differentiates between Susceptible and Resistant rats. Susceptible rats markedly increased RWA in a largely indiscriminate manner across both the dark and light phases, compared to a blunted increase in dark phase RWA displayed by Resistant rats (1700-2200h all *p*s<.0188; **Figure 2.5 I**), with light-phase increases in RWA largely restricted to anticipation of food access. Analysis of mean hourly RWA in ABA as a proportion of total daily RWA (**Figure 2.5 L**), to control for the significant individual differences in *overall* RWA between groups in this phase, highlights the deliberate and motivated nature of the ramping of RWA over the latter portion of the light cycle by Resistant rats. This was consistent with a specific and marked increase in FAA for Resistant rats that was evident regardless of whether proportional raw values or change from habituation values were examined (both *ps*<.0001; **Figure 2.5 L** and **M**). In fact, running in anticipation of food during ABA, defined in these studies as the penultimate hour before lights off (coinciding with food access for ABA rats), was substantially increased in Resistant rats (Resistant > Susceptible *p*<.0001, Resistant > RW *p*=.0002; **Figure 2.5 N**) and “true” FAA during ABA needs to account for each animal’s RWA during this hour when food access was not scheduled (i.e. in the habituation phase). This adjusted RWA measure confirms that elevated FAA is a specific feature of Resistant rats, far greater than Susceptible rats (*p*<.0001) and RW rats (*p*=.0019) maintained on *ad libitum* feeding, with these latter groups not differing (*p*=.5396; **Figure 2.5 O**).

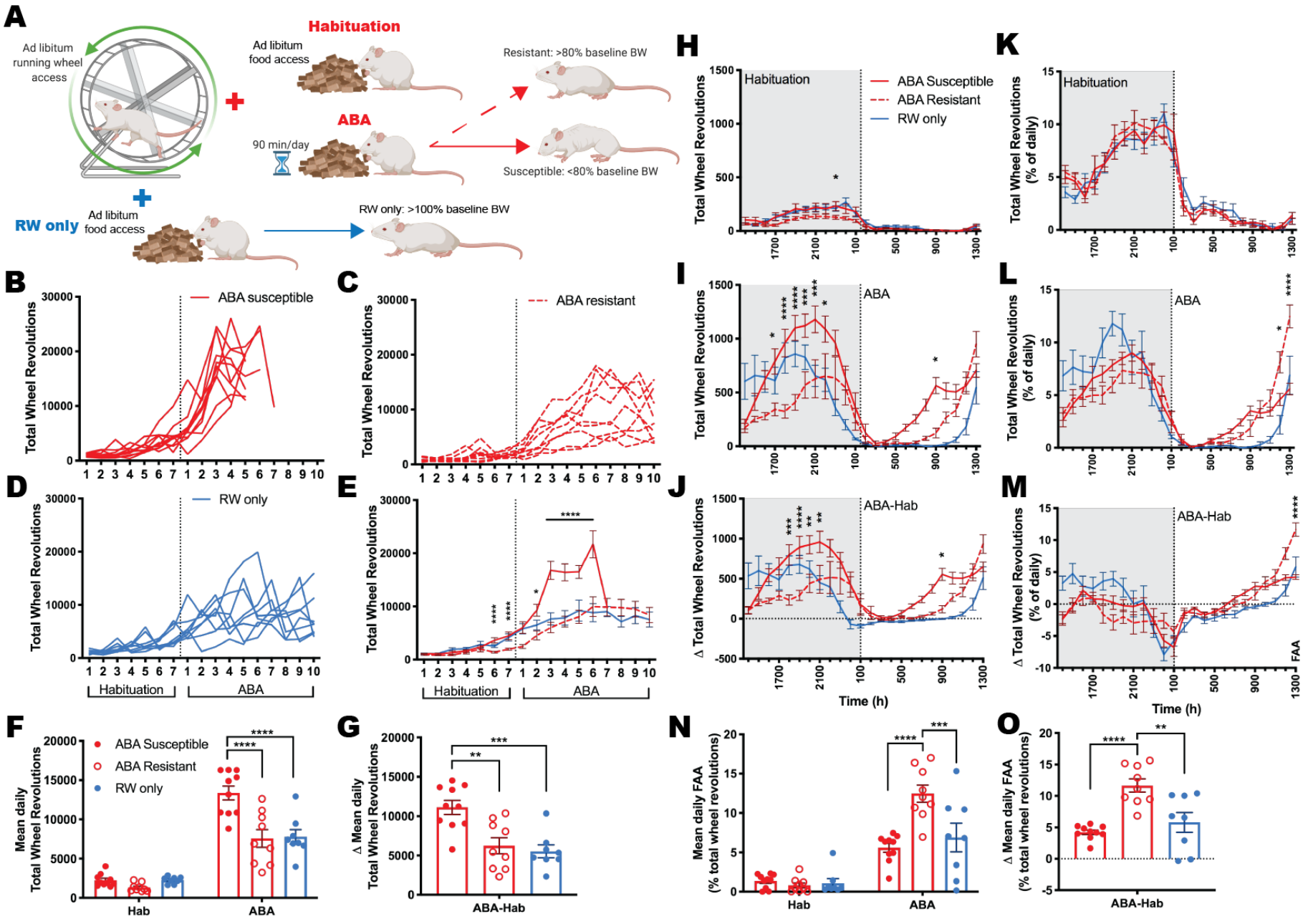
A
Habituation
Ad libitum running wheel access
Ad libitum food access
Resistant: >80% baseline BW
ABA
90 min/day
Susceptible: <80% baseline BW
RW only
Ad libitum food access
RW only: >100% baseline BW
B
ABA susceptible
C
ABA resistant
D
RW only
Habituation
ABA
E
F
ABA Susceptible
ABA Resistant
RW only
Mean daily Total Wheel Revolutions
Hab
ABA
G
Δ Mean daily Total Wheel Revolutions
ABA-Hab
H
Habituation
ABA Susceptible
ABA Resistant
RW only
Total Wheel Revolutions
I
ABA
J
ABA-Hab
Δ Total Wheel Revolutions
Time (h)
K
Total Wheel Revolutions (% of daily)
L
M
FAA
N
Mean daily FAA (% total wheel revolutions)
O
Δ Mean daily FAA (% total wheel revolutions)

Figure 2.5 RWA for rats with wheel access either maintained on *ad libitum* feeding (RW only) or combined with food restriction (ABA). **A)** Schematic showing the three groups. Group mean ± SEM **(E)** of daily RWA for individual rats that were either Susceptible to ABA **(B)**, Resistant to ABA **(C)**, or had wheel access but were maintained on *ad libitum* feeding (RW) **(D)**. **E** and **H-M)** Longitudinal analyses were only performed on ABA groups, RW are only for visual comparison; Data are group mean ± SEM. Two-way RM ANOVA followed by post-hoc multiple comparisons with a Bonferroni correction. **F** and **N)** Individual animals (circles) and group mean (bars) ± SEM. Two-way RM ANOVA followed by post-hoc multiple comparisons, Tukey's for between groups or with a Bonferroni correction for between phase. **G** and **O)** Individual animals (circles) and group mean (bars) ± SEM. One-way ANOVA followed by Tukey's post-hoc multiple comparisons. **E)** Habituation: Time $F(6, 102)=20.24$, $p<.0001$; Outcome $F(1, 17)=8.378$, $p=.0101$; Interaction $F(6, 102)=6.850$, $p<.0001$. Rats that went on to be susceptible to ABA ran significantly more on days 6 and 7 of habituation than rats that went on to be resistant to ABA (both *p*s<.0001). ABA: Mixed-model ANOVA (due to missing values from Susceptible rats removed from the experiment over time): Time $F(9, 109.996)=20.644$, $p<.0001$, Outcome $F(1, 20.878)=24.739$, $p<.0001$, Interaction $F(6, 110.337)=5.431$, $p<.0001$. Susceptible rats had significantly greater RWA than Resistant rats on ABA day 2 ($p=.014$) and days 3-6 (all *p*s<.0001). **F)** Mean daily RWA. Phase $F(1, 24)=204.3$, $p<.0001$ (all groups $p<.0001$); Group $F(2, 24)=11.66$, $p=.0003$ (Susceptible > Resistant $p=.0004$; Susceptible > RW $p=.0038$); Interaction $F(2, 24)=11.33$, $p=.0003$. Susceptible rats ran significantly more, on average, per day in the ABA phase than both Resistant and RW rats (both *p*s<.0001). **G)** Change in mean daily RWA from habituation to ABA, $F(2, 24)= 11.33$, $p=.0003$. The magnitude of increase in mean daily RWA from habituation to ABA was significantly greater in Susceptible rats than both Resistant ($p=.0022$) and RW ($p=.0008$) rats. Absolute **(H-J)** and proportional **(K-M)** mean hourly RWA over habituation **(H and K)**, ABA **(I and L)** and the change from habituation to ABA **(J and M)**. **H)** Time $F(23, 391)=23.55$, $p<.0001$; Outcome $F(1, 17)=8.378$, $p=.0101$; Interaction $F(23, 391)=1.867$), $p=.0095$. Susceptible rats ran significantly more than Resistant rats at 2300h ($p=.0138$). **I)** Time $F(23, 391)=25.18$, $p<.0001$; Outcome $F(1, 17)=16.64$, $p=.0008$; Interaction $F(23, 391)=3.981$, $p=.0095$. Susceptible rats ran significantly more than Resistant rats at 1700h ($p=.0159$), 1800h, 1900h (both *p*s<.0001), 2000h, 2100h (both *p*s=.0006), 2200h ($p=.0188$), and 0900h ($p=.0177$). **J)** Time $F(23, 391)=18.93$, $p<.0001$; Outcome $F(1, 17)=13.06$, $p=.0021$; Interaction $F(23, 391)=3.574$, $p<.0001$. Susceptible rats had a significantly greater increase in hourly RWA from habituation to ABA than Resistant rats at 1800h ($p=.0003$), 1900h ($p<.0001$), 2000h ($p=.0036$), 2100h ($p=.0063$), and 0900h ($p=.0156$). **K)** Time $F(23, 391)=53.88$, $p<.0001$; Outcome $F(1, 17)=0.07041$, $p=.7939$; Interaction $F(23, 391)=0.7915$), $p=.7429$. **L)** Time $F(23, 391)=29.80$, $p<.0001$; Outcome $F(1, 17)=0.9184$, $p=.3513$; Interaction $F(23, 391)=3.531$, $p<.0001$. Resistant rats ran significantly more than Susceptible rats at 1200h ($p=.0132$) and 1300h ($p<.0001$). **M)** Time $F(23, 391)=22.33$, $p<.0001$; Outcome $F(1, 17)=0.1744$, $p=.6814$; Interaction $F(23, 391)=3.433$, $p<.0001$. Resistant rats had a significantly greater increased in mean hourly RWA from habituation to ABA than Susceptible rats at 1300h ($p<.0001$). **N)** Mean daily FAA. Phase $F(1, 24)=146.6$, $p<.0001$ (Susceptible $p=.0007$, Resistant and RW $p<.0001$); Group $F(2, 24)=5.870$, $p=.0084$ (Resistant > Susceptible $p=.0093$, Resistant > RW $p=.0430$); Interaction $F(2, 24)=14.84$), $p<.0001$. Resistant rats had significantly greater FAA in the ABA phase than both Susceptible ($p<.0001$) and RW ($p=.0002$) rats. **O)** Change in mean daily FAA, $F(2, 24)= 14.84$, $p<.0001$. The magnitude of increase in mean daily FAA from habituation to ABA was significantly greater in Resistant rats than both Susceptible ($p<.0001$) and RW ($p=.0019$) rats. *$p<.05$, **$p<.01$, ***$p<.001$, ****$p<.0001$.

2.3.9 The relationships between ABA-associated outcomes are different for Susceptible and Resistant rats

The relationships between ABA-associated outcomes, namely RWA, food intake and weight loss were similar for all animals *except* rats susceptible to developing ABA. For control animals, daily food intake was moderately *negatively* correlated with coincident/concurrent daily body weight % *loss* (r=.4434, R^2=.1966, p<.0001; purple **Figure 2.6 A**), i.e. the higher food intake in any single day the greater the amount of body weight gained on that same day. This was also the case for RW animals (r=.4486, R^2=.2012, p<.0001; blue) and was especially strong for FR animals (r=.8712, p<.0001; green), with variation in 90-minute food intake accounting for a sizeable 76% (R^2=.7590) of the variation in daily body weight % loss. This relationship was also very strong for Resistant rats (r=.7248, R^2=5254, p<.0001; black) with a breakdown of this relationship between food intake and body weight % loss only occurring in Susceptible rats, (r=.1953, R^2=.03816, p=.1610; red).

While daily RWA was moderately *positively* correlated with daily body weight % loss across the same 24-hour period for both Susceptible (r=.4970, R^2=.2470, p=.0002; red **Figure 2.6 B**) and RW (r=.3553, R^2=.1263, p=.0012; blue) rats, there was no relationship between these variables for Resistant rats (r=.1539, R^2=.02367, p=.1476; black). Moreover, daily RWA was not related to daily food intake for RW rats (r=.1986, R^2=.03945, p=.0774; blue **Figure 2.6 C**) whereas there was a strong *positive* association for both Susceptible (r=.5574, R^2=3107, p<.0001; red) and Resistant (r=.5508, R^2=3034, p<.0001; black) rats. Finally, FAA was moderately *negatively* associated with subsequent daily body weight % loss for Resistant rats (r=.4569, R^2=2088, p<.0001; black **Figure 2.6 D**) and moderately *positively* associated with subsequent 90-minute food intake (r=.4876, R^2=2378, p<.0001; black **Figure 2.6 E**), such that greater levels of FAA were associated with subsequently higher 90-minute food intake and less body weight % loss over the following day. Conversely, there was no relationship for Susceptible rats between FAA and either body weight % loss (r=.2823, R^2=.07969, p=.0666; red **Figure 2.6 D**) or 90-minute food intake (r=.0139, R^2=.0007975, p=.9293; red **Figure 2.6 E**).

2.3.10 Anxiety-like behaviour, general locomotion, and compulsivity do not predict subsequent ABA susceptibility

The duration of time spent in different zones of the EPM did not differentiate between rats that went on to be susceptible or resistant to ABA (p=.9979; **Figure 2.6 G**); however, rats that went on to be susceptible to ABA entered and exited zones more often than rats that went on to be resistant to ABA (p=.0053; **Figure 2.6 H**), including more frequent crosses into the centre zone (p=.0355), - these differences did not result in an increase in the total distance travelled (p=.1215; **Figure 2.6 I**). The number of exploratory rears (p=.6048; **Figure 2.6 J**) or head dips in the EPM (p=.2709; **Figure 2.6 K**) did not differ between rats that went on to be susceptible or resistant to ABA, nor did any of the compulsivity measures in the MBT (latency to dig p=.2109, **Figure 2.6 M**; marble interactions p=.0551, **Figure 2.6 N**; total digging time p=.5630, **Figure 2.6 O**), or locomotor activity (total distance travelled p=.8629; **Figure 2.6 Q**), anxiety-like behaviour (duration in centre zone p=.5724, **Figure 2.6 R**; entries into centre zone p=.6799, **Figure 2.6 S**) or exploratory rears in the OF (p=.7875; **Figure 2.6 T**). Of note, given RWA in habituation was predictive of ABA susceptibility but general locomotor activity was not, there was no relationship between total distance travelled in the OF and mean daily RWA during habituation (Susceptible r=.4866, R^2=.2368, p=.1538; Resistant r=.2435, R^2=.05931, p=.5278; All ABA r=.1152, R^2=.01326, p=.6387; **Figure 2.6 U**).

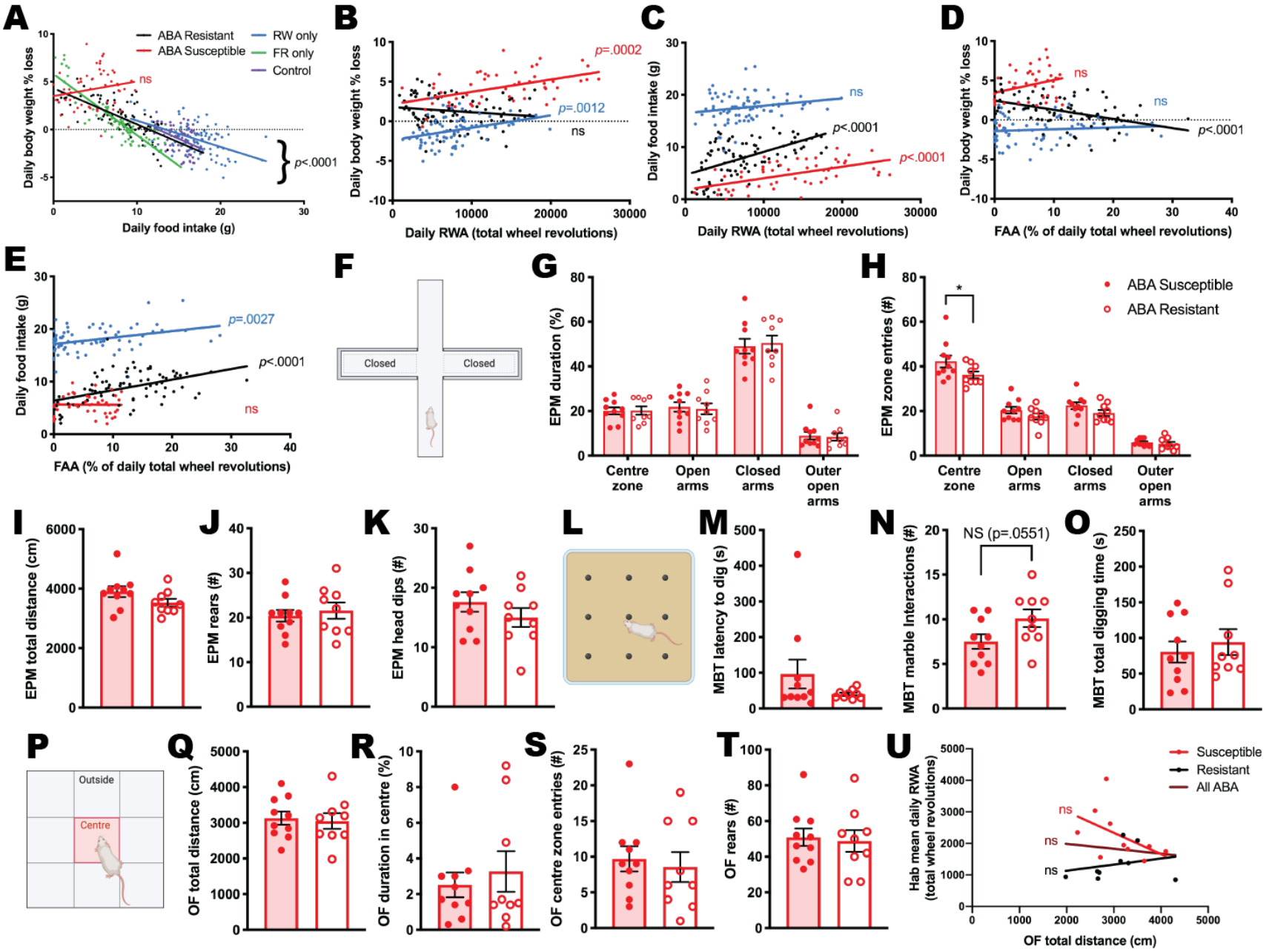
A
ABA Resistant
ABA Susceptible
RW only
FR only
Control
ns
p<.0001
Daily body weight % loss
Daily food intake (g)
B
p=.0002
p=.0012
ns
Daily body weight % loss
Daily RWA (total wheel revolutions)
C
ns
p<.0001
p<.0001
Daily food intake (g)
Daily RWA (total wheel revolutions)
D
ns
ns
p<.0001
Daily body weight % loss
FAA (% of daily total wheel revolutions)
E
p=.0027
p<.0001
ns
Daily food intake (g)
FAA (% of daily total wheel revolutions)
F
Closed
Closed
G
EPM duration (%)
Centre zone
Open arms
Closed arms
Outer open arms
H
*
ABA Susceptible
ABA Resistant
EPM zone entries (#)
Centre zone
Open arms
Closed arms
Outer open arms
I
EPM total distance (cm)
J
EPM rears (#)
K
EPM head dips (#)
L
M
MBT latency to dig (s)
N
NS (p=.0551)
MBT marble Interactions (#)
O
MBT total digging time (s)
P
Outside
Centre
Q
OF total distance (cm)
R
OF duration in centre (%)
S
OF centre zone entries (#)
T
OF rears (#)
U
Susceptible
Resistant
All ABA
ns
ns
ns
Hab mean daily RWA (total wheel revolutions)
OF total distance (cm)

Figure 2.6 Linear regression of body weight %, food intake and RWA over 10 days of exposure to components of the ABA paradigm, and pre-exposure behavioural test results. A-E) Linear regression with line of best fit and all individual data points. **A)** Daily food intake vs. daily body weight % loss. ABA resistant r=.7248, R^2=.5254, p<.0001, Y=-0.3769*X + 4.315; ABA susceptible r=.1935, R^2=.03816, p=.1610, Y=0.1543*X + 3.496; RW only r=.4486, R^2=.2012, p<.0001, Y=-0.2741*X + 3.670; FR only r=.8712, R^2=.7509, p<.0001, Y=-0.6389*X + 5.803; Control r=.4434, R^2=.1966, p<.0001, Y=-0.3882*X + 4.863. **B)** Daily RWA vs. Daily body weight % loss. ABA resistant r=.1539, R^2=.02367, p=.1476, Y=-6.416e-005*X + 1.778; ABA susceptible r=.4970, R^2=.2470, p=.0002, Y=0.0001551*X + 2.159; RW only r=.3553, R^2=.1263, p=.0012, Y=0.0001550*X - 2.366. **C)** Daily RWA vs. Daily food intake. ABA resistant r=.5508, R^2=.3034, p<.0001, Y=0.0004417*X + 4.671; ABA susceptible r=.5574, R^2=.3107, p<.0001, Y=0.0002202*X + 1.830; RW only r=.1986, R^2=.03945, p=.0774, Y=0.0001417*X + 16.51. **D)** Daily FAA vs. Daily body weight % loss. ABA resistant r=.4569, R^2=.2088, p<.0001, Y=-0.1161*X + 2.452; ABA susceptible r=.2823, R^2=.07969, p=.0666, Y=0.1615*X + 3.453; RW only r=.0957, R^2=.009167, p=.4237, Y=0.02232*X - 1.404. **E)** Daily FAA vs. Daily food intake. ABA resistant r=.4876, R^2=.2378, p<.0001, Y=0.2002*X + 6.384; ABA susceptible r=.0139, R^2=.0007975, p=.9293, Y=-0.007589*X + 5.681; RW only r=.3484, R^2=.1214, p=.0027, Y=0.1226*X + 17.14. **F)** Schematic of the EPM. **G)** Duration (%) spent in each of the EPM zones. Two-way RM ANOVA: Zone F(3, 68)=111.2, p<.0001; ABA outcome F(1, 68)=7.200e-006, p=.9979; Interaction F(3, 68)=0.08631, p=.9673. **H)** Number of entries into each of the EPM zones. Two-way RM ANOVA: Zone F(3, 68)=155.5, p<.0001; ABA outcome F(1, 68)=8.307, p=.0053; Interaction F(3, 68)=0.9624, p=.4156. Post-hoc multiple comparisons with a Bonferroni correction revealed that ABA susceptible rats made significantly more entries into the centre zone than ABA resistant rats (p=.0355). **I-K, M-O, Q-T)** Independent samples t-test. **I)** Total distance travelled (cm) in the EPM, t(17)=1.630, p=.1215. **J)** Number of exploratory rears during EPM, t(17)=0.5274, p=.6048. **K)** Number of exploratory head dips during EPM, t(17)=1.138, p=.2709. **L)** Schematic of the MBT test set up. **M)** Latency (s) to dig in the MBT, t(17)=1.300, p=.2109. **N)** Number of interactions with marbles during the MBT, t(17)=2.060, p=.0551. **O)** Total digging time (s) in the MBT, t(17)=0.5899, p=.5630. **P)** Schematic of the OF test chamber and zones. **Q)** Total distance travelled (cm) in the OF, t(17)=0.1753, p=.8629. **R)** Duration (%) spent in the centre zone of the OF, t(17)=0.5757, p=.5724. **S)** Number of entries into the centre zone of the OF, t(17)=0.4199, p=.6799. **T)** Number of exploratory rears during OF, t(17)=0.2738, p=.7875. **U)** Total distance travelled (cm) in the OF vs. Habituation mean daily RWA. Linear regression with line of best fit and one data point per animal. ABA Susceptible r=.4866, R^2=.2368, p=.1538, Y=-0.6747*X + 4362; ABA Resistant r=.2435, R^2=.05931, p=.5278, Y=0.1927*X + 750.7; All ABA r=.1152, R^2=.01326, p=.6387, Y=-0.1556*X + 2299. **G-K, M-O, Q-T)** Data show individual animals (circles) and group mean (bars) ± SEM. NS = not significant p>.05, *p<.05.

2.4 Discussion

The experiments detailed in this Chapter sought to investigate whether the development of a transient anhedonia and/or individual variability in the behavioural domains of anxiety, general locomotion and compulsivity could explain the heterogeneity of susceptibility to ABA-induced body weight loss in wildtype Sprague-Dawley rats. In Experiment 1, access to running wheels resulted in the immediate and universal development of an exercise-induced taste aversion to both saccharin and sucrose, despite familiarity with and prior preference for the sweetened solutions. However, seven days of access to unlocked running wheels *before* initial sucrose exposure prevented the formation of this aversive response, enabling evaluation of hedonic choice before, during and after development of ABA. Contrary to expectation, susceptibility to ABA was *not* associated with the development of a transient anhedonia that would recover with weight regain. In Experiment 2, assessment of anxiety-like behaviour, general locomotion, and compulsiveness prior to ABA exposure did not differentiate between rats that went on to be susceptible or resistant to ABA. However, in both experiments, RWA *before* the onset of food restriction was significantly elevated for rats that went on to be susceptible to ABA, confirming that future susceptibility can be predicted by propensity to run in the wheel at baseline (potentially before allocation to treatment groups). Further, following the onset of timed food restriction, resistance to body weight loss was associated with the development of marked FAA without the accompanying overall increase in daily RWA that was characteristic of the Susceptible phenotype.

The development of a conditioned taste aversion (CTA) when rats were exposed to sweetened water *in combination with* first exposure to a running wheel, but not in response to any other aspect of the paradigm (novelty of chambers, handling or even food restriction), is in keeping with existing literature demonstrating the development of exercise-induced CTAs. In fact, CTA to a flavor often develops when paired with wheel running, whether running is voluntary (99, 113-118) or forced (motorized wheel) (99, 116), which is explicitly an *exercise-induced* CTA that does not occur as a consequence of exposure to, or confinement in, locked wheels, but instead results from the action of running (115, 118). Importantly, the timing of first exposure to wheel running and the paired taste (e.g. sweet sucrose) or flavour (e.g. orange Kool-aid) plays a distinct role in whether a CTA will develop. In these previous studies, the flavour was presented first, immediately followed by exposure to the wheel, although one study showed that

taste exposure up to an hour prior to wheel running still produced a conditioned aversion (118). The results presented in this Chapter suggest that rats require 7 days of wheel running prior to the first exposure to sweetened water in order to prevent a CTA, although this only occurred with sucrose and not with saccharin. Other studies have shown that allowing wheel running for 1 hour prior to a novel flavour was sufficient to suppress the degree of CTA that subsequently developed (114), and that pre-exposure to wheel running for 1-hour per day for 7 days eliminated the development of an exercise-induced CTA (119). While these studies all used flavoured liquids, food is equally capable of generating exercise-induced CTA (120-122) with the same importance attributed to the order of exposure to the wheel. Simultaneous exposure to running wheels and a high-fat or high-sugar diet is shown to produce an exercise-induced CTA, which can be prevented by pre-exposure to the running wheel (120). This raises the question of whether wheel running could itself produce a conditioned aversion to regular chow and therefore play a bigger role in the development of ABA than previously thought, especially as many ABA protocols only unlock wheels at the same time as the initiation of food restriction. What seems to be the case is that exercise-induced CTA only occurs in ABA when wheel running is paired with the introduction of a *novel* food, because no aversion was shown to develop when animals were maintained on familiar chow (122). It has also been demonstrated that wheel running simultaneously produced an exercise-induced CTA to a paired liquid flavour whilst increasing chow intake in *ad libitum* fed animals (117), indicating that the ABA phenotype is unlikely to be generated by the development of a conditioned aversion to chow.

The findings presented here also challenge our understanding of reward-related deficits as contributors to the ABA phenotype. Although a transient anhedonia, or a dissolution in the experience of pleasure associated with rapid weight loss, was not shown to drive susceptibility to ABA in the present study, findings from both experiments suggest that resistance to ABA is conferred by maintaining the *motivation* to eat when food is available, while susceptibility to ABA results from a deficit in motivational processes. While it would be very interesting to follow up these results with an explicit examination of motivation throughout the development of ABA, the concern is that the requirements of partial food restriction and/or palatable rewards necessary for progressive ratio operant tasks would likely confound the reward processing landscape of exposed animals to influence subsequent feeding behaviour and weight loss in ABA. However, multiple findings from the current experiments indicate that FAA is a reliable index of the motivation to eat, in line with other recent evidence suggesting that motivational state drives FAA (123), and is associated with resistance to ABA. Specifically, Resistant rats

exhibited FAA that was positively associated with subsequent food intake, suggesting it could be used as an indicator of motivation to eat in the ABA paradigm. Moreover, the negative association between FAA and weight loss in Resistant rats suggests the degree to which rats develop *motivated* running (i.e. in anticipation of food, rather than overall running) is a determinant of survival. Contrary to the indiscriminate increase in RWA across the day exhibited by Susceptible rats, the specific increase in running prior to food access displayed by Resistant rats was a deliberate, motivated and goal-directed behaviour in anticipation of food availability and resulted in significantly greater food intake during the ABA exposure period and resistance to weight loss.

This interpretation of the FAA as a motivated and goal-directed behaviour relying on intact higher-level reward processing is supported on multiple fronts. While hunger from food restriction alone is involved in the generation of FAA, with FAA also developing in sedentary rats on a restricted feeding schedule (RFS) (124, 125) being modulated, at least in part, by ghrelin signaling (126), hunger is not necessary for the development of FAA (124, 125). FAA, albeit to a lesser degree and shorter duration, is also exhibited by *ad libitum* fed rats, with or without access to running wheels, that are on a palatable feeding schedule (PFS) where they receive a palatable treat (e.g. chocolate) at the same time every day (124, 125, 127). Importantly, compared to a RFS, rats on a PFS have increased c-Fos expression in the hours preceding and at the time of treat presentation in reward-related brain areas (e.g. PFC, Acb core and shell), while those on RFS also have increased activity in homeostatic/metabolic regions (hypothalamus) (124) compared to *ad libitum* fed rats. These findings underscore the involvement of FAA in hedonic/reward processes either in concert with or independent from hunger/metabolic factors, with a cumulative effect such that FAA occurs at a higher level and over a longer time course when feeding is restricted (124, 125, 127, 128).

More generally, studies that manipulate reward circuitry or alter food palatability have been implicated in the development of ABA. Firstly, provision of a high fat diet (HFD) in addition to chow is shown to attenuate ABA-induced body weight loss (129) and provision of sweet HFD or pure vegetable fat (not sweet) in addition to chow both prevented and reversed ABA-induced body weight loss, whereas access to saccharin or sucrose in addition to chow does not alter ABA susceptibility (130). Moreover, administration of the natural cannabinoid receptor agonist tetrahydrocannabinol (THC) during ABA induction has been demonstrated to attenuate body weight loss, decrease RWA, and while not increasing food intake overall, is shown to transiently

increase food intake at the initiation of ABA (129). However, when THC administration was *combined* with access to a HFD during ABA, there was once again an attenuation of body weight loss, a decrease in RWA, and an increase in both chow and HFD food intake, over the first two days of ABA (129). These data suggest that administration of THC and HFD produce a combinatorial effect on attenuation of body weight loss greater than that produced by either THC administration or provision of HFD alone. Furthermore, administration of either THC or the synthetic cannabinoid receptor agonist CP has been shown to attenuate ABA-induced body weight loss without inducing an overall effect on susceptibility (131). Importantly, this study utilized the same four treatment groups as in Experiment 2 and found that neither THC nor CP had any significant effect on body weight, food intake, or RWA in these non-ABA groups, indicating that the effects of THC and its synthetic analogues are specific to the altered reward conditions of ABA (131).

With respect to the dopaminergic influence on reward-processing in ABA, treatment with a non-selective dopamine (DA) antagonist was shown to attenuate ABA-induced body weight loss by increasing food intake and decreasing RWA and general locomotor activity, although reductions in both activity measures were also shown in *ad libitum* fed rats and suggests a blunting of activity that is not specific to ABA (132). Treatment with non-specific DA 2/3 receptor antagonists or specific D2 antagonists is also shown to increase survival in ABA by increasing food intake, whereas specific D3 receptor antagonist had no effect on weight loss, food intake, RWA or FAA, suggesting the role of the D2 autoreceptor is of primary importance in the reward feedback from feeding and running during exposure to ABA (133). Overall, the role of DA in hyperactivity is well established, where treatments that increase DA signaling (e.g. amphetamines) increase physical activity (134), whereas those that decrease or block DA (e.g. olanzapine) reduce physical activity (135). However, the specific role of DA signaling in mediating FAA is unclear with many reports proffering conflicting findings (123, 127, 136-138). We do know that chemogenetic activation of DA neurons projecting from the ventral tegmental area (VTA) of the midbrain to the shell subregion of the nucleus accumbens (AcbSh), both prevented and rescued rats from ABA-induced body weight loss, significantly increasing survival, food intake and FAA despite no overall change in daily RWA (112). This latter finding adds evidence to the above conclusion that resistance to ABA is conferred by development of FAA which is a behavioural marker of increased motivation to eat in ABA.

The most interesting behavioural characteristic to note from Experiment 2, in the context of the pursuit of behavioural predictors of ABA susceptibility, is that general locomotor activity in the OF was not associated with ABA outcome. This discrepancy highlights a key point - that general locomotor activity and RWA are two distinct behaviours and there was no relationship between locomotor and running activity in this study. Wheel running is inherently rewarding (139), induces cFos expression in the Acb (140, 141), is capable of generating a conditioned preference (140, 142-144) and rats are willing to work for the ability to run in wheels (143, 145, 146). Whereas general locomotor activity is a relatively constant behaviour, wheel running is a deliberate and driven behaviour requiring a conscious decision to engage in, and effort to perform (139).

In both experiments described in this Chapter, Susceptible rats displayed marked starvation-induced hyperactivity with the onset of food restriction, exhibiting significantly greater RWA over the course of the day during ABA compared to Resistant rats, and showed a specific increase when individual RWA during ABA was compared to baseline running during habituation. This is a consistent finding in the ABA literature, with hyperactivity being characteristic of the ABA phenotype and largely determining survival – hence the name *activity-based* anorexia (84). There are multiple theories as to the cause of starvation-induced hyperactivity, one being that it is an inappropriate expression of foraging behaviour in the laboratory that would be otherwise adaptive and beneficial in the wild where moving from a place with scarce food availability to one of food abundance is necessary for survival (147-150). However, the continuation of this behaviour despite the negative consequences in the ABA setting is not so easily reconciled – in this situation rats seem unable or willing to adapt to reduce running activity to maintain homeostatic energy balance when emaciation begins. The exact mechanisms underlying this paradoxical behaviour are complicated and appear to be modulated by overlapping systems and neurocircuitry involving an interaction between the control of behaviour and its rewarding properties, which makes sense when conceived of as an environment inappropriate expression of an otherwise adaptive behaviour with evolutionary advantage (147-150).

Unsurprisingly, some of the major homeostatic metabolic regulatory systems also mediate physical activity, but once again in a seemingly paradoxical way when considering ABA. Administration of leptin, a peptide that circulates in the blood in proportion to fat mass, has been shown to both prevent the onset of semi-starvation induced hyperactivity (SIH) and reverse already established SIH when treatment was delayed in a rodent model in which

calories are restricted to 60% of *ad libitum* intake (151). In the ABA model, leptin administration is demonstrated to decrease RWA and general locomotor activity, with no effect on these measures in *ad libitum* fed animals (152). However, leptin treatment also decreased food intake and increased thermogenic energy expenditure in this study, resulting in a trend towards decreased body weight, all undesirable outcomes in ABA (152). Acute leptin administration (into the lateral ventricle or directly into the VTA) in ABA has also been shown to decrease general locomotor and running activity with no effect on food intake (153). These discrepancies may be explained by the link between leptin and midbrain dopamine signalling, which is known to play a role in the development of the ABA phenotype (112). DA neurons in the VTA express the leptin receptor (154, 155) and neuronal firing rate is suppressed by leptin binding (155), which results in decreased DA levels in the Acb (156). Furthermore, rats with a specific genetic knockout of the leptin receptor in the VTA display increased general locomotor activity (155), and prevention of leptin signalling in VTA DA neurons via genetic knockout of part of the post-binding pathway also increases general locomotor activity and RWA (144). It has therefore been proposed that in the anorectic state (low leptin levels) the mesolimbic DA system is overactive resulting in hyperactivity and that leptin administration in ABA decreases hyperactivity by suppressing VTA DA neuronal firing, although the exact mechanism has not been determined (152, 153, 157). This is at odds with the evidence that increasing the activity of VTA-AcbSh DA neurons protects against ABA-induced weight loss (112). However, this manipulation concerns a subpopulation of VTA neurons with precisely defined projections and therefore provides a clearer insight into the role of DA in the development of ABA, with a mechanism defined within this specific pathway.

To summarize, altered reward processing was found to be associated with susceptibility to ABA-induced weight loss, not via the development of a transient anhedonia, but rather by a decrement of the motivation to eat during the restricted feeding period. Susceptibility was also associated with increased RWA at baseline which became exacerbated with the onset of food restriction resulting in marked starvation-induced hyperactivity. On the other hand, resistance to ABA was characterized by the maintained motivation to eat which manifested in the development of FAA, greater subsequent food intake, and the suppression of non-specific starvation-induced hyperactivity. These findings are in keeping with the data from human studies of patients with AN where pre-existing elevated activity levels are a risk factor for development of AN with activity further increasing with the onset of food restriction and body weight loss. The present study was the first to look at the association between behavioural

correlates of anxiety and compulsivity *before* exposure to ABA with the intention to determine whether they predict susceptibility to ABA – previous studies have found increased anxiety-like behaviour *after* exposure to ABA but did not interrogate whether there was systematic variation in the level of ABA body-weight loss and anxiety. Anxiety-like behaviour, determined on the EPM and OF, and compulsivity, determined with the MBT, did not differentiate between rats that went on to be Susceptible or Resistant. While there was a trend toward Resistant rats having more interactions with marbles during the MBT than Susceptible rats, this difference did not reach significance and considering the lack of differences across other MBT measures is perhaps more reflective of increased exploratory behaviour than compulsive behaviour. This is contrary to the expectation from findings in the human literature that pre-existing levels of anxiety and OCD/OCP symptoms are risk factors for developing AN. It may be premature, however, to exclude such predisposing behaviour on the basis of the tests performed in our studies to date. These may lack the sensitivity or acuity to unveil underlying differences that will align with susceptibility to ABA. Alternatively, there may be no single behavioural measure that predicts susceptibility to ABA, but rather a constellation of small differences that independently fail to predict susceptibility but, in concert, comprise a cumulative burden which promotes susceptibility to ABA. This would fit with the difficulties in finding predictive markers for AN in the human condition and also explain the deficiencies of current treatments that often target only a subset of AN pathologies. Future studies could investigate this hypothesis by employing a battery of behavioural tests before ABA and generating cumulative/composite scores across a wide range of outcomes, which may unveil a summative effect of minor individual perturbations that together predict ABA susceptibility.

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CHAPTER THREE

Corticostriatal Control of Body Weight and Motivated Behaviour

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3.1 Introduction

Brain imaging studies have shown beyond doubt that anorexia nervosa (AN) has a neurobiological basis with both structural and functional contributions to the aetiology of the condition. Disruptions within multiple brain regions have been implicated in AN that underlie cognitive and behavioural domains ranging from cognitive control to reward processing and interoceptive bodily awareness (1-3). Together with the growing understanding of the genetic origins of the disorder, as well as the established, but often overemphasized, role of psychosocial and cultural factors in the genesis of AN, the appreciation and acceptance of a neurobiological underpinning has transformed the shape of the AN landscape. A recent focus on the most consistent findings from brain imaging studies in both acutely ill and weight-recovered patients has led to the conceptualization that AN results from an imbalance between cognitive control and reward processing, where underactivity in reward processing networks combined with overactivity in executive/cognitive control networks leads to the paradoxical and maladaptive behaviours inherent within AN pathology (1-3).

Patients with AN demonstrate increased activity in the prefrontal cortex (PFC), which is associated with cognitive control and decision-making (4-6) and this prefrontal hyperactivity leads to ascetic behaviour and the ability to delay or completely reject receipt of rewards (7, 8). Specifically, during both the anticipation of and after receipt of a reward, patients weight-recovered from AN (REC-AN) display increased activity in the dorsolateral prefrontal cortex (dlPFC), a key region within the cognitive control domain, indicating increased regulatory control over reward-related processing (4). Further, during a delay discounting task using monetary reward, REC-AN patients failed to show the differential functional brain activity displayed by healthy controls when either hungry (i.e. increased reward circuit activation, including the ventral striatum) or sated (i.e. increased control circuit activation, including the ventrolateral PFC and insula) (5). This indicates that even after body weight recovery, individuals having experienced AN have reward valuation that is insensitive to hunger or metabolic state (5). Moreover, unlike healthy controls, REC-AN patients do not differentially

encode positive and negative feedback to a monetary task, suggesting that they are also unable to accurately distinguish reward valence (i.e. emotional significance) for outcomes that are not dependent on hunger or food cues (9). REC-AN patients do not display the distinct pattern of activity in the ventral striatum that is associated with positive and negative feedback on this task (i.e. wins and losses) in healthy weight control participants, but instead demonstrate increased activity in the dorsolateral PFC, indicating an altered approach to reward based decision making that is biased towards cognitive control rather than pleasurable or hedonistic outcomes (9).

It also appears that AN may be associated with a 'recalibration' of reward valence towards illness relevant stimuli rather than a global dampening of reward processing (10). This 'bias' applies to both subjective pleasantness ratings as well as objective measures including, but not limited to, functional brain activity, reaction time, and startle response upon contemplation of illness-relevant stimuli. These stimuli exist along a continuum, e.g. between high- and low-calorie foods (11-13), varied body weight/shape (11, 14-16), and physically active versus passive actions (14, 17). Specifically, in acutely ill patients with AN, viewing images of underweight female bodies elicits increased activity in the ventral striatum (15, 16), while stimuli depicting physically active female bodies resulted in greater PFC activity during a task requiring prepotent response inhibition, implying higher reward valence with greater PFC activation required to inhibit responding (17).

The potential contribution of perturbed interoceptive awareness in the aetiology of AN is highlighted by findings of disrupted and diminished neural activity in the insula, in both acutely ill and weight-restored patients with AN. The insula, which includes the primary gustatory (taste) cortex, is the site of interoceptive processing responsible for relating, integrating and consolidating internal states (e.g. hunger, reward and motivation) with external stimuli to raise this information to consciousness and derive the subjective experience of "how you feel" (18). Activity in the anterior insula and striatum is shown to be decreased in REC-AN following administration of sucrose (a sweet and normally pleasant taste), and the normal association between subjective pleasantness ratings and insular activity is not present in these patients (19, 20). Further, simply viewing images of high-calorie food produced decreased activity in the anterior insula relative to control participants in both AN and REC-AN when hungry, a finding that persisted in AN when satiated, and demonstrates both a multimodal dysfunction in

processing food stimuli and an insensitivity to hunger signaling during the acute phase of the illness (12).

It is therefore thought that increased signaling in prefrontal circuits associated with ‘top-down’ processing mediates disrupted reward signaling in patients with AN, enabling their ascetic behaviour even if hedonic processing remains intact; in short, overactive prefrontal circuitry enables excessive self-control to override hedonic drives toward food consumption (1, 21, 22). Additionally, patients with AN may experience dysregulated interoceptive awareness and an inability to integrate their internal states with their conscious experience, both of which could perpetuate the paradoxical and maladaptive behaviours that characterize AN and contribute to the long-term maintenance of the disorder (19, 20).

One of the considerable challenges to understanding the aetiology of AN is the design of studies and composition of study groups. Considerable challenges include controlling for body weight, physical activity levels, and the effects of starvation that are independent of AN illness. The former is almost impossible, but constitutionally thin women represent the closest body weight comparison group, although only a few studies include them as a control group (23-25). Controlling for physical activity levels is more straight forward and accommodated by including a comparison group of athletes or healthy non-athletes that also have very high levels of exercise - again this experimental design option is not often utilized (17, 26). The predominant strategy used to experimentally dissect the effects of starvation present in the acutely ill AN population as a confound in investigations of AN specific morbidity is to either solely use REC-AN patients or include both AN and REC-AN groups. However, it remains possible that neurophysiological differences identified in REC-AN patients are ‘scars’ from their prior acute AN status, and without longitudinal data from patients before AN onset it is difficult to conclude whether changes identified in REC-AN were present before and contributed to illness onset (3). To this end, an appropriate animal model is required to be able to investigate and probe potential underlying neurobiological factors that are causally involved in the onset and maintenance of anorectic behavior in AN. The activity-based anorexia (ABA) rodent model recapitulates the core features of the human condition, namely decreased food intake and voluntary hyperactivity resulting in marked body weight loss (27, 28). The use of this model in combination with the use of experimentally-incisive techniques such as chemogenetics to modulate specific neural circuits in awake, behaving animals allows us to reveal pathways in the brain that are causally involved in the development of pathological weight loss.

We have previously demonstrated that activity within the primary reward pathway, comprised of dopaminergic (DA) cell bodies in the ventral tegmental area (VTA) that project to the nucleus accumbens (Acb), is involved in both the development and rescue of pathological weight loss in ABA (29).The Acb and VTA also receive direct glutamatergic (Glut) projections from the PFC (30-32), which influence reward perception and processing (33, 34). Thus, the PFC projections influence both indirectly (Acb) and directly (VTA) the mesolimbic dopaminergic reward network (32). As such, the Acb acts as an integration hub of DA projections from the VTA and Glut projections from the PFC (35). Given the reciprocal reward/control imbalance hypothesis outlined above in relation to the aetiology of AN, we sought to investigate the extent to which the PFC to Acb pathway is involved in body weight maintenance in ABA.

To investigate any causative role of cognitive control circuitry on the development of ABA and associated behaviours, this Chapter describes experiments utilizing chemogenetic modulation of neurons in the medial PFC that project to the shell subregion of the nucleus accumbens (i.e. mPFC-AcbSh; **see Methods section 3.2.2**). These approaches will help to identify, through either stimulation or suppression of mPFC-AcbSh, the impact of altered reward context on changes in feeding and exercise behavior in the ABA paradigm. Moreover, related experimental designs involving exposure of rats to *ad libitum* fed conditions, with and without access to a running wheel, or exposure to novel environments will help to define the specific contribution of the mPFC-AcbSh circuit to ABA.

3.2 Methods

3.2.1 Pathway specific chemogenetics - DREADDs

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are used here as part of a dual-viral strategy involving microinjection of two viral vectors into brain regions that are connected through a direct projection. Essentially, a retrogradely transported adeno associated virus expressing Cre recombinase was injected in the vicinity of the nerve terminals of the targeted projection and a second Cre-dependent viral vector was injected, in the same animal, into the brain region containing the parent cell bodies of those terminals (see below). Recombination of the viral vectors allows expression of the transported DREADD receptors which are then recruited (activated or inhibited) by daily intraperitoneal (i.p.) injections of the DREADD designer-ligand clozapine-n-oxide (CNO). This approach allows for anatomical and temporal specificity in activating or inhibiting (determined by the type of DREADD injected) the targeted pathway.

3.2.1.1 Viruses and drugs

The retrogradely transported Cre-expressing virus designated AAV5-pmSyn1-eBFP-Cre (Addgene, USA plasmid #51507; AAV-Cre) was injected into the AcbSh which contains the nerve terminals of cell bodies in the mPFC. In addition, Cre-dependent viral vectors, tagged with mCherry, that either encoded DREADDs (see below) or, in the case of the control virus, lacked DREADDs but still expressed mCherry (AAV5-hSyn1-mCherry, Addgene, USA plasmid #50459), were injected into the mPFC. DREADD-containing viruses were either inhibiting (AAV5-hSyn1-mCherry- hM4D(Gi); abbreviated hM4Di; UNC Vector Core, USA) or activating (AAV5-hSyn1-mCherry-hM3D(Gq); abbreviated hM3Dq; UNC Vector Core, USA) and contained the floxed inverted sequence of hM4D(Gi)-mCherry or hM3D(Gq)-mCherry, respectively (36, 37). These sequences reorient in the presence of Cre and as such DREADD receptors are only expressed in neurons that contain AAV-Cre (36, 37). Once effectively expressed, DREADDs can be “exclusively activated by designer drugs” as the name suggests – the designer drug being clozapine-n-oxide (CNO) (36, 38, 39). When CNO binds to inhibiting DREADDs the Gi-coupled signaling pathway is initiated, hyperpolarizing, and effectively silencing neuronal firing (36, 39, 40). Conversely, when CNO binds to the activating DREADDs the Gq-coupled signaling pathway is initiated, depolarizing the neuron and increasing its firing

rate (38, 39); see also **Results section 3.3.1** and additional published electrophysiology data (41). CNO was administered at 0.3 mg/kg (rats expressing hM3Dq) or 3.0 mg/kg (rats expressing hM4Di; mCherry injected control rats were split between doses), diluted in 0.9 % sterile saline containing 5% dimethyl sulfoxide (DMSO).

Potential off target effects of CNO and appropriate controls: In recent years it has become apparent that CNO can be metabolized to clozapine introducing a potential confound whereby clozapine, unlike its parent compound, may bind to endogenous receptors causing off target physiological or behavioral effects (42). In order to test whether such effects could be detected in the present study, a CNO dose-response experiment was undertaken in non DREADD-expressing animals and no behavioural, motor (running wheel activity), food intake or body weight changes were found exceeding, and including, our highest dose of 3.0 mg/kg (See **Supplementary Figure S3.1**). In addition, in all experiments described in this Chapter there was systematic control for potential off-target CNO effects through the use of a control virus (AAV-mCherry), that does not express a DREADD receptor, and administration of CNO to all animals.

3.2.2 Surgical procedure

Rats were anaesthetized by inhalation of 2.5% isoflurane with oxygen as a carrier gas. This gaseous anaesthetic was maintained while rats were placed in stereotaxic apparatus (Kopf Instruments, CA, USA), and injected bilaterally with 300nl of AAV-Cre ($6.0x10^9$ genomic copies/µl) into the AcbSh (from bregma: AP: +1.6mm, ML: ±0.8mm, DV: -6.6mm), and either hM4Di ($4.5x10^{12}$ genomic copies/µl), hM3Dq or AAV-mCherry ($4.0x10^9$ genomic copies/µl) into the mPFC. In these experiments the mPFC comprised both the prelimbic (PrL) and infralimbic (IL) cortices; from bregma; AP: +2.9mm, ML: ±0.5mm, DV: -3.4/-3.2mm (150nl at each depth). Viruses were infused over 5 min through micropipettes pulled from borosilicate glass capillary tubes (ID=0.700mm) using a vertical pipette puller (Stoelting, IL, USA) with a tip diameter of ~40µm. Micropipette tips remained at the injection site for a further 5 min to allow time for virus to diffuse away from the point of injection.

Prior to viral infusion, each rat received a subcutaneous injection of Meloxicam analgesic (0.2mg/kg; Boehringer Ingelheim, Germany) and the antibacterial agent Enrofloxacin (25mg/ml; Bayer, Germany) was administered via the drinking water (0.5ml/L) for 48h post-surgery. Rats

were group-housed in standard wire-topped opaque polypropylene cages (32x32x40 cm) following surgery with *ad libitum* access to food and water.

3.2.3 Animals and housing

All experimental procedures were approved by the Monash Animal Resource Platform Ethics Committee (MARP-2015-046 & MARP-2018-026). A singly housed male rat was present in all experimental rooms to synchronise the estrous cycles of the female rats via the Whitten effect (43).

3.2.3.1 mPFC-AcbSh circuit modulation in activity-based anorexia

To investigate the involvement of mPFC projections to the ventral reward network, specifically to the AcbSh, on the development of ABA and associated behaviours, 42 female Sprague-Dawley rats underwent stereotaxic surgery (body weight at surgery 140-180g) as described above (mPFC virus injected: hM3Dq *n*=16; hM4Di *n*=13; mCherry *n*=13). Following recovery from surgery, all rats were exposed to ABA conditions (as described in **Chapter 2 Methods section 2.2.1.3** without the acclimatization phase and enduring for Resistant rats for the full 10 days) and administered CNO daily (0.3-3.0 mg/kg; i.p) to determine the effects of mPFC-AcbSh circuit modulation on food intake, running wheel activity (RWA), food anticipatory activity (FAA), body weight loss and concomitant ABA survival.

3.2.3.2 mPFC-AcbSh circuit modulation with ad libitum feeding

In order to determine whether effects of mPFC-AcbSh circuit modulation were specific to outcomes under ABA conditions, the impact of perturbed activity of this circuit was examined in the absence of restricted feeding in a separate cohort of 26 female Sprague-Dawley rats. These animals underwent the same surgical procedure as described above (body weight at surgery 150-200g; mPFC virus injected: hM3Dq *n*=9; hM4Di *n*=9; mCherry *n*=8) but were then placed in individual activity living chambers with *locked* running wheels for 7 days. Subsequently, rats were allowed access to running wheels (7+10 days) and administered CNO (0.3-3.0 mg/kg; i.p) daily (final 10 days); however, food was available *ad libitum* throughout this study.

Following 3 days of undisturbed habituation to the activity living chambers (without wheel access), body weight and food intake were recorded daily for the duration of the experiment. Bodyweight was recorded 30 min before lights off (10:30h), with i.p. injections of CNO or saline administered at this same time when required. We recorded food intake across three different time periods beginning at lights off: 1) 90-min intake (11:00-12:30h), to compare with ABA food intake; 6-hr intake (11:00-17:00h), which encompasses the biological half-life of CNO (44); and 3) 24-hr (daily) food intake. Over the final 4 days of before wheel access rats received saline (days 1 and 2) followed by CNO (0.3-3.0mg/kg; days 3 and 4). Wheels were then unlocked for 17 days where days 1-7 were analogous to the ABA habituation period and days 8-17 were analogous to the ABA restricted feeding period (except *ad libitum* food access was maintained throughout). During this period of running wheel access, rats were administered saline before day 7 and CNO before days 8-17.

To determine the effects of mPFC-AcbSh modulation on general locomotor and feeding behaviour in a novel environment, at the end of the running wheel experiment all rats were subjected to a 10-min open field (OF) test and returned to their cages with food removed for an overnight fast. The following day rats were subjected to a combined light/dark emergence and novelty suppressed feeding (NSF) test. Both tests were conducted in the same order and at approximately the same time of day to ensure the same length of fast for all rats. Rats were administered CNO 30 min before each test and all behavioural tests were recorded with an overhead camera.

3.2.3.2.1 Open field test

The OF test was conducted as described in **Chapter 2 Methods section 2.2.2.3** except animal tracking in the present study was conducted with Ethovision XT software (Version 14; Noldus, Netherlands) tracking the rats' centre of gravity. The centre zone was restricted to the central square of a 3x3 grid (20x20cm; See **Figure 3.6 A**). The total distance (cm) travelled, the number of transitions between the centre and outside zone, as well as the proportion (%) of time spent in the centre versus outside zone were all measured.

3.2.3.2.2 Novelty suppressed feeding test

The NSF test was conducted in a light/dark box (45cm long [30cm light compartment + 15cm dark compartment] x 29cm wide x 29cm high), with the rat initially placed inside the dark compartment (solid opaque black plastic internal wall with a 15x10 cm hole separating light and dark compartments), and an ~1g standard laboratory chow pellet placed on a small piece of filter paper (9cm diameter; Whatman) in the open area on the far wall opposite the opening to the dark area (See **Figure 3.6 G**). The latency from the time the lid was placed on the box until the emergence of the animal's **a)** head, **b)** head and both shoulders, and **c)** full body, and until **d)** the rat picked up the pellet of food and bit into it, were all recorded with the test terminating upon the rat biting the food or after 10 minutes, whichever happened first. The NSF videos were manually scored using timestamps on the videos to determine latency durations.

3.2.4 Immunohistochemistry and imaging

At the completion of each experiment all rats were deeply anaesthetized with pentobarbitone sodium (Lethabarb, Virbac, Australia; >100mg/kg i.p.) and perfused transcardially with 200ml saline followed by 200ml 4% paraformaldehyde in phosphate buffer (PFA-PB). Brains were excised and placed into 4% PFA-PB solution overnight, followed by submersion in 30% sucrose in PB solution at 4ºC for 3-4 days. Brains were sectioned at 35µm thickness using a cryostat (CM1860, Leica Biosystems, Germany). Brain sections were collected in multiwell cell culture plates in a 1:4 series and stored in cryoprotectant fluid (PB containing 30% Ethylene glycol and 20% Glycerol) for immunohistochemical processing. Sections were washed in PB and subsequently blocked and permeabilized in PB containing 10% normal horse serum (NHS) and 0.3% Triton for 30 min. Sections were then incubated overnight at room temperature with primary antibodies including mouse anti-DsRed (sc-390909, 1:1000; Santa Cruz Biotechnology, Dallas, TX), chicken anti-GFP (ab13970, 1:2000; Abcam) and rabbit anti-cfos (sc-52, 1:2000; Santa Cruz Biotechnology) in PB containing 1% NHS and 0.3% Triton. Sections were then washed (2x15 min) in PB before a 90-min incubation in secondary antibodies conjugated with Alexa Fluor dyes (either 594 [red], 488 [green] or 647 [far red], both diluted in PB 1:1000; Abcam, Cambridge, United Kingdom). Following two final 15 min washes in PB slices were mounted on glass slides covered in mounting medium (Fluoromount-G, SouthernBiotech) and coverslipped. Imaging was conducted by **Dr Claire Foldi** using a Leica SP5 5 channel confocal microscope (with 20X magnification, resolution 1024x1024 pixels) running LAS AF software

(Leica Microsystems, Wetzlar, Germany). Tiled, z-stacked images (4x2 tiles, z=9) were captured of the rostral portion of the brain, containing mPFC, from 4x35µm sections per brain from a subset of animals from the ABA cohort for confirmation of the action of CNO on mCherry+ neurons (see below).

3.2.4.1 Injection site hit or miss criteria

Analysis of viral injection sites revealed the presence and extent of Cre and DREADD labeling in AcbSh and mPFC. Rats with brains containing little or no mCherry-labeled neurons, or with neurons labelled outside the bounds of the medial portion of the PFC that includes both PrL and IL were excluded from all analyses (See **Methods section 3.2.5.3** for details). Moreover, if there was evidence of Cre expression (through detection of eBFP associated with the AAV-Cre vector, which cross reacts with the anti-GFP antibody) dorsal to the AcbSh in the medial septum, these animals were also excluded. Injection site evaluation was conducted by **Dr Claire Foldi** who was blinded to animal identity during evaluation.

3.2.4.2 Fos and DREADD colocalization in mPFC

A subset (hM3Dq *n*=3; hM4Di *n*=3, mCherry *n*=4) of rats from the ABA cohort were administered CNO 90 minutes before transcardial perfusion with aldehydes in order to investigate the extent to which activation or inhibition could be detected in DREADD-expressing (mCherry-positive) neurons in the mPFC. This approach relied on the immunohistochemical staining of the proto-oncogene cFos as a (nuclear) marker of neuronal activation. Essentially colocalization of green (Alexa 647) – tagged Fos protein present in the nuclei of activated cells and mCherry expression (enhanced with anti- DS red) in the cytoplasm of those cells undergoing recombination were assessed quantitatively as a guide to the efficacy of the approach. Blinded counts were made from confocal microscope images using ImageJ version 1.49u (2015; National Institutes of Health, Bethesda, MD). It should be noted (see **Results section 3.3.1**) that relatively high levels of background activity in this neuronal population allowed the detection of reduced Fos activity after CNO treatment of animals receiving inhibitory (hM4Di) DREADDs.

3.2.4.3 Whole-cell patch clamp electrophysiology in mPFC

In order to more directly evaluate the extent of activation and inhibition of DREADD-expressing neurons after CNO treatment and the possibility of "off target" CNO effects, whole-cell patch clamp electrophysiology was used in tissue slices from DREAAD-injected animals (hM3Dq *n*=2; hM4Di *n*=2, mCherry *n*=2) three weeks after viral injections. Essentially, a series of current steps were injected into cells (hM3Dq *n*=10; hM4Di *n*=9, mCherry *n*=9) at baseline and at the peak of the response to bath-applied CNO (1-10 μM). The time to peak response following CNO application was typically 3-5 min, after which current injections were made to determine current-voltage relationships, input resistance and rheobase. Membrane potential was recorded when stable just prior the application of CNO, and at the peak of the CNO response. Firing frequency was determined by measuring the number of action potentials fired in the 60 seconds following the peak CNO response (hM3Dq-expressing cells). Input resistance was determined by the membrane voltage response to a small hyperpolarizing step (-30 to -60 pA) while cells were held at approximately -60 mV. Rheobase was obtained by holding cells to -60 mV, then injecting positive current steps of 2 pA increment until the cell first fired at two concordant current measures. For data analysis, the signal was digitized at 2-10 kHz and analyzed utilizing pClamp10 (Axon Instruments). Electrophysiological validation was carried out by **Dr Paul Mirabella** (41).

3.2.5 Statistical analysis and data exclusion

3.2.5.1 Data management of ABA outcomes

Due to the nature of the ABA protocol susceptible rats are removed from the experiment once they reach criterion while resistant rats remain in the experiment for its' duration. This creates a bias in group means towards values representative of resistant rats as they come to predominate and eventually exclusively comprise group membership as ABA progresses. As such, for outcome measures that differ largely between susceptible and resistant rats this means that longitudinal analysis of data is either inappropriate or misrepresentative (although it may still be insightful if interpreted with this caveat in mind), whereas it is applicable for outcomes where there is not a large difference between rats with different ABA outcomes. By definition, body weight differentiates susceptible and resistant rats and longitudinal analysis of group data is thus prohibited. RWA and food intake do not necessarily systematically align with

susceptibility and thus group means have been generated and analyzed. However, in all cases the most appropriate method to analyze these outcomes is to generate mean daily values which take into consideration, for each individual animal, how many days they remained in the ABA paradigm.

3.2.5.2 Statistical analysis

Graphing and statistical analyses were performed in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA), and significance for all tests was set at $p<.05$. Survival in ABA was assessed with log-rank (Mantel-Cox) χ^2 tests using a Bonferroni correction for multiple comparisons. One-way ANOVA followed by Tukey's post-hoc multiple comparisons was used to analyze: mCherry and cFos colocalization; ABA mean daily body weight loss (g and %), food intake, RWA change and FAA change; *ad libitum* change in mean daily RWA and FAA, OF distance travelled and zone transitions, and all NSF emergence and feeding latencies. Mixed-effect models were used to analyze ABA daily food intake and RWA. Two-way ANOVA followed by post-hoc multiple comparisons (Tukey's for between group, or with a Bonferroni correction for within group) was used to analyze: ABA mean daily RWA, hourly RWA, FAA; *ad libitum* daily body weight, mean food intake (locked wheels, open wheels, CNO locked vs. open wheels) across all feeding windows, mean daily RWA, hourly RWA, FAA and OF zone duration. Paired t-tests (two-tailed) were used to compare *ad libitum* baseline (saline) mean food intake locked vs. open wheels across all feeding windows. Linear regression correlations were used to analyze the relationship between *ad libitum* OF distance travelled and RWA.

3.2.5.3 Animal and data exclusion

Rats with misdirected viral injections, or that showed aberrant feeding behaviour in the form of food hoarding were excluded from the data set leaving the final cohort size for ABA experiments $n=35$ (hM3Dq $n=13$; hM4Di $n=10$; mCherry $n=12$). Exclusion of rats with misdirected viral injections from experiments with *ad libitum* access to food produced a final cohort size of $n=23$ (hM3Dq $n=7$; hM4Di $n=8$; mCherry $n=8$). Additionally, all feeding data from three rats (one from each group) and locked wheel feeding data from one hM4Di rat were excluded from analysis due to food hoarding. Further, OF video from one hM3Dq rat was corrupted preventing analysis.

3.3 Results

3.3.1 Validation of injection sites and CNO effects on DREADD expressing mPFC-AcbSh projecting neurons

In order to better interpret the behavioral outcomes of DREADD interventions it was important to validate the extent to which CNO was effective in either activating or inhibiting mPFC-AcbSh projecting neurons shown to have undergone Cre-induced recombination (and incorporation of DREADD receptors) by their expression of mCherry. This was undertaken in two ways, firstly fluorescence immunohistochemistry (**Figure 3.1 A**) utilizing the protein Fos, as a marker of neuronal activity, and secondly relying on patch clamp electrophysiology (**Figure 3.1 D**) to evaluate directly the excitation or inhibition of DREADD-expressing neurons in tissue slice preparations of mPFC after CNO application.

In the first approach, comparison of Fos-positivity induced by i.p. CNO in mCherry positive neurons in the mPFC after either local injections of control AAV-mCherry or AAV-mCherry-DREADDs (**Figure 3.1 B**), showed an elevation of colocalization in neurons expressing activating-hM3Dq-DREADDs (p=.0186) and a reduction in those expressing inhibiting-hM4Di-DREADDs (p=.0001) compared to controls, with a significant difference also shown between DREADD groups (p<.0001; **Figure 3.1 C**).

In the second validation approach, bath application of CNO (1 μM hM3dq; 10 μM hM4Di) to tissue slices containing the mPFC from rats that had undergone the dual AAV procedure revealed whole cell current clamp electrophysiological responses that were distinctly bidirectional depending on the incorporation of either activating or inhibiting DREADDs. Patch clamp recordings from all (n=8) mPFC neurons expressing activating-hM3Dq-DREADDS showed a marked depolarization of membrane potential and increased firing rate (**Figure 3.1 E)**. Conversely, there was a marked hyperpolarization and decreased firing in all (n=9) cells expressing inhibiting-hM4Di-DREADDs with no change in activity in control cells expressing only mCherry (n=8; **Figure 3.1 E**). This latter finding confirmed that the approximately 50% colocalization of Fos+ and mCherry+ following control injections was due to high constitutive Fos activity in the mPFC and not off-target effects of CNO. Parenthetically, this relatively high level of “background” Fos activity enabled the detection of reduced double labelling in the inhibitory DREADD situation (**Figure 3.1 C**).

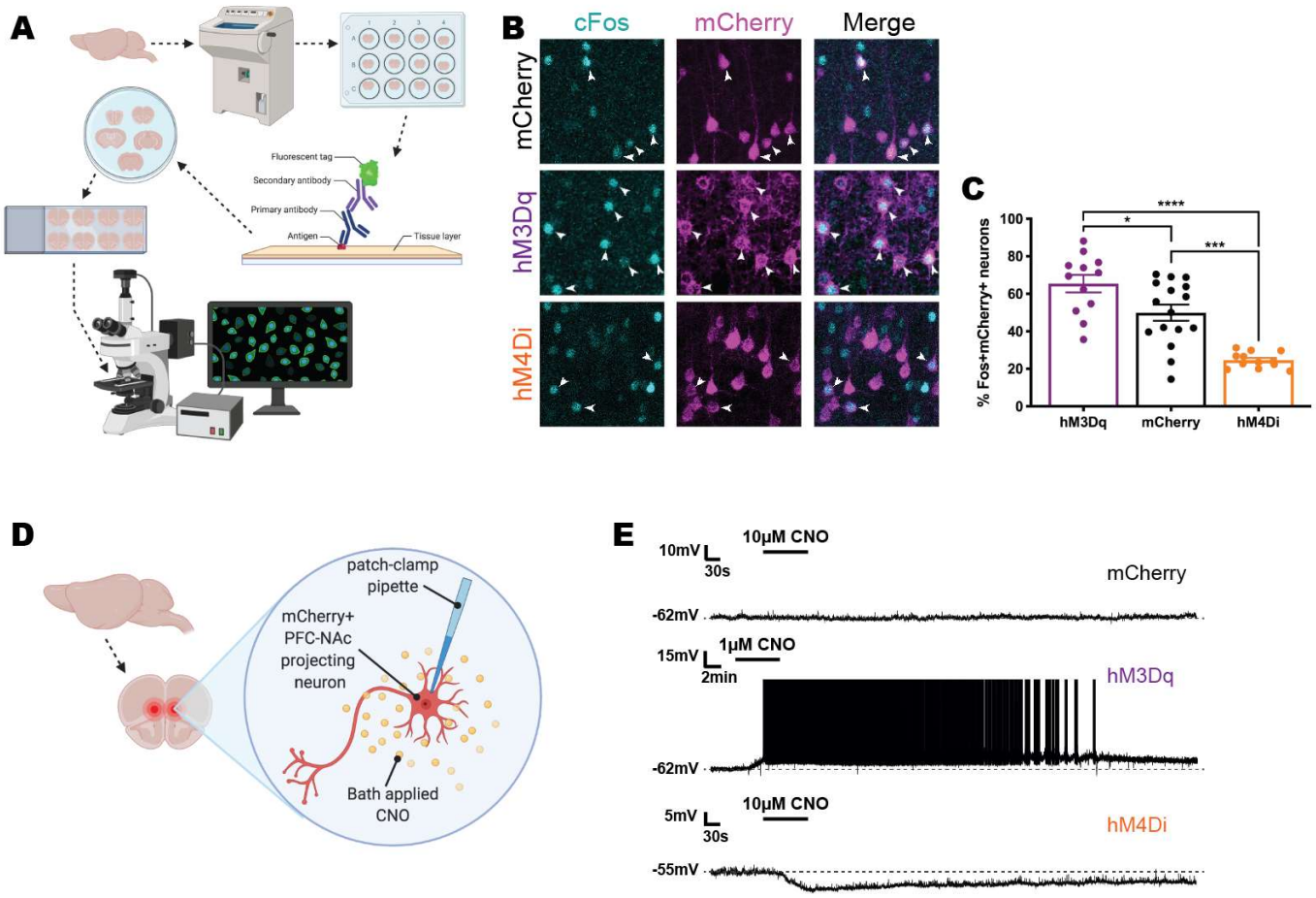

Figure 3.1 Immunohistochemical staining and electrophysiological characterisation of the effects of CNO on DREADD-expressing vs control injected mPFC-AcbSh neuronal projections. A) Schematic showing the broad steps involved in processing of brain tissue for immunohistochemical staining. **B)** Representative images of mPFC sections of brains from each DREADD group, showing immunofluorescence for mCherry+ (magenta) and Fos+ (cyan) cells and mCherry+/Fos+ colocalization (white arrow heads). **C)** Quantification of colocalization between mCherry+ and Fos+ in neuronal cell bodies from each section examined. One-way ANOVA followed by Tukey's post-hoc multiple comparisons: $F(2, 37)=25.39$, $p<.0001$; hM4Di < mCherry $p=.0001$, mCherry < hM3Dq $p=.0186$, hM4Di < hM3Dq $p<.0001$. Data show individual sections (circles) and group mean ± SEM (bars). *$p<.05$, ***$p<.001$, ****$p<.0001$. **D)** Schematic showing whole cell patch-clamp electrophysiology. **E)** Representative electrophysiological traces following bath administration of CNO for each receptor type.

3.3.2 mPFC-AcbSh pathway inhibition prevents body weight loss in ABA

In order to determine the role of mPFC-AcbSh projecting neurons in the development of ABA, the activity of this circuit was modulated during exposure to the ABA paradigm **(Figure 3.2 A)**. This process essentially involved rats that, given their pretreatment, expressed either excitatory (hM3Dq) or inhibitory (hM4Di) DREADDs or a control AAV injection that expressed mCherry. Following a 7-day habituation period with free access to running wheels and *ad libitum* access

to food there was a 10-day experimental period during which there was exposure to ABA conditions and daily administration of CNO. Chemogenetic inhibition with hM4Di prevented the typical ABA induced body weight loss resulting in complete resistance to ABA with 100% (10/10) survival which was significantly greater than that seen in rats undergoing chemogenetic activation of these same projections (38% [5/12]; *p*=.0030, **Figure 3.2 B**) or those with control (mCherry) expression (58% [7/12]; *p*=.0243).

When individual responses were evaluated in each of the groups there was a mixture of susceptible and resistant responses within the circuit activated and control cohorts with approximately half of each group displaying the typical body weight free fall characteristic of susceptibility to ABA (solid lines in **Figure 3.2 C** and **E**), compared to those in the circuit inhibited group where there was a uniform flattening of the trajectory of body weight loss resulting in all rats maintaining body weight above 80% of baseline (**Figure 3.2 D**).

Differences in survival potential were therefore aligned with differences between and within groups in the amount of weight lost in the ABA protocol. There was a significant difference in mean daily ABA-induced body weight loss when measured either in absolute terms (*p*=.0213; **Figure 3.2 G**) or as a percentage of baseline body weight (*p*=.0246; **Figure 3.2 H**) with circuit inhibited rats, on average, losing significantly less body weight per day than circuit activated rats on both measures (grams *p*=.0161, percent *p*=.0193). In fact body weight loss may be considered a predictor of survival with all rats that lost less than 5g or 3% of their body weight /day ultimately showing resistance to ABA (*n*=20), with only 2/15 that lost weight at a greater rate showing resistance to ABA and 6/6 rats that lost more than 10g or 5% of their body weight / day succumbing to ABA. Therefore, in broad terms, to ensure survival during ABA rats need to lose less than 5g or 3% body weight per day.

The two components of the ABA model, that will likely impact body weight and may be shifted by chemogenetic modulation of the prefrontal circuit, are time-restricted access to food and voluntary unrestricted access to a running wheel and are considered below.

3.3.3 mPFC-AcbSh pathway inhibition increases food intake during ABA

In order to identify which facets of the ABA protocol were impacted by circuit modulation, and drove the divergent effects on body weight maintenance, we compared the effects of circuit

modulation on food intake and running wheel activity (RWA) across the 10-day ABA period. Individual daily food intake is plotted for all animals separated by group and then combined to allow direct visualization of the entire cohort (**Figure 3.2 I-L**). There was no overall difference in daily food intake between groups when analyzed longitudinally (p=.4244; **Figure 3.2 M**). However, analysis of mean daily food intake did reveal a significant difference (p=.0252), whereby, on average, mPFC-AcbSh circuit inhibited hM4Di rats (100% resistant) ate significantly more per day than hM3Dq rats with this circuit activated (38% resistant; p=.0229; **Figure 3.2 N**).

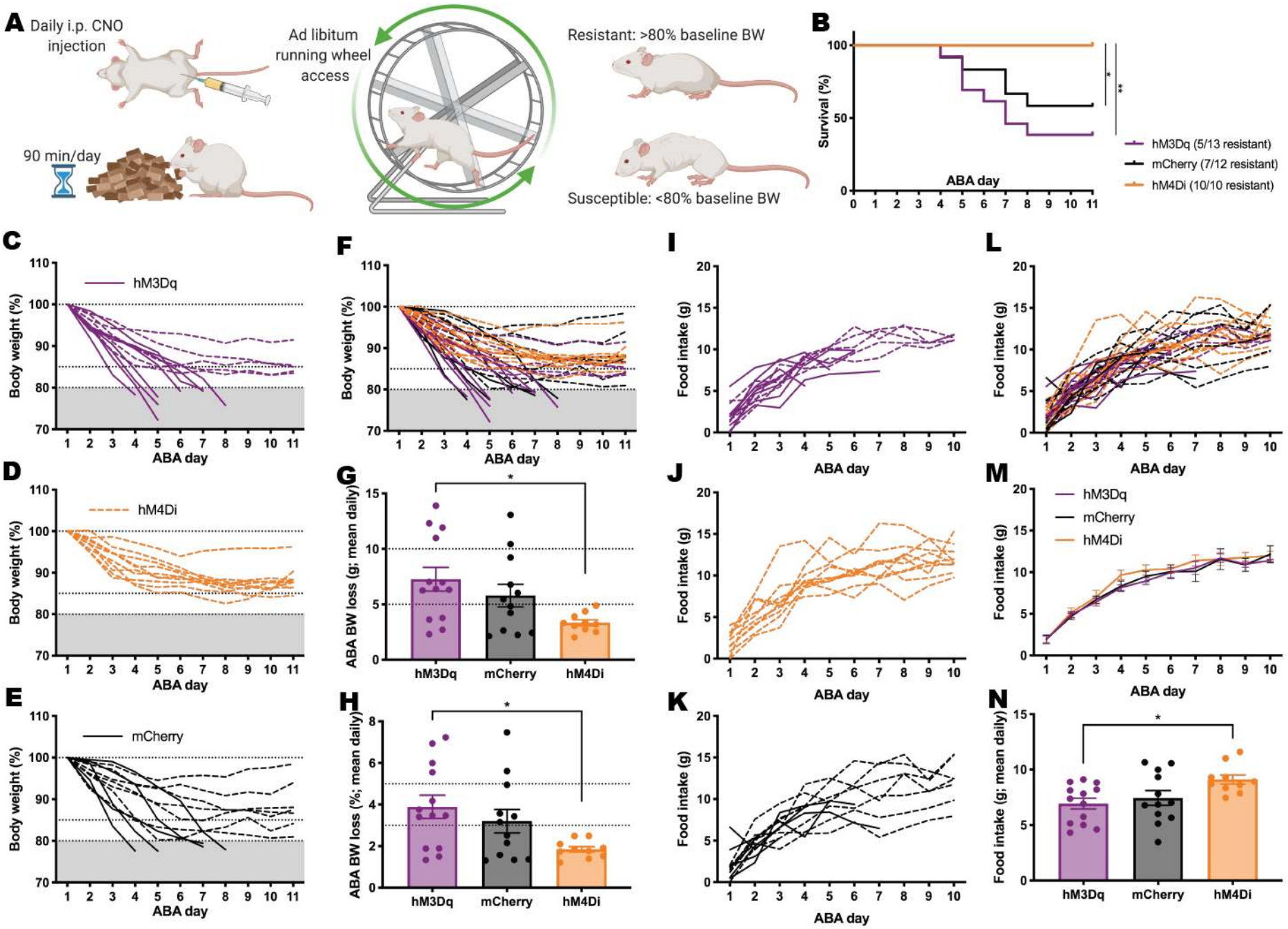
A
Daily i.p. CNO injection
90 min/day
Ad libitum running wheel access
Resistant: >80% baseline BW
Susceptible: <80% baseline BW
B
Survival (%)
ABA day
hM3Dq (5/13 resistant)
mCherry (7/12 resistant)
hM4Di (10/10 resistant)
C
hM3Dq
Body weight (%)
ABA day
D
hM4Di
E
mCherry
F
G
ABA BW loss (g; mean daily)
hM3Dq
mCherry
hM4Di
H
ABA BW loss (%; mean daily)
I
Food intake (g)
J
K
L
M
N
Food intake (g; mean daily)

Figure 3.2 The impact of modulation of mPFC-AcbSh projecting neurons on the development of the ABA phenotype. Following 7 days of habituation to running wheels, rats with inhibiting-hM4Di-DREADDs (*n*=10; orange), activating-hM3Dq-DREADDs (*n*=13; purple), or AAV-mCherry (control; *n*=12; black) expressed in the cell bodies of mPFC neurons projecting to the AcbSh were exposed to a maximum of 10 days of ABA conditions **(A)** consisting of daily i.p. injections of CNO and food access limited to the first 90 minutes of the dark phase. **B)** Percentage survival after the 10 days of exposure to the ABA protocol. Log-rank (Mantel-Cox) χ^2 tests using Bonferroni correction for multiple comparisons: hM4Di > mCherry χ^2=5.075, *p*=.0243; hM4Di > hM3Dq χ^2=8.832, *p*=.0030. **C-F)** Individual rats' body weight % change from baseline (ABA day 1) across 10 days of exposure to the ABA protocol (susceptible; solid lines, resistant; dashed lines). Mean daily ABA-induced body weight loss in grams (**G**) and as a percentage of baseline body weight (%; **H**) over the 10-day procedure. **G-H**) One-way ANOVA analyses applied to both absolute weight loss ($F(2, 32)=4.351$, $p=.0213$) and % weight loss ($F(2, 32)=4.171$, $p=.0246$) together with Tukey's post-hoc multiple comparisons revealed hM4Di lost significantly less body weight (g) and % weight loss on average per day than hM3Dq ($p=.0161$ and $p=.0193$ respectively). **I-L)** Individual 90-minute food intake over 10 days of exposure to the ABA protocol, indicating susceptible (solid lines) and resistant (dashed lines) rats. **M)** Group mean ± SEM of data from **L**. Mixed-effects model: Time $F(2.313, 57.05)= 130.1$, $p<.0001$; Group $F(2, 32)= 0.8803$, $p=.4244$; Interaction $F(18, 222)= 0.5001$, $p=.9564$. **N**) Mean daily food intake over a maximum of 10 days of ABA. One-way ANOVA: $F(2, 32)=4.137$, $p=.0252$. Tukey's Post-hoc multiple comparisons revealed that hM4Di rats ate significantly more, on average, per day than hM3Dq rats $p=.0229$. **G, H, N)** Circles are individual animals, bars are group mean ± SEM. *$p<.05$, **$p<.01$

3.3.4 mPFC-AcbSh pathway activation exacerbates starvation-induced hyperactivity

Chemogenetic activation of mPFC-AcbSh projecting neurons facilitated starvation-induced hyperactivity with daily RWA increasing from the onset of food restriction (**Figure 3.3 A**), whereas there was a slower and less dramatic increase in both rats with this circuit inhibited (**Figure 3.3 B)** and mCherry control**s** (**Figure 3.3 C**). Despite the large variability in RWA within each group, the overall difference between groups is evident when the individual data of the entire cohort are overlaid (**Figure 3.3 D**).

While all groups increased their daily RWA over time (p<.0001), and there was no statistically significant difference between DREADD modulation groups over the entire course of running activity (p=.2620), the onset of food restriction uncovered a significant interaction between mPFC modulation and wheel running over time (p=.0027; **Figure 3.3 E**), despite this longitudinal analysis not differentiating between the habituation and ABA phases and being susceptible to bias towards resistant values arising from susceptible rats being removed from ABA over time as detailed in **Methods section 3.2.5.1**. Therefore, mean daily RWA within each phase was calculated for each rat and group means were compared. While all groups significantly increased RWA with the onset of food restriction (all ps<.0001), the magnitude of this RWA increase was dependent upon the mode of mPFC-AcbSh circuit modulation (p=.0059), such that RWA during habituation was similar across groups whereas the circuit activated hM3Dq group ran significantly more, on average, per day in ABA than the control mCherry group (p=.0265; **Figure 3.3 F**). To account for baseline variability in RWA that is not a result of circuit modulation, we calculated the change in mean daily RWA resulting from food restriction (mean daily ABA RWA – mean daily Habituation RWA). This revealed an overall effect of mPFC-AcbSh circuit modulation (p=.0059), whereby circuit activated hM3Dq rats increased RWA following the onset of food restriction significantly more than both control mCherry (p=.0126) and circuit inhibited hM4Di (p=.0180) rats (**Figure 3.3 G**).

In the ABA phase, the RWA of circuit activated hM3Dq rats displayed a circadian shift from that seen during habituation, with the formation of a sustained peak across the first half of the dark period and a steady incline towards a sharp peak across the latter half of the light period, a pattern which developed more rapidly and to a greater magnitude than for either the circuit inhibited hM4Di or control mCherry groups (**Figure 3.4**).

The timing of RWA for rats is largely dependent upon the phase of the light cycle, with greater activity during the dark phase than the light phase. This cyclic pattern of RWA is evident during the habituation phase, when rats have *ad libitum* access to food and there is no circuit modulation, where RWA is predominantly observed in the dark phase (14:00-01:00h; grey shaded area **Figure 3.3 H** and **K**), particularly in the latter half, rather than the light phase (02:00-13:00h; unshaded area), and is very similar across groups**.** However, during ABA when food availability is restricted to 90 minutes per day at the onset of the dark phase (14:00h-15:30h) and the mPFC-AcbSh circuit is modulated by CNO administration, differences in the overall volume and in the timing of RWA as a result of circuit modulation emerge. All groups increased RWA in the dark phase (shaded grey area in **Figure 3.3 I**) with peak RWA in this phase shifted forward to occur during the first half (shaded grey area in **Figure 3.3 J**), reflected by a relative increase (positive values) in RWA in the early dark phase and a relative decrease (negative values) in the late dark phase (shaded grey area in **Figure 3.3 M**). Further, RWA in the latter half of the light phase progressively increased to match or exceed that of the dark phase, and is evident in both absolute and relative RWA (unshaded area in **Figure 3.3 I, J, L** and **M**). The importance of looking at the relative measure of RWA is demonstrated within the ABA phase and in comparing the change from habituation to ABA, where absolute RWA for circuit activated hM3Dq rats during some 1-hour blocks was significantly greater than both control mCherry rats (^) and circuit inhibited hM4Di ($) rats (**Figure 3.3 I** and **J**), but this effect is negated when looking at relative RWA (**Figure 3.3 L** and **M**), indicating that this difference is not due to a change in the circadian rhythm of RWA for circuit activated hM3Dq rats' RWA but merely reflects their increased daily volume of RWA reported above (**Figure 3.3 E**).

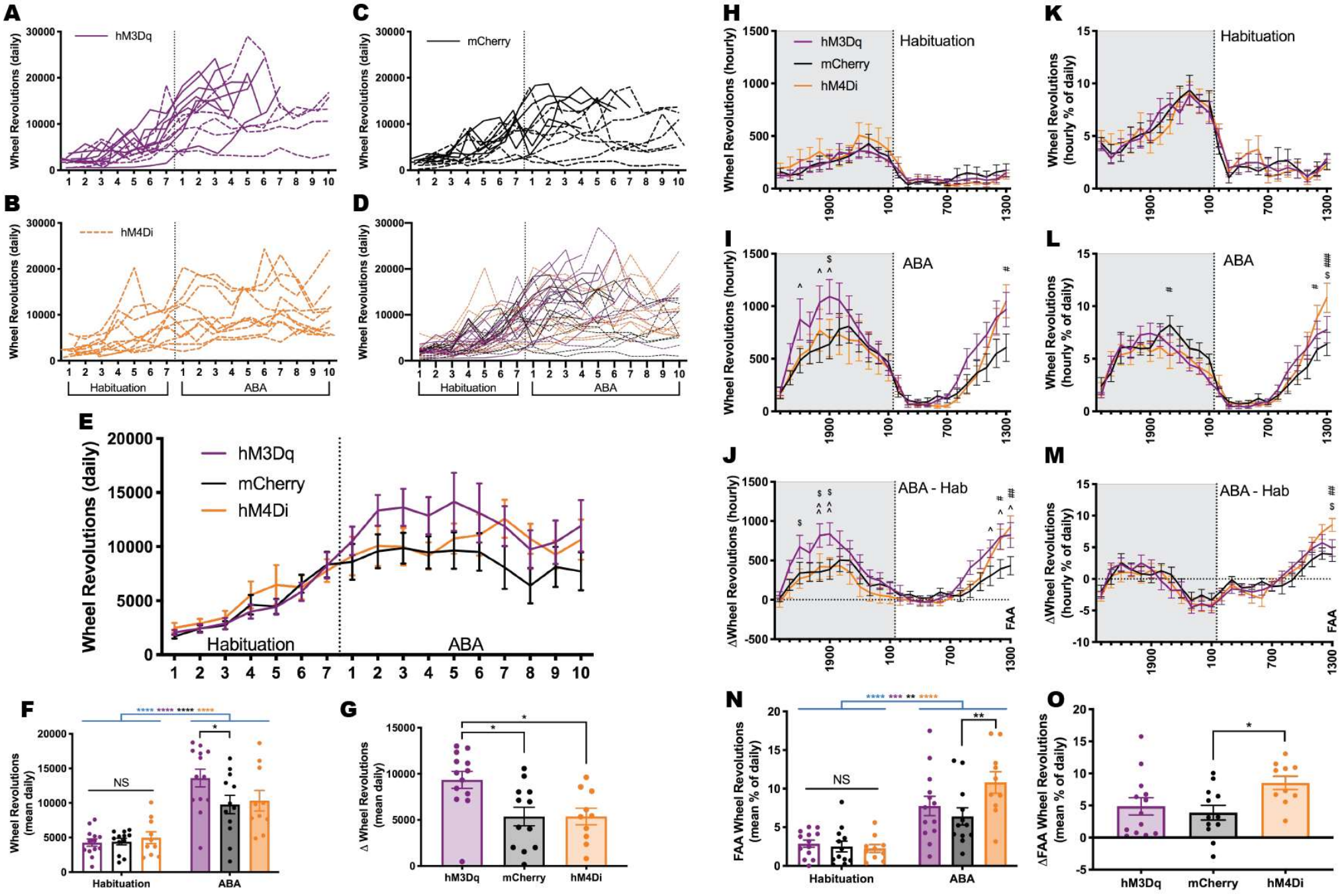
A
hM3Dq
Wheel Revolutions (daily)
B
hM4Di
Habituation
ABA
C
mCherry
D
E
hM3Dq
mCherry
hM4Di
Wheel Revolutions (daily)
Habituation
ABA
F
Wheel Revolutions (mean daily)
NS
Habituation
ABA
G
Δ Wheel Revolutions (mean daily)
hM3Dq
mCherry
hM4Di
H
Habituation
Wheel Revolutions (hourly)
I
ABA
J
ABA - Hab
ΔWheel Revolutions (hourly)
FAA
K
Wheel Revolutions (hourly % of daily)
L
M
ΔWheel Revolutions (hourly % of daily)
N
FAA Wheel Revolutions (mean % of daily)
O
ΔFAA Wheel Revolutions (mean % of daily)

Figure 3.3 Effects of chemogenetic modulation of mPFC-AcbSh projecting neurons on ABA-related outcomes. Running wheel activity (RWA; total wheel revolutions) over 7 days of habituation and a maximum of 10 days of ABA. All rats including control mCherry (*n*=12, black) and those with either inhibiting-hM4Di-DREADDs (*n*=10; orange) or activating-hM3Dq-DREADDs (*n*=13; purple) expressed in the cell bodies of mPFC neurons projecting to the AcbSh were administered CNO to modulate the circuit only throughout the ABA phase. **A-D)** Individual rat's daily RWA, indicating whether rats were susceptible (solid lines) or resistant (dashed lines) to ABA body weight loss. **E)** Group mean ± SEM of data in **D**. Mixed-effects model: Time $F(5.450, 152.3)=43.08$, $p<.0001$; Group $F(2, 32)=1.397$, $p=.2620$; Interaction $F(32, 447)=1.895$, $p=.0027$. **F**) Mean daily RWA over the habituation and ABA phases. Two-way ANOVA: Time $F(1, 32)= 145.3$, $p<.0001$; Group $F(2, 32)= 1.082$, $p=.3510$; Interaction $F(2, 32)= 6.063$, $p=.0059$. Post-hoc multiple comparisons with a Bonferroni correction revealed that all groups had significantly greater mean daily RWA during ABA than Habituation (all *p*s<.0001). Tukey's post-hoc multiple comparisons revealed that hM3Dq rats ran significantly more than mCherry rats in the ABA phase ($p=.0265$). **G**) Change in mean daily RWA from habituation to ABA calculated as mean daily RWA in ABA minus mean daily RWA in habituation. One-way ANOVA: $F(2, 32)= 6.063$, $p=.0059$. Tukey's Post-hoc multiple comparisons revealed that hM3Dq rats increased RWA with the onset of food restriction significantly more than both mCherry ($p=.0126$) and hM4Di ($p=.0180$) rats. **H-M)** Group mean hourly RWA over 24 hours during habituation (*ad libitum* access to food; **H** and **K**), ABA (90 minutes access to food + CNO administration to modulate mPFC-AcbSh circuit; **I** and **L**), and the change in activity from habituation to ABA calculated by subtracting hourly habituation RWA from hourly ABA RWA (**J** and **M**), measured as absolute wheel revolutions (total wheel revolutions; **H-J**) and relative/proportionate RWA (hourly RWA as a percentage of daily RWA; **K-M**). Positive change values in **J** and **M** indicate an increase in RWA in ABA compared to habituation and negative change values indicate a decrease in RWA in ABA compared to habituation. **H**) Two-Way ANOVA: Time $F(2.913, 93.22)= 13.42$, $p<.0001$; Group $F(2, 32)= 0.3543$, $p=.7044$; Interaction $F(46, 736)= 0.5736$, $p=.9899$. **I**) Two-Way ANOVA: Time $F(23, 736)= 23.01$, $p<.0001$; Group $F(2, 32)= 2.458$, $p=.1016$; Interaction $F(46, 736)= 0.9972$, $p=.4799$. Tukey's post hoc multiple comparisons revealed that hM3Dq rats had significantly greater RWA than mCherry at 1600h ($p=.0346$), 1800h ($p=.0156$) and 1900h ($p=.0117$) and than hM4Di at 1900h ($p=.0472$), and hM4Di rats had significantly greater RWA than mCherry at 1300h ($p=.0250$). **J**) Two-Way ANOVA: Time $F(23, 736)= 16.50$, $p<.0001$; Group $F(2, 32)= 6.063$, $p=.0059$; Interaction $F(46, 736)= 1.332$, $p=.0730$. Tukey's post hoc multiple comparisons revealed that hM3Dq rats had significantly greater RWA than mCherry at 1800h ($p=.0035$), 1900h ($p=.0048$), 1100h ($p=.0423$), 1200h ($p=.0112$) and 1300h ($p=.0176$) and than hM4Di rats at 1600h ($p=.0193$), 1800h ($p=.0226$), and 1900h ($p=.0142$), whilst hM4Di rats had significantly greater RWA than mCherry at 1200h ($p=.0373$) and 1300h ($p=.0036$).**K**) Two-Way ANOVA: Time $F(4.137, 132.4)= 22.57$, $p<.0001$; Group $F(2, 32)= 0.1668$, $p=.8471$; Interaction $F(46, 736)= 0.4817$, $p=.9986$. **L**) Two-Way ANOVA: Time $F(23, 736)= 26.10$, $p<.0001$; Group $F(2, 32)= 0.9877$, $p=.3835$; Interaction $F(46, 736)= 1.152$, $p=.2315$. Post hoc multiple comparisons with a Bonferroni correction revealed that hM4Di rats had significantly greater RWA than mCherry at 2100h ($p=.0406$), 1200h ($p=.0282$) and both mCherry ($p=.0007$) and hM3Dq ($p=.0256$) at 1300h. **M**) Two-Way ANOVA: Time $F(23, 736)= 19.61$, $p<.0001$; Group $F(2, 32)= 1.024$, $p=.3706$; Interaction $F(46, 736)= 0.8752$, $p=.7068$. Tukey's post hoc multiple comparisons revealed that hM4Di rats had significantly greater RWA than both hM3Dq ($p=.0399$) and mCherry ($p=.0068$) at 1300h. **N-O)** Food anticipatory activity (RWA in the penultimate hour before food availability [1300h-1400h]) as a proportion of total daily RWA during habituation (**N** left) and ABA + CNO administration to modulate mPFC-AcbSh circuit (**N** right) and as a change from habituation to ABA showing the effect of the onset of food restriction (ABA-Hab; **O**). **N)** Two-way ANOVA: Time $F(1, 32)= 67.45$, $p<.0001$; Group $F(2, 32)= 1.721$, $p=.1950$; Interaction $F(2, 32)= 3.779$, $p=.0336$. Post-hoc multiple comparisons with a Bonferroni correction revealed that all groups had significantly greater FAA during ABA than Habituation, but that the magnitude of the increase was different (hM3Dq $p=.0005$; mCherry $p=.0081$; hM4Di $p<.0001$). Tukey's post-hoc multiple comparisons revealed that hM4Di rats had significantly greater FAA during ABA than mCherry $p=.0082$ **O)** One-way ANOVA: $F(2, 32)=3.779$, $p=.0336$. Tukey's post-hoc multiple comparisons revealed that the increase in FAA with the onset of food restriction was significantly greater for hM4Di rats compared to mCherry $p=.0333$. **F, G, N, O)** Individual rats (circles) and group mean ± SEM.*$p<.05$, **$p<.01$, ***$p<.001$, ****$p<.0001$. **H-M)** Group mean ± SEM. hM3Dq vs. mCherry: ^$p<.05$, ^^$p<.01$; hM4Di vs. mCherry: #$p<.05$, ##$p<.01$, ###$p<.001$; hM3Dq vs. hM4Di: $$p<.05$, $$$p<.01$.

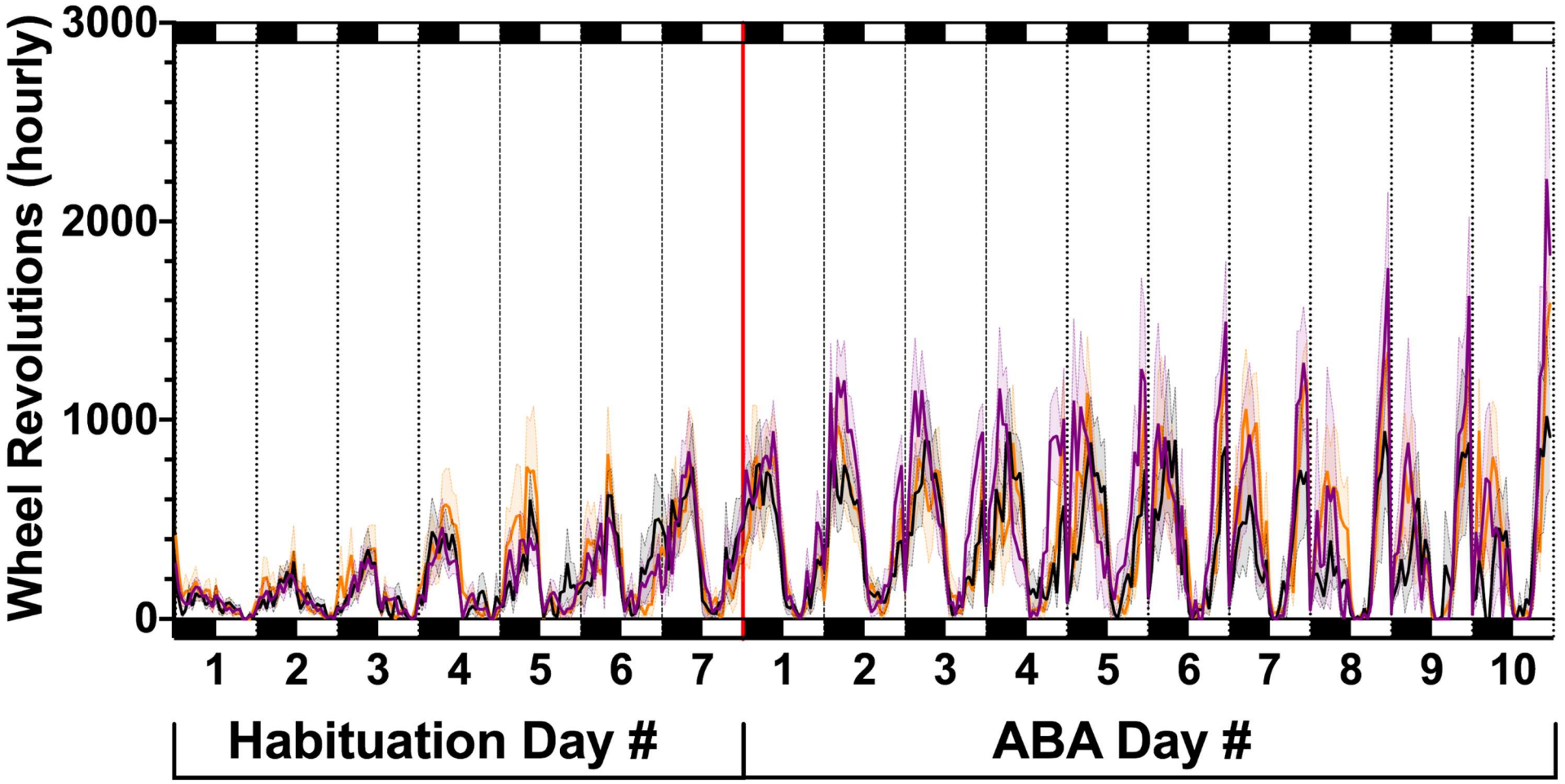

Figure 3.4 Running wheel activity during Habituation and ABA periods. RWA measured hourly during 7 days of habituation and 10 days of exposure to the ABA protocol with either activating-hM3Dq-DREADDs (purple), inhibiting-hM4Di-DREADDs (orange) or mCherry (black) in mPFC-AcbSh projecting neurons. CNO administered to modulate the circuit throughout the ABA phase. Black boxes represent the dark cycle. Data are mean ± SEM.

3.3.5 mPFC-AcbSh pathway inhibition increases food anticipatory activity in ABA

Conversely, circuit inhibited hM4Di rats selectively and dramatically increased RWA preceding food availability, termed food anticipatory activity (FAA), peaking in the penultimate hour, where they ran significantly more than control mCherry (#) rats (p=.0276, **Figure 3.3 I**; p=.0038, **Figure 3.3 J**). This is a preferential shift in the timing of RWA as this peak is maintained and emphasized when calculated as relative RWA (to account for the overall volume of RWA) where circuit inhibited hM4Di rats increased RWA significantly more than both circuit activated hM3Dq rats ($; ABA p=0284, ABA-Hab p=0452). and control mCherry rats (#; ABA p=.0007, ABA-Hab p=.0072) during this hour (**Figure 3.3 L** and **M**).

When FAA is examined in isolation, which was defined as RWA in the penultimate hour before food access (RWA between 13:00h and 14:00h), an overall increase in FAA following the onset of food restriction (p<.0001) was revealed, and was influenced by circuit modulation (p=.0336**)** resulting in a differing magnitude of increase in FAA across groups (hM3Dq p=.0005; mCherry p=.0081; hM4Di p<.0001; **Figure 3.3 N**). mPFC-AcbSh circuit inhibition produced greater FAA during ABA (p=.0082; **Figure 3.3 N**) and a more substantial increase in FAA from habituation to ABA (p=.0333; **Figure 3.3 O**) compared to control.

3.3.6 mPFC-AcbSh pathway activity does not affect ABA related outcomes when food is available *ad libitum*

In order to determine whether those divergent effects of chemogenetic mPFC-AcbSh circuit modulation on body weight maintenance, food intake and RWA seen in ABA were specific to the ABA conditions we replicated the same circuit modulation in rats with free access to the running wheel and *ad libitum* access to food (**Figure 3.5 A**). Circuit modulation did not alter body weight maintenance under *ad libitum* feeding conditions (**Figure 3.5 C**), with all groups steadily increasing body weight over the duration of the experiment (p<.0001), in a similar fashion (p=.4568), with no differential effect of circuit modulation (p=.5362; **Figure 3.5 D**), greatly contrasting with the effects seen during ABA (**Figure 3.2 C-H**).

With wheels unlocked 90-minute food intake remained consistent across time (**Figure 3.5 E**) with no significant circuit modulation effects during this period (all ps≥.3086; **Figure 3.5 F**).

Access to running wheels resulted in a significant increase in 90-minute food intake under baseline conditions (saline administration; p=.0181, **Figure 3.5 G**), but there were no effects resulting from CNO-mediated circuit modulation (all $ps \geq .1652$, **Figure 3.5 H**), contrary to that observed following circuit modulation in ABA conditions (**Figure 3.2 I-N**).

As expected RWA steadily increased over time for all groups (**Figure 3.5 I**) and analysis of mean daily RWA revealed that this increase was consistent across the cohort (all $ps \leq .0013$, **Figure 3.5 J**) with no difference between groups (p=.7653) nor interaction of circuit modulation (p=.9077), resulting in a consistent change in mean daily RWA from saline to CNO phases (p=.9077, **Figure 3.5 K**), notably different from the effects of circuit modulation with the simultaneous onset of food restriction observed in ABA (**Figure 3.3 A-G**). The final hour of the light phase is crucial for comparison with the analogous FAA window in ABA, we consider if an FAA-proxy that accounts for all the effects of all experimental variables other than upcoming food access on RWA (e.g. anticipation of lights off). We therefore analyzed proportional FAA-proxy, which revealed a significant *decrease* in FAA-proxy following chemogenetic circuit modulation across the cohort (p=.0277; **Figure 3.5 L**), resulting in the circuit inhibited hM4Di group displaying significantly *less* FAA-proxy than the control mCherry group (p=.0230). Most importantly, modulation of the mPFC-AcbSh circuit had no effect on the change in FAA-proxy from baseline (p=.3591, **Figure 3.5 M**), in stark contrast to the significant increase in “true” FAA following mPFC-AcbSh circuit inhibition in ABA (**Figure 3.3 L-O**).

A detailed investigation of the differential impact of RW access and CNO administration was also conducted to ensure effects seen in ABA were specific to the paradigm (see **Supplementary sections 3.6.1** to **3.6.4,** and **Supplementary Figures S3.2** and **S3.3** for details).

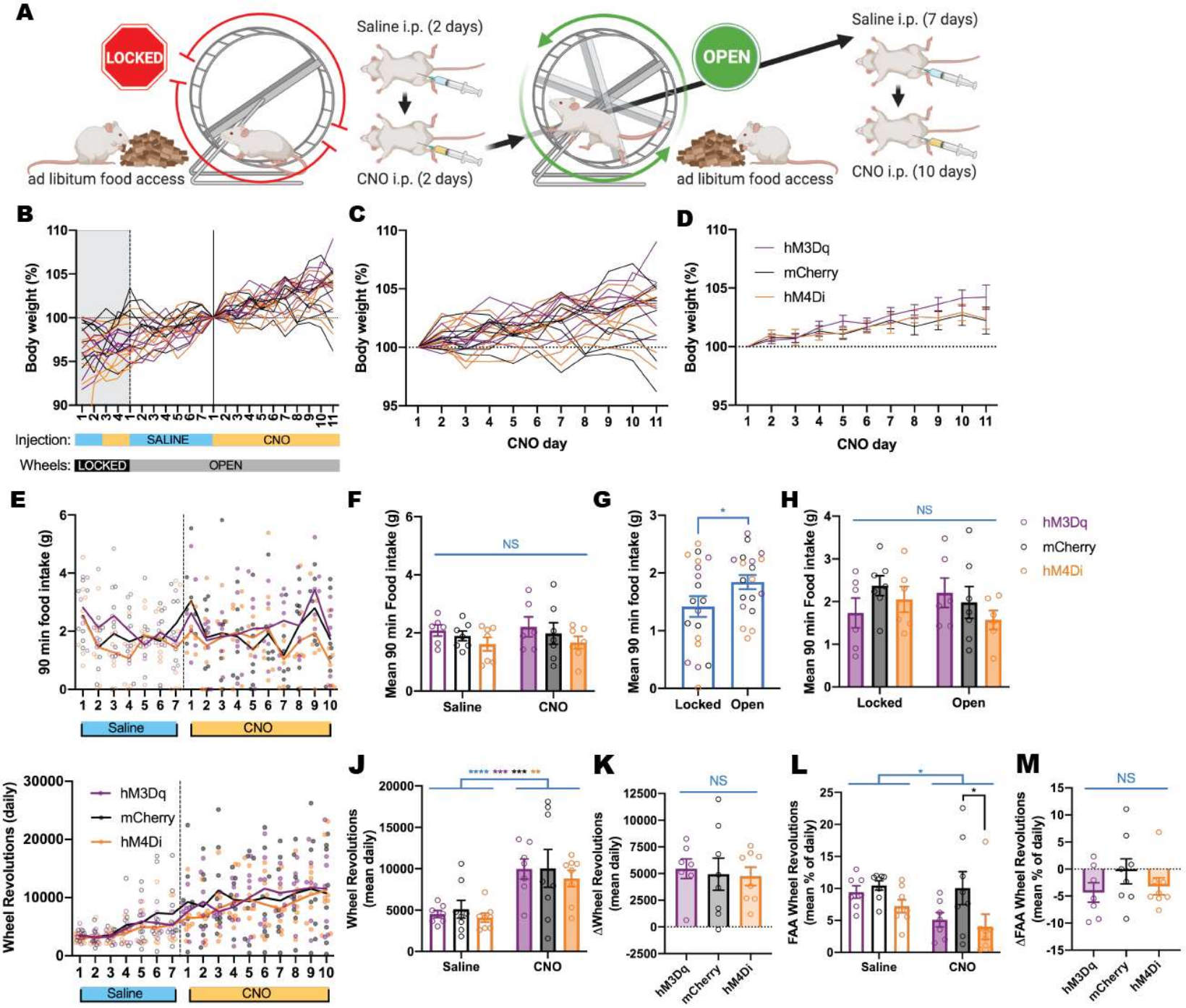
A
LOCKED
ad libitum food access
Saline i.p. (2 days)
CNO i.p. (2 days)
OPEN
ad libitum food access
Saline i.p. (7 days)
CNO i.p. (10 days)
B
Body weight (%)
Injection:
SALINE
CNO
Wheels:
LOCKED
OPEN
C
Body weight (%)
CNO day
D
hM3Dq
mCherry
hM4Di
Body weight (%)
CNO day
E
90 min food intake (g)
Saline
CNO
F
Mean 90 min Food intake (g)
NS
Saline
CNO
G
Mean 90 min food intake (g)
*
Locked
Open
H
Mean 90 min Food intake (g)
NS
Locked
Open
hM3Dq
mCherry
hM4Di
I
Wheel Revolutions (daily)
hM3Dq
mCherry
hM4Di
Saline
CNO
J
Wheel Revolutions (mean daily)
Saline
CNO
K
ΔWheel Revolutions (mean daily)
NS
hM3Dq
mCherry
hM4Di
L
FAA Wheel Revolutions (mean % of daily)
*
*
Saline
CNO
M
ΔFAA Wheel Revolutions (mean % of daily)
NS
hM3Dq
mCherry
hM4Di

Figure 3.5 Effects of chemogenetic modulation of mPFC-AcbSh projecting neurons when food is available *ad libitum*. **A)** Schematic illustrating the experimental schedule of wheel access and drug administration across the *ad libitum* feeding experiment for rats with either inhibiting-hM4Di-DREADDs (*n*=8; orange), activating-hM3Dq-DREADDs (*n*=7; purple), or AAV-mCherry (control; *n*=8; black) expressed in the cell bodies of mPFC neurons projecting to the AcbSh. **B)** Individual rats' body weight as a percentage of baseline (wheels open + CNO day 1) with wheels locked for 4 days, then unlocked for 17 days, and with either saline or CNO administration. **C)** Individual rats' body weight percentage across 10 days of daily CNO administration with wheels open which is analogous to the ABA wheel access and drug administration schedule. **D)** Group mean ± SEM from **C**. Two-way ANOVA: Time $F(3.528, 70.55)= 16.52$, $p<.0001$; Group $F(2, 20)= 0.8151$, $p=.4568$; Interaction $F(20, 200)= 0.9409$, $p=.5362$. **E)** Individual rats (circles) and group mean (lines) 90-minute food intake with wheels open following 7 days of saline (open circles) and 10 days of CNO (solid circles) administration; circles show individual animals line shows group mean. **F, H, J** and **L)** Two-way ANOVA followed by post-hoc multiple comparisons when required (Tukey's for between groups, with a Bonferroni correction for within group). **K** and **M)** One-way ANOVA. **F)** Mean daily 90-minute food intake. Drug $F(1, 17)= 0.2748$, $p=.6069$; Group $F(2, 17)= 1.261$, $p=.3086$; Interaction $F(2, 17)= 0.01337$, $p=.9867$. **G** and **H)** Mean daily 90-minute food intake with wheels open versus locked following saline **(G)** or CNO **(H)** administration. **G)** Cohort mean due to baseline conditions (i.e. no circuit modulation). Paired t-test (two-tailed), $t(18)= 2.600$, $p=.0181$. **H)** Wheels $F(1, 16)= 0.4060$, $p=.5330$; Group $F(2, 16)= 0.5541$, $p=.5852$; Interaction $F(2, 16)= 2.019$, $p=.1652$. **I)** Individual rats (circles) and group mean (lines) daily running wheel activity (RWA; total wheel revolutions) over 7 days of saline (open circles) and 10 days of CNO (solid circles) administration. **J)** Mean daily RWA within each phase. Phase $F(1, 20)= 57.41$, $p<.0001$ (hM3Dq $p=.0006$; hM4Di $p=.0013$; mCherry $p=.0009$); Group $F(2, 20)= 0.2711$, $p=.7653$; Interaction $F(2, 20)= 0.09735$, $p=.9077$. **K)** Change in mean daily RWA from Saline to CNO. $F(2, 20)= 0.09735$, $p=.9077$. **L** and **M)** Mean hourly RWA as a proportion of total daily RWA in the hour before lights off (analogous to the food anticipatory activity [FAA] window in ABA) within each phase **(L)** and the change from habituation to CNO **(M)**. **L)** Phase $F(1, 20)= 5.639$, $p=.0277$; Group $F(2, 20)= 3.714$, $p=.0425$ (hM4Di > mCherry $p=.0358$); Interaction $F(2, 20)= 1.078$, $p=.3591$. In the CNO phase FAA was significantly lower for hM4Di than mCherry $p=.0230$. **M)** $F(2, 20)=1.078$, $p=.3591$. Bar graphs show individual animals (circles) and group mean ± SEM (bars) following saline (unfilled bars), CNO (dark filled bars) or change from saline to CNO (light filled bars). NS not significant $p>.05$, $*p<.015$, $**p<.01$, $***p<.001$, $****p<.0001$.

3.3.7 mPFC-AcbSh pathway activity does not affect activity and feeding in response to novelty

In order to determine whether the effects of chemogenetic mPFC-AcbSh circuit modulation on feeding and RWA seen in ABA could be the result of underlying differences in general locomotor activity or feeding in response to novelty, rats underwent both the open field (OF) test (**Figure 3.6 A**) and a combined light/dark emergence and novelty suppressed feeding (NSF) test (**Figure 3.6 G**) following circuit modulation. There were no effects of circuit modulation on total distance travelled (p=.1613; **Figure 3.6 B**) nor the number of zone crossings (p=.1434; **Figure 3.6 C**) in the OF. All groups were similar (p=.1646) and displayed the typical pattern of significantly greater time spent in the outside zone of the OF compared to the aversive inside zone (p<.0001) with the magnitude of this difference consistent across groups (all ps<.0001) and not impacted by circuit modulation (p=.5590; **Figure 3.6 D**). Interestingly, it was found that there was no relationship between distance travelled in the OF following circuit modulation and mean daily RWA either at baseline (all ps>.2013; **Figure 3.6 E**) or following circuit modulation (all ps>.0902; **Figure 3.6 F**), indicating that general locomotor behavior and RWA are distinct forms of physical activity.

The combined light/dark emergence and novelty suppressed feeding test revealed no effects of circuit modulation on any emergence latencies (head, p=.8905, **Figure 3.6 H**; upper body, p=.5161, **I**; full body, p=.3717, **J**), nor latency to start eating the lone offered food pellet (p=.7703; **Figure 3.6 K**), despite the rats' acute hunger following an overnight fast.

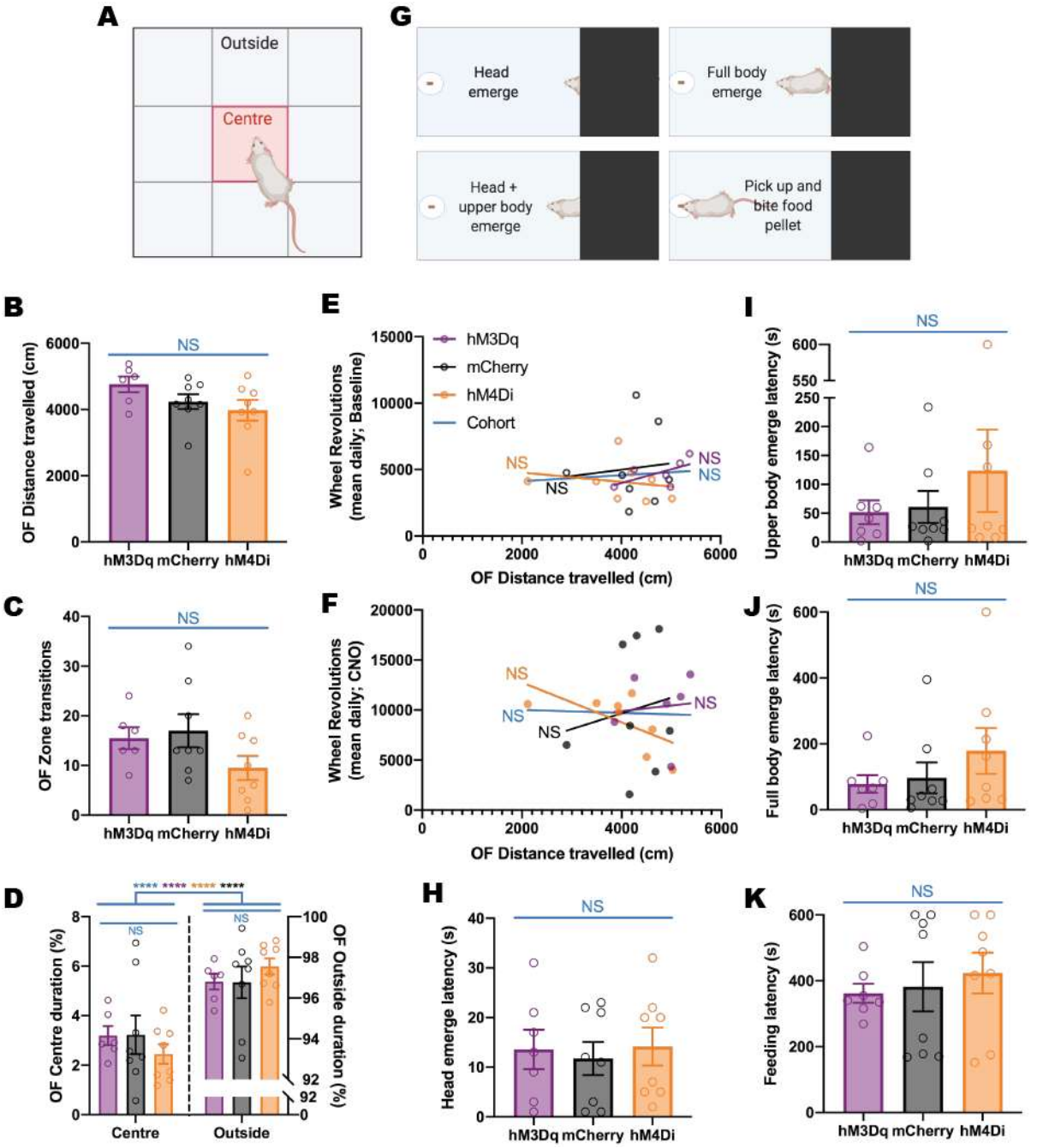

Figure 3.6 Effects of chemogenetic modulation of mPFC-AcbSh projecting neurons on outcomes in the Open Field and Novelty Suppressed Feeding tests. Illustrations of the 10-minute open field (OF; **A**) and novelty suppressed feeding (NSF; **G**) tests undertaken by control mCherry rats (*n*=8; black) and rats with either inhibiting-hM4Di-DREADDs (*n*=8; orange) or activating-hM3Dq-DREADDs (OF *n*=6, NSF *n*=7; purple) expressed in the cell bodies of mPFC neurons projecting to the AcbSh, following CNO administration to modulate the circuit. Total distance travelled (cm; **B**), total number of zone transitions **(C)** and the duration of time spent in each zone **(D)** during the 10-minute OF test **(A)**. **B)** One-way ANOVA: $F(2, 19)= 2.011$, $p=.1613$. **C)** One-way ANOVA: $F(2, 19)= 2.155$, $p=.1434$. **D)** Two-way ANOVA followed by Bonferroni post-hoc multiple comparisons. Zone $F(1, 19)= 19268$, $p<.0001$ (all groups $p<.0001$); Group $F(2, 19)= 1.987$, $p=.1646$; Interaction $F(2, 19)= 0.5998$, $p=.5590$. Linear regression correlation between total distance travelled in the OF and mean daily RWA during the saline **(E)** and CNO **(F)** phases; circles are individual animals, lines are of best fit; blue indicates the cohort as a whole. **E)** hM3Dq: Y = 1.034*X - 155.1, $r=.61$, $R^2=.37$, $p=.2013$. hM4Di: Y = -0.3537*X + 5487, $r=.21$, $R^2=.05$, $p=.6130$. mCherry: Y = 0.4822*X + 3061, $r=.10$, $R^2=.01$, $p=.8096$. Cohort: Y = 0.2250*X + 3676, $r=.08$, $R^2=.01$, $p=.7104$. **F)** hM3Dq: Y = 0.5633*X + 7641, $r=.10$, $R^2=.01$, $p=.8560$. hM4Di: Y = -1.991*X + 16739, $r=.64$, $R^2=.40$, $p=.0902$. mCherry: Y = 1.568*X + 3403, $r=.15$, $R^2=.02$, $p=.7144$. Cohort: Y = -0.1458*X + 10301, $r=.03$, $R^2=.00$, $p=.9119$. Latency during the 10-minute

novelty suppressed feeding test **(G)** for emergence of the rat's head **(H)**, upper body **(I)** and full body **(J)**, and latency to eat the offered food pellet **(K);** an X indicates a rat that failed to engage in the behaviour within the maximum allowed 10-minute test duration. **H)** One-way ANOVA: $F(2, 20)= 0.1166$, $p=.8905$. **I)** One-way ANOVA: $F(2, 20)= 0.6839$, $p=.5161$. **J)** One-way ANOVA: $F(2, 20)= 1.040$, $p=.3717$. **K)** One-way ANOVA: $F(2, 20)= 0.2644$, $p=.7703$. B-D and H-K) Data are individual animals (circles and Xs) with group mean ± SEM (bars). NS not significant $p>.05$, ****$p<.0001$.

3.4 Discussion

Elevated levels of activity within regions of the PFC and dysfunction of corticostriatal circuitry have been demonstrated in patients with AN (1, 2, 45), and the condition is associated with disruptions to specific cognitive abilities, such as cognitive flexibility (46, 47) and reward-related decision making (4, 48). However, human neuroimaging studies are unable to determine whether this plays a causative role in the development or maintenance of AN pathologies. Nevertheless, identification of 'state' markers of AN (i.e. those associated with the acute phase of the illness; e.g. hormonal and endocrine abnormalities) is possible with comparison of AN and REC-AN groups, but it remains difficult to determine whether 'trait' markers (i.e. those that persist after body weight recovery; e.g. impaired cognitive flexibility) common to both AN and REC-AN but not healthy controls precede illness onset or are an enduring consequence of AN (1, 3, 45). The only way to determine the role that activity within corticostriatal circuitry plays in the *generation* of pathological weight loss is to use well-established and validated animal models where these circuits can be experimentally perturbed. Here, we show in the ABA rat model that suppressing activity in mPFC-AcbSh projecting neurons prevented the development of the anorectic phenotype by increasing food intake when food is available and promoting the development of FAA. Conversely, we show that stimulation of the mPFC-AcbSh pathway exacerbated weight loss during ABA by increasing hyperactivity in response to timed-food restriction. Importantly, these effects were *specific* to the maladaptive behaviours expressed under ABA conditions, effects that were not present when food was available *ad libitum*. The direction of these effects fits neatly with what is known about PFC dysfunction in patients with AN and highlights the utility of the ABA model for interrogating the neurobiological bases of the human condition.

Our data showing the behavioral sequelae of experimental perturbations of PFC circuits in ABA align with observations made in the human condition. Given the impact of such experimental manipulations on the development or retardation of ABA, the present data are consistent with a causative role for PFC activity in the aetiology of AN. Of note, PFC dysfunction in AN includes structural changes including increased dlPFC and decreased right anterior insula cortex grey matter volume in acutely ill patients with AN (49), as well as both 'state' and 'trait' cortico-limbic-striatal grey matter volume changes in both AN and REC-AN. In addition to the functional disturbances described in the introduction to this Chapter, both patients with AN and REC-AN exhibit increased medial PFC activity compared to healthy controls when viewing images of

food, with this difference not registered in response to emotionally aversive non-food images (50, 51). Further, acutely ill patients with AN also show increased neural activity in dlPFC when required to actively think about eating the foods depicted in images presented to them in contrast to when passively viewing these images (52). These observations support a food-specific trigger of increased mPFC activity, rather than a general response to emotionally salient cues. Critically, body weight recovery restores subjective evaluation of pleasantness of food images to ratings from healthy controls, yet REC-AN still exhibit elevated mPFC activity, highlighting that *neuronal responses* to food imagery remain impaired following body weight recovery in AN (50, 51). Considered together with data, outlined in the Introduction, showing increased activity in the PFC of patients with AN (4, 6) and shifts in delayed discounting tasks used to interrogate impulsiveness vs cognitive control (4, 7, 8), it is likely that the overactive PFC in patients with AN dominates reward based decisions, enabling persistent food restriction and excessive exercise despite emaciation. Moreover, these functional changes in activity promoting maladaptive behaviours are further enhanced in AN by an insensitivity to interoceptive signaling normally driving food intake in response to hunger and negative energy state (5, 19, 20).

As noted above, the current experiments clearly indicate a *causal* role for a PFC-mediated impact on physical activity in the development of the anorexic phenotype. Specifically, activation of mPFC-AcbSh projecting neurons exacerbates starvation-induced hyperactivity promoting body weight loss and ABA susceptibility, whereas circuit inhibition reduced susceptibility to ABA by promoting increased food intake and preventing non-specific starvation-induced hyperactivity. Critically, inhibition of these prefrontal inputs to the reward network specifically increased running activity in anticipation of food (FAA) and subsequent food intake, consistent with an increased motivation to eat. However, the striking improvement in survival following inhibition of the mPFC-AcbSh circuit is incompletely explained by the small increases in food intake and FAA. Considering that RWA and food intake were not significantly different from control levels, this suggests the involvement of additional “hidden” factors influencing body weight maintenance that we did not measure, for example metabolism.

The question then arises as to the possible neural substrate(s) that may underpin the relationship between activity in the mPFC and running wheel activity, whether the latter be in anticipation of food (increased following inhibition) or over the entire circadian cycle (increased following excitation). One potential candidate that has been proposed to explain behavioral

shifts in ABA is the extent of GABAergic input to pyramidal neurons in the prelimbic cortex (53). In that study, there was a negative correlation between the size of GAD- positive (GABA) terminals on pyramidal neurons in the prelimbic cortex and the amount of RWA during the feeding window, and a positive correlation between the increase in GAD-positive terminal contact lengths and FAA (during the final 6 hours of the light cycle) (53). Our data can be interpreted in terms of these findings such that the observed increase in GABA'ergic input (both in terms of terminal size and contact length) to prelimbic cortical neurons that occurs contemporaneous with improved ABA outcomes could underlie the behavioral outcomes of increased food intake and FAA that are generated after chemogenetic inhibition of this pathway in our hands. While further removed from our current focus on the PFC it has also been shown by the same group that hyperactivity in response to food restriction and subsequent body weight loss and susceptibility to ABA was associated with a reduction of GABAergic inputs to *hippocampal* pyramidal neurons (54, 55). Conversely, resistance to the development of increased locomotor activity and therefore resistance to ABA was associated with enlargement of GABAergic terminals on pyramidal cell bodies in the hippocampus (54, 55). This, perhaps, represents a more generalized reliance on GABA'ergic synaptic changes to explain shifts in locomotor activity and thus ABA outcomes.

The other major transmitter candidate that may mediate the behavioral outcomes driven by modulating prefrontal circuits is dopamine – here the published data lacks consistency. The majority of PFC projections are excitatory and glutamatergic (56) with widespread cortical, subcortical and midbrain nuclei targets (57-59). Stimulation of the PFC increases both glutamate (60) and dopamine (60, 61) release in the Acb. Increased DA in the Acb is associated with spontaneous generalized locomotor hyperactivity (62, 63) and RWA (64) During ABA, extracellular Acb DA is elevated during feeding but not during the development of FAA, i.e. associated with food consumption but not anticipation (65), whereas we previously found that chemogenetic activation of predominantly dopaminergic VTA to AcbSh projecting neurons increased survival, food intake and FAA in ABA (29). Multiple studies have tested the effects of selective or non-selective dopamine receptor antagonists in ABA, resulting in very different findings. Selective D1/2 antagonists increased food intake and body weight and decreased dark phase RWA with no effect on FAA (66). D1-like antagonists increased food intake and FAA with no effect on total RWA or survival (67). Two nonselective D2/3 antagonists produced differing effects, whereby both increased food intake and survival, but one increased and the other decreased RWA, and one had no effect on FAA while the other decreased FAA

(67).Selective D3 receptor antagonist increased survival but without changes in any other behavioural measure while selective D2 antagonist increased survival, body weight, food intake, RWA and FAA (67). It is interesting to note that in both these studies (66, 67) the effects were restricted to the ABA condition with no differences resulting from DA receptor antagonism during baseline (*ad libitum* feeding) conditions, and in this way paralleled our data showing a lack of behavioural effect of modulating of mPFC-AcbSh projecting neurons outside ABA. While the conflicting direction of these findings makes interpretation of the role of DA hard, the consistent finding, including in the present experiments, of effects specific to the ABA condition fits with a DA based mechanism of action reliant upon the altered reward conditions of ABA. Given the large heterogeneity that exists within DA subpopulations and circuitry, along with the multitude of different receptor subtypes and dopamine release dynamics (e.g. tonic, phasic, ramping), it is not surprising that complicated response dynamics are implicated and experimental findings conflict both within and between studies (68). Systematic and targeted investigation of individual DA cell subpopulations, circuits, and receptor subtypes will be invaluable in determining the complimentary and dissociable roles of DA in ABA relevant behaviours.

Of critical importance in interpreting our findings is the complete absence of any effect of mPFC-AcbSh circuit modulation outside of the ABA paradigm. In other words, the effects of circuit modulation on food intake or running wheel activity were specific to the altered reward conditions arising from the combination of wheel access and restricted feeding in ABA. This highlights the importance of these prefrontal inputs to the reward network in behavioural adaptation to changes in the environment. Wheel running in a confined cage is intrinsically rewarding when food is available *ad libitum* (69), but food restriction typically leads to the development of starvation-induced hyperactivity, the extent to which is consistently shown to produce the ABA phenotype (27, 70, 71). Therefore, rats must learn to modify this previously rewarding behavior to be able to maintain their body weight under ABA conditions. Further, recognition of and adaptation to the restricted feeding schedule itself is critical to the maintenance of body weight in ABA, as prior exposure to the same schedule of food access before wheel access prevents the development of excessive RWA and ABA (72, 73). Interestingly, pre-exposure to the running wheel before the onset of food restriction, as occurs in our ABA protocol, has been found to exacerbate development of ABA (73), which makes our 100% survival rate following inhibition of mPFC-AcbSh projecting neurons that much more

striking. Necessarily then, rats that are more readily able to adapt their feeding and RWA in accordance with changes in food availability are more likely to be resistant to ABA.

While these data as presented are clear in relation to the involvement of mPFC-AcbSh projecting neurons in the susceptibility to ABA there are some considerations that need to be highlighted. Firstly, our mPFC region encompasses both the PrL and IL, which have been found to have dissociable roles in reward related behaviours including reversal learning (74-76), conditioned place preference (77), and goal-directed actions (78). As such we cannot resolve any independent contribution of these areas on the alterations in behavior resulting from circuit modulation. Secondly, while our dual viral strategy specifically targeted mPFC neurons that directly project to the AcbSh, we cannot exclude the potential for collateral projections to other brain regions relevant to reward and adaptive behaviour, for example the hippocampus, basolateral amygdala (BLA) and the ventral tegmental area (VTA) (58). These pathways may be coincidentally modulated, given that the DREADD receptors are located in the neuronal cell bodies of the mPFC and our studies utilized systemic administration of CNO. However, of the identified population of PFC neurons projecting to the nucleus accumbens, only 7% and 3% were found to have collateral projections to the BLA and VTA, respectively, with the greatest proportion of collateral projections (13%) terminating in the contralateral PFC (58). As such, any complication of the data by recruitment of collateral branches to sites outside of the AcbSh is likely to be small.

In conclusion, the data presented in this Chapter extend our understanding of the aetiology of ABA and as such the human condition. The results presented here and those from previous studies support the notion that the mPFC-AcbSh pathway is integrally involved in the determination of behavioral outcomes arising from ABA. The findings highlight the potential that the activity of this circuit impacts the variability in behavioural adaptability to changing reward conditions that underlies the spectrum of ABA susceptibility. Depending on the polarity of its action, the mPFC-AcbSh circuit may cause cognitive rigidity predisposing to ABA susceptibility, and cognitive flexibility promoting resistance to ABA. These concepts will be interrogated more directly in the following Chapter.

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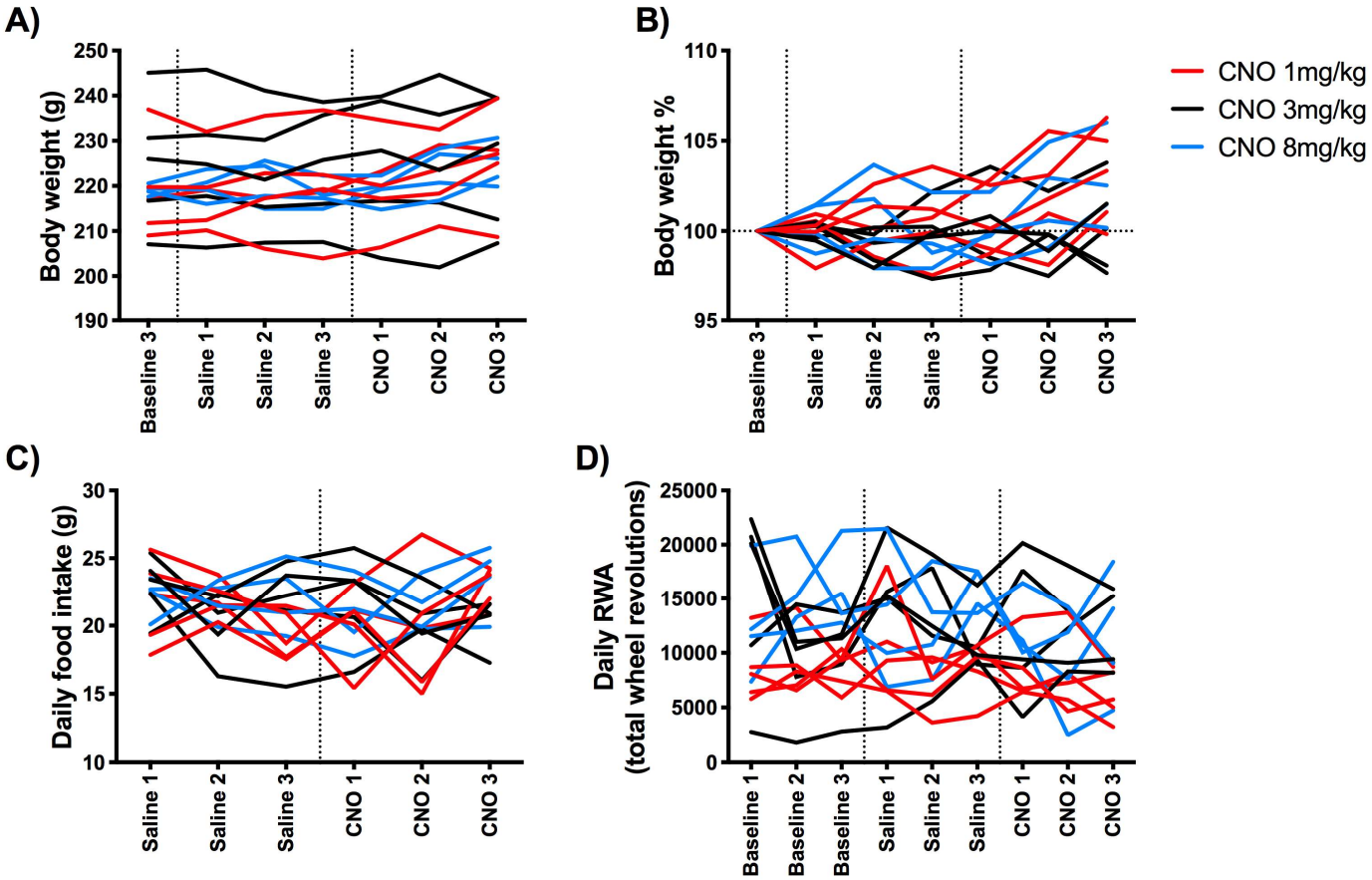
3.6 Supplementary material

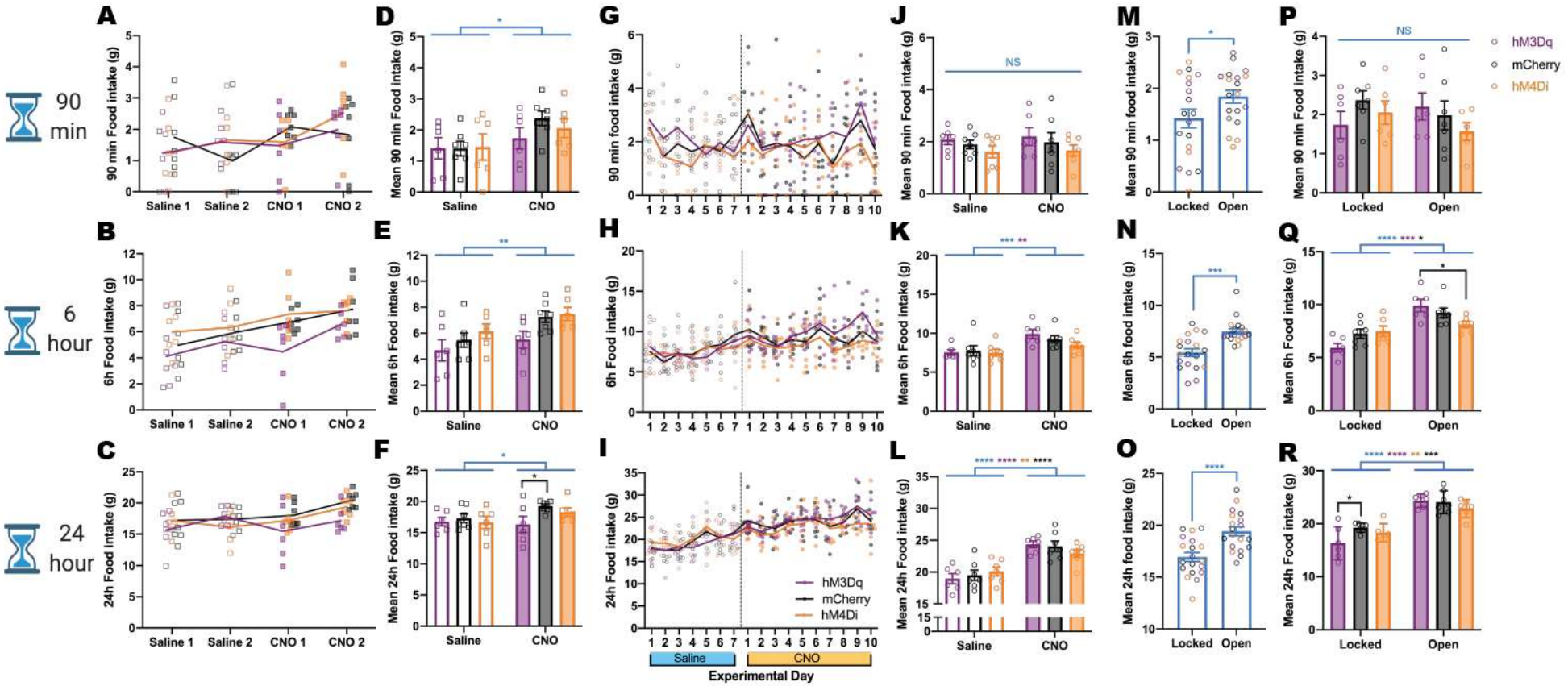


Supplementary Figure S3.1 CNO dose response experiment. There was no systematic effect of CNO administration at 1mb/kg (red; *n*=5), 3mg/kg (dose used for inhibiting DREADDs in all experiments; black; *n*=5), or 8mg/kg (blue; *n*=4) on body weight (grams; A), body weight % (B), daily food intake (C) or total daily RWA (D) in female wild type Sprague-Dawley rats.

3.6.1 mPFC-AcbSh pathway activity does not affect *ad libitum* feeding with or without access to wheels

Although there was an overall significant increase in *ad libitum* food intake after CNO-mediated mPFC-AcbSh circuit modulation for all feeding windows (**Figure S3.2 D** 90-minute p=.0180; **E** 6-hour p=.0086; **F** 24-hour p=.0397), this was an overall effect across the cohort and not specific to any individual group (hM3Dq all ps≥.9199; hM4Di all ps≥.1962; mCherry all ps≥.0659). While there were some significant group differences (see figure legend), for example following CNO in 24-hour food intake (**Figure S3.2 F**), all group differences appear to be an artifact driven by unusually low food intake on CNO day 1 by two hM3Dq rats, resolved on CNO day 2, rather than a true CNO mediated effect (**Figure S3.2 A-C**). Further, there were no interactions between group and whether the circuit was modulated or not (i.e. CNO vs. Saline) for any feeding window (all ps≥.1080; **Figure S3.2 D-F**), indicating that CNO-mediated circuit modulation does not underlie changes in *ad libitum* food intake over time.

With open running wheels, food intake increased steadily across time for both the 6-hour (**Figure S3.2 H**) and 24-hour (**Figure S3.2 I**) windows, but remained consistent in the 90-minute window (**Figure S3.2 G**) with no significant circuit modulation effects during this period (all ps>.3086; **Figure S3.2 J**). Following mPFC-AcbSh circuit modulation there was a cohort wide significant increase in both 6-hour (p=.0002, **Figure S3.2 K**) and 24-hour (p<.0001, **Figure S3.2 L**) food intake, with group only significantly interacting with circuit modulation for the 24-hour window (p=.0435). While all groups significantly increased 24-hour food intake following circuit modulation (hM3Dq p<.0001, hM4Di p=.0016, mCherry p<.0001, **Figure S3.2 L**), there were no significant differences between groups within any phase for any feeding window (all ps≥.4050; **Figure S3.2 J-L**), indicating that this increase in food intake was not a result of circuit modulation **Figure S3.2 I**.



Supplementary Figure S3.2 *Ad libitum* food intake following mPFC-AcbSh modulation in 90-minute, 6-hour and 24-hour feeding windows. Food intake for control mCherry rats (*n*=7; black) and rats with either inhibiting-hM4Di-DREADDs (*n*=6; orange) or activating-hM3Dq-DREADDs (*n*=6; purple) expressed in the cell bodies of mPFC neurons projecting to the AcbSh. 90-minute (**top row**), 6-hour (**middle row**), and 24-hour (**bottom row**) food intake with wheels locked (**A-F; M-R left**) or wheels open (**G-L; M-R right**) following either saline (unfilled squares and bars) or CNO (filled squares and bars) administration. **D-F, J-L** and **P-R)** Two-way ANOVA followed by post-hoc multiple comparisons when required (Tukey's for between groups, with a Bonferroni correction for within group). **M-O)** Paired t-test (two-tailed). **Wheels locked: D)** Drug $F(1, 16)= 6.951$, $p=.0180$; Group $F(2, 16)= 0.4659$, $p=.6358$; Interaction $F(2, 16)= 0.6333$, $p=.5436$. **E)** Drug $F(1, 16)= 8.956$, $p=.0086$; Group $F(2, 16)= 3.786$, $p=.0451$ (hM3Dq < hM4Di $p=.0442$); Interaction $F(2, 16)= 0.4114$, $p=.6696$. **F)** Drug $F(1, 16)= 5.015$, $p=.0397$; Group $F(2, 16)= 1.612$, $p=.2303$; Interaction $F(2, 16)= 2.566$, $p=.1080$. Following CNO 24-hour food intake was significantly lower for hM3Dq than mCherry ($p=.0348$). **Wheels open: J)** Drug $F(1, 17)= 0.2748$, $p=.6069$; Group $F(2, 17)= 1.261$, $p=.3086$; Interaction $F(2, 17)= 0.01337$, $p=.9867$. **K)** Drug $F(1, 17)= 22.37$, $p=.0002$ (hM3Dq $p=.0037$); Group $F(2, 17)= 0.9427$, $p=.4090$; Interaction $F(2, 17)= 1.502$, $p=.2507$. **L)** Drug $F(1, 17)= 118.3$, $p<.0001$ (hM3Dq $p<.0001$; hM4Di $p=.0016$; mCherry $p<.0001$); Group $F(2, 17)= 0.05601$, $p=.9457$; Interaction $F(2, 17)= 3.790$, $p=.0435$. **Wheels locked vs. open with Saline:** cohort mean due to baseline conditions (i.e. no circuit modulation). **M)** $t(18)= 2.600$, $p=.0181$. **N)** $t(18)= 4.469$, $p=.0003$. **O)** $t(18)= 5.569$, $p<.0001$. **Wheels locked vs. open with CNO: P)** Wheels $F(1, 16)= 0.4060$, $p=.5330$; Group $F(2, 16)= 0.5541$, $p=.5852$; Interaction $F(2, 16)= 2.019$, $p=.1652$. **Q)** Wheels $F(1, 16)= 30.22$, $p<.0001$ (hM3Dq $p=.0001$; mCherry $p=.0253$); Group $F(2, 16)= 0.6138$, $p=.5536$; Interaction $F(2, 16)= 5.675$, $p=.0137$. With wheels open 6-hour food intake was significantly higher for hM3Dq than hM4Di

(*p*=.0284). **R)** Wheels *F*(1, 16)= 85.73, *p*<.0001 (hM3Dq *p*<.0001; hM4Di *p*=.0025; mCherry *p*=.0008); Group *F*(2, 16)= 1.767, *p*=.2026; Interaction *F*(2, 16)= 3.082, *p*=.0737. With wheels locked 24-hour food intake was significantly lower for hM3Dq than mCherry (*p*=.0268). Data show individual rats (squares and circles) with group mean over days (lines; **A-C** and **G-I**) and group mean ± SEM of individual animal mean intake per phase (bars; **D-F** and **J-R**). NS not significant *p*>.05, **p*<.015, ***p*<.01, ****p*<.001, *****p*<.0001.

3.6.2 Access to running wheels increases food intake independent of mPFC-AcbSh pathway modulation

As expected, access to running wheels increased food intake across all time windows at baseline (i.e. after saline administration; **Figure S3.2 M** 90-minute p=.0181; **N** 6-hour p=.0003; **O** 24-hour p<.0001) and following CNO-mediated mPFC-AcbSh circuit modulation in both the 6-hour (p<.0001; **Figure S3.2 Q**) and 24-hour (p<.0001; **Figure S3.2 R**) feeding windows, but not for 90-minute food intake (p=.4060, **Figure S3.2 P**). Importantly, all groups ate similar amounts of food following chemogenetic modulation (all ps≥.2026, **Figure S3.2 P-R**), indicating no systematic effect of circuit modulation on *ad libitum* food intake. All identified significant interactions or group differences (see figure legend), except for hM3Dq > hM4Di 6-hour intake (p=.0284, **Figure S3.2 Q),** appear to again be driven by the previously mentioned unusually low food intake with wheels locked for two hM3Dq rats. Crucial for comparison with ABA food intake was an absence of any effects of circuit modulation in the 90-minute feeding window (all ps≥.1652, **Figure S3.2 P**).

3.6.3 mPFC-AcbSh pathway activity does not systematically alter RWA with *ad libitum* feeding

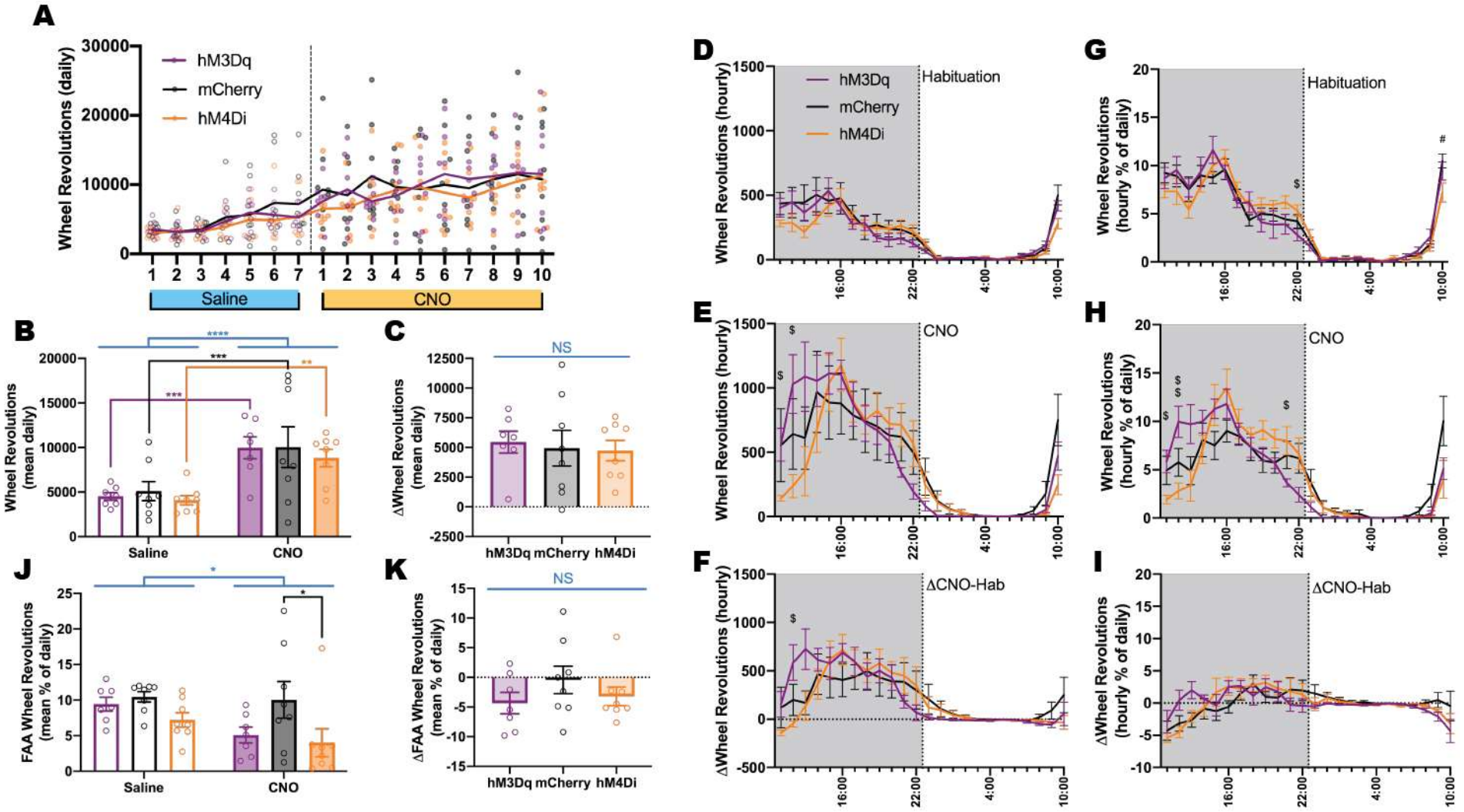
Daily RWA increased steadily over the duration of the experiment (**Figure S3.3 A**) leading to a significant increase in mean daily RWA from the saline to the CNO administration periods for the entire cohort (p<.0001) and each group independently (hM3Dq p=.0006; hM4Di p=.0013; mCherry p=.0009). RWA was not different between groups (p=.2711) **Figure S3.3 B**), and there was no effect of chemogenetic mPFC-AcbSh circuit modulation (p=.9077; **Figure S3.3 C**).

Observation of the hourly pattern of RWA over the day is insightful, and provides a key contrast to that seen in ABA with the initiation of food restriction. When food access was maintained *ad libitum*, we saw a CNO-mediated effect on RWA in the two hours following administration, whereby the circuit activated hM3Dq group had significantly higher RWA than the circuit inhibited hM4Di group measured either as wheel revolutions (**Figure S3.3 E** 11:00h p=.0388, 12:00h p=.0298) or as a percentage of daily RWA (**Figure S3.3 H** 11:00h p=.0352, 12:00h p=.0089). resulting in a significant absolute increase in wheel revolutions from habituation to ABA as a result of circuit activation compared to circuit inhibition 2 hours post circuit modulation (p=.0324; **Figure S3.3 F**). Evaluating RWA as a proportion of daily RWA accounts for variability

in overall RWA and allows us to see the circadian pattern of RWA and how this might change with circuit modulation. At baseline (saline) we see that the hM4Di group ran significantly more in the last hour of the dark phase than the hM3Dq group (22:00h p=.0262; **Figure S3.3 H**), while following circuit inhibition in ABA the hM4Di group ran significantly more than the circuit activated hM3Dq group in the preceding hour (21:00h p=.0461; **Figure S3.3 I**).

3.6.4 mPFC-AcbSh pathway inhibition significantly decreased RWA in the hour before lights off with *ad libitum* feeding

While the mCherry group ran significantly more than the hM4Di group in the final hour of the light phase at baseline (10:00h p=.0498; **Figure S3.3 G**), there were no differences between groups at this time point following mPFC-AcbSh circuit modulation (**Figure S3.3 H**). However, this final hour of the light phase is crucial for comparison with the analogous FAA window in ABA, we consider if an FAA-proxy that accounts for all the effects of all experimental variables other than upcoming food access on RWA (e.g. anticipation of lights off). We therefore analyzed proportional FAA-proxy in isolation from the rest of the time points, which revealed a significant *decrease* in FAA-proxy following chemogenetic circuit modulation across the cohort (p=.0277; **Figure S3.3 J**), resulting in the circuit inhibited hM4Di group displaying significantly *less* FAA-proxy than the control mCherry group (p=.0230). Most importantly, modulation of the mPFC-AcbSh circuit had no effect on the change in FAA-proxy from baseline (p=.3591, **Figure S3.3 K**), in stark contrast to the significant increase in “true” FAA following mPFC-AcbSh circuit inhibition in ABA (**Figure 3.3 L-O**).



Supplementary Figure S3.3 Running wheel activity following mPFC-AcbSh modulation where food was available *ad libitum*. Running wheel activity for control mCherry rats (*n*=8; black) and rats with either inhibiting-hM4Di-DREADDs (*n*=8; orange) or activating-hM3Dq-DREADDs (*n*=7; purple) expressed in the cell bodies of mPFC neurons projecting to the AcbSh. **A)** Individual rats (circles) and group mean (lines) daily running wheel activity (RWA; total wheel revolutions) over 7 days with saline administration and 10 days with CNO administration. **B)** Mean daily RWA within each phase. Two-way ANOVA followed by post-hoc multiple comparisons with a Bonferroni correction: Phase $F(1, 20)= 57.41$, $p<.0001$ (hM3Dq $p=.0006$; hM4Di $p=.0013$; mCherry $p=.0009$); Group $F(2, 20)= 0.2711$, $p=.7653$; Interaction $F(2, 20)= 0.09735$, $p=.9077$. **C)** Change in mean daily RWA from Saline to CNO. One-way ANOVA: $F(2, 20)= 0.09735$, $p=.9077$. Absolute **(D-F)** and proportional **(G-I)** mean hourly RWA over 7 days of habituation (saline i.p. on day 7; **D** and **G**), 10 days of CNO (**E** and **H**), and the change from habituation to CNO (**F** and **I**) where a positive value denotes an increase from habituation to CNO and a negative value denotes a decrease. **D-I)** Two-way ANOVA followed by Tukey's post-hoc multiple comparisons. **D**) Time $F(3.308, 66.15)= 39.27$, $p<.0001$; Group $F(2, 20)= 0.4916$,

p=.6189; Interaction *F*(46, 460)= 1.167, *p*=.2178. **E**) Time *F*(3.246, 64.91)= 24.41, *p*<.0001; Group *F*(2, 20)= 0.1817, *p*=.8352; Interaction *F*(46, 460)= 1.513, *p*=.0197. Hourly RWA was significantly higher for hM3Dq than hM4Di at 11:00 (*p*=.0388) and 12:00 (*p*=.0298). **F**) Time *F*(3.934, 78.69)= 12.17, *p*<.0001; Group *F*(2, 20)= 0.09735, *p*=.9077; Interaction *F*(46, 460)= 1.323, *p*=.0826. Hourly RWA was significantly higher for hM3Dq than hM4Di at 12:00 (*p*=.0324). **G**) Time *F*(4.750, 95.01)= 81.49, *p*<.0001; Group *F*(2, 20)= 0.6932, *p*=.5116; Interaction *F*(46, 460)= 1.537, *p*=.0162. Hourly RWA was significantly higher for hM4Di than hM3Dq at 22:00 (*p*=.0262), and significantly lower for hM4Di than mCherry at 10:00 (*p*=.0498). **H**) Time *F*(4.421, 88.43)= 35.31, *p*<.0001; Group *F*(2, 20)= 0.02558, *p*=.9748; Interaction *F*(46, 460)= 2.545, *p*<.0001. Hourly RWA was significantly higher for hM3Dq than hM4Di at 11:00 (*p*=.0352) and 12:00 (*p*=.0089), and for hM4Di than hM3Dq at 21:00 (*p*=.0461). **I**) Time *F*(4.568, 91.35)= 5.948, *p*=.0001; Group *F*(2, 20)= 0.01590, *p*=.9842; Interaction *F*(46, 460)= 1.241, *p*=.1412. Mean hourly RWA as a proportion of total daily RWA in the hour before lights off (analogous to the food anticipatory activity [FAA] window in ABA) within each phase **(J)** and the change from habituation to CNO **(K)**. **J)** Two-way ANOVA followed by post-hoc multiple comparisons (Tukey's for between group, with a Bonferroni correction for within group): Phase *F*(1, 20)= 5.639, *p*=.0277; Group *F*(2, 20)= 3.714, *p*=.0425 (hM4Di > mCherry *p*=.0358); Interaction *F*(2, 20)= 1.078, *p*=.3591. In the CNO phase FAA was significantly lower for hM4Di than mCherry *p*=.0230. **K)** One-Way ANOVA: *F*(2, 20)=1.078, *p*=.3591. **B, C, J** and **K)** Data show individual rats (circles) and group mean ± SEM (bars); NS not significant *p*>.05, **p*<.015, ***p*<.01, ****p*<.001, *****p*<.0001. **D-I)** Data are group mean ± SEM of individual rat means; hM3Dq vs. hM4Di $*p*<.05, $$*p*<.01; hM4Di vs. mCherry #*p*<.05.

CHAPTER FOUR

Corticostriatal Regulation and Rapid Assessment of Cognitive Flexibility

4.1 Introduction

Cognitive or behavioural flexibility, terms that are often used interchangeably, refers to the capacity to flexibly modify decision-making and behavioural choice to meet the demands of a changing environment (1-3). Cognitive flexibility is crucial to optimal functioning because we live in environments that are often uncertain and therefore the selection of appropriate responses will differ with context and circumstance (including changes in internal states) (4, 5). As such, adaptive success requires the ability to appropriately identify and modify behaviour in response to positive (rewarding) and negative (punishing) outcomes (3, 4, 6). An inability to appropriately update decision-making and behavioural choice by accurately incorporating outcome feedback leads to maladaptive behaviours that are damaging to an organism's health and survival. This often involves an inappropriate transition from goal-directed to habitual behaviours, where the former are more flexible and the latter more resistant to change (1, 7). Impaired cognitive flexibility is a phenotype common to a range of psychiatric illnesses such as schizophrenia (8, 9), obsessive compulsive disorder (OCD) (1), and addictive disorders (10, 11), which are characterized by stereotypical patterns of rigid behaviours that persist despite negative consequences and are resistant to change (1, 10).

In broad terms, cognitive flexibility is a facet of executive control that is primarily controlled by the prefrontal cortex (PFC) (2, 12); however, the nature of this control is complex. The PFC is comprised of multiple subregions, defined both structurally and functionally, that exhibit complementary and dissociable roles in the control of specific aspects of executive function including cognitive flexibility (12-17). These responses are mediated via widespread reciprocal projections from the PFC to, and from, many subcortical regions involved in reward processing including the dorsal and ventral striatum (e.g. nucleus accumbens [Acb]), ventral tegmental area (VTA), insula and basolateral amygdala (BLA) (13). Studies in human patients and non-human primates (17-20), and comparison with more incisive data generated from optogenetics and chemogenetics in rats and mice (21-24), show a broad conservation of PFC control of executive function that extends to rodents (15-17). Similarly complicated is the range of

neurotransmitters that are thought to be utilized in the mediation of cognitive flexibility, a list which includes dopamine (DA), serotonin (5-HT), glutamate (Glut) and GABA (12), among other molecules, which have region-, circuit- and receptor- specific effects on learning and cognition. Apart from consideration of the role of dopamine (see **Discussion section 4.4** of this Chapter), a more comprehensive examination of the individual contributions of different transmitters and receptors is beyond the scope of this Chapter and as such the reader is referred to comprehensive published reviews (12, 25).

Given the overarching role of the PFC in mediating cognitive flexibility, it follows that PFC dysfunction is a common consideration in the aetiology of psychiatric illnesses where this trait is typical. Therefore, it is not surprising that patients with a current or previous diagnosis of anorexia nervosa (AN), who consistently demonstrate both structural and functional abnormalities in PFC (26-28), also have accompanying cognitive deficits including impaired cognitive flexibility, often exhibiting rigid and rule-based behaviours and perfectionist tendencies, especially surrounding illness relevant stimuli such as feeding and exercise (29-33). A specific cognitive deficit common in patients with AN, both currently ill and weight recovered (REC-AN), is impaired set-shifting – the ability to switch between multiple stimuli-response contingencies or cognitive ‘sets’ (34). This deficit is often identified through impaired performance on the Wisconsin Card Sorting Test (WCST) (29, 33). In the WCST participants must sort stimuli cards into one of four multidimensional categories by inferring the sorting rule (colour, shape or number) based on feedback after card selection. After a predetermined number of consecutive correct card allocations, the sorting rule is changed without warning and participants must determine the new sorting dimension, i.e. they must shift to a new stimuli-response strategy (35). Poor performance across multiple WCST outcome measures, including perseverative errors (failure to switch sorting category following negative feedback) and perseverative responses (persistent responses using an incorrect sorting category) is demonstrated in AN (29, 31-33, 36-38), REC-AN (29, 32, 37), and in unaffected healthy sisters of patients with AN (31, 32, 37). This reflects an inability to adapt to changing action-outcome contingencies, and impairments in set-shifting and cognitive flexibility are seen across a broad range of neurocognitive tasks in all three participant groups (30, 37, 39). While cognitive rigidity is most severe in patients acutely ill with AN and likely contributes to perpetuating the condition (29, 32), the persistence of cognitive impairments following weight recovery and in unaffected sisters of patients with AN suggests that it is involved in the aetiology of AN (32, 33, 37). While we can be confident in the premise that cognitive rigidity is a fundamental trait of AN, a recent

systematic review of patients with AN and REC-AN compared to healthy controls highlighted that measures of cognitive flexibility, including the WCST, are prone to inconsistent findings between studies, a complication that is likely to be amplified by large discrepancies in participant demographics and experimental approaches (40).

The question then arises as to how measures of cognitive flexibility derived from the clinical situation relate to similar measures that can be interrogated in experimental animals. In this respect, two major aspects of cognitive flexibility, namely perseveration and set-shifting, can be assessed in rodents using reversal learning paradigms and the attentional set-shifting test (ASST), respectively. Importantly, these tasks are very similar (or in some instances identical) to those utilized in human and non-human primate studies, which gives them high translational value for studying the neurobiology underlying cognitive flexibility (6, 25, 41). Reversal learning paradigms come in many forms but the basic task requires rats (or mice) to learn to discriminate between two stimuli, one of which delivers a reward while the other is unrewarded or “punished”. Following acquisition, the reward contingencies are reversed without an accompanying signal or cue to indicate the change and the animal must inhibit responding to the previously rewarded stimulus and instead respond to the other stimulus to earn rewards (6, 42-44). The ASST is more complex and encompasses multiple stimulus modalities and several reward contingency shifts, both within and between stimulus dimensions, and also includes traditional reward contingency reversals (6, 45, 46). A number of studies support the essential involvement of the rodent PFC in mediating cognitive flexibility where PFC dysfunction or ablation (e.g. by chemical blockade) results in impaired reversal learning (47-49) and set-shifting on the ASST (45, 50), with some studies highlighting a crucial role for dopamine signaling (51-56). Moreover, impairments in cognitive flexibility are demonstrated in animal models of human pathologies characterized by PFC dysfunction including schizophrenia (8, 9), OCD (1), addiction (10, 11) and, most relevant to this thesis, the activity-based anorexia (ABA) model of AN (57). Specifically, exposure to ABA conditions impairs reversal learning on the ASST compared to rats with equal body weight loss resulting from food restriction alone – these ABA-induced reversal learning deficits normalize following weight regain (57).

Clearly correlating behavioral responses in human subjects and experimental animals is complicated by a number of situational and methodological differences that have been, until recently, peculiar to each. The study of cognition and behaviour in rodents has benefited in recent years from some major advances in technology that have increased the translational

capacity of rodent models of human pathologies (58, 59). A major contribution in this area has been the incorporation of touchscreens displaying visual stimuli in rodent test batteries that closely mimic those used for human cognitive testing (59). Visual stimuli can be manipulated in diverse ways (e.g. quantity, presentation duration, intensity/brightness, similarity/difference between stimuli) that are not feasible with tests that require mechanical stimuli (42-44, 59); critically, some touchscreen tests are *identical* for humans and rodents (59). Such uniformity of approach goes a long way toward standardization of interpretation of data; however, in the case of animals, testing using touchscreens, to date, has required experimenter intervention to transfer subjects to and from the testing chamber. This invokes the well-known dictate that experimenter involvement necessarily influences experimental outcomes, particularly so for behavioural studies. Specifically, along with stress from handling, which varies between experimenters and differentially impacts upon task performance (60-62), manual transfer to test chambers disrupts normal social behaviour and is insensitive to the current motivational state of the animal (63, 64). While the wide adoption of touchscreen cognitive testing has already yielded substantial benefits for behavioural neuroscience, the next frontier lies in the automation of the role of the experimenter in gatekeeping touchscreen access (63, 64). This is coming to fruition with modular and automated systems such as the PhenoSys animal sorting system. In such a system, group housed rats or mice can voluntarily move to and from the home cage to the touchscreen chamber via an automated sorting device. The PhenoSys uses radiofrequency identification (RFID) technology to identify and record an individual's movements throughout the system, allow selective entrance into the touchscreen chamber and track operant task performance (63, 64). The ability to utilize translationally-relevant cognitive tasks, without the confounds of experimenter intervention, has enormous potential for delineating the neurobiological underpinnings of cognitive flexibility in the ABA model, and ultimately how cognitive deficits drive the development of AN.

Given the links between the PFC and its subcortical projections and the likely involvement in cognitive flexibility detailed above, this Chapter investigates the potential that the improved ability of rats to adapt to the restricted feeding schedule and maintain body weight following suppression of neurons projecting from the medial prefrontal cortex (mPFC) to the shell of the nucleus accumbens (AcbSh), described in **Chapter 3** as the mPFC-AcbSh pathway, is mediated by such changes in cognitive control and enhanced flexibility. The involvement of this neuronal circuit in cognitive flexibility will be directly assessed using pathway-specific DREADD modulation of mPFC-AcbSh projection neurons during a touchscreen serial reversal learning

task, a task which will subsequently be established and optimized in the PhenoSys. This communal living and behavioral testing module represents state of the art technology that, to our knowledge, has not been established for rat studies anywhere else in the world. Its utility will prove invaluable in the rapid examination of cognitive flexibility and its relationship to ABA susceptibility in the same animals in future studies.

4.2 Methods

All experimental procedures involving animals were approved by the relevant Monash University (MARP-2018-026) or La Trobe University (AEC-18-17) Animal Ethics committees.

4.2.1 Experiment 1: Chemogenetic modulation of mPFC-AcbSh projecting neurons with traditional touchscreen testing

4.2.1.1 Animals and housing

Female Sprague-Dawley rats (*n*=40; 10-weeks-old at surgery, 140-180g) were used to examine the effects of mPFC-AcbSh pathway activity on cognitive flexibility in a touchscreen reversal learning paradigm. Rats received bilateral injections of AAV5-pmSy1n-EBFP-Cre into the AcbSh and either hM4Di (*n*=14), hM3Dq (*n*=14) or AAV-mCherry (control; *n*=12) DREADD viruses into the mPFC (prelimbic [PrL] and infralimbic [IL] cortices) to recruit the mPFC-AcbSh pathway, as described in **Chapter 3 Methods section 3.2.1**. Before and immediately after surgery rats were housed in groups of 5 in individually ventilated cages with *ad libitum* access to food and water in a temperature (21-22º) and humidity (60%) controlled room with a reversed 12h light/dark cycle (lights off 08:00h). After recovery from surgery and throughout touchscreen testing, rats were pair-housed in standard wire topped cages with free access to water and an amount of food restricted to maintain ~85-90% of their expected body weight if they were allowed *ad libitum* access to food.

4.2.1.2 Testing conditions

Touchscreen testing was conducted during the dark phase in an adjacent room with the same environmental conditions with each rat tested in the same chamber at approximately the same time each day. Food (standard laboratory chow; Barastoc, Australia) was provided in the home cage immediately following completion of touchscreen testing. Thirty minutes before their scheduled touchscreen test each rat was weighed and (if required) administered either saline (0.9% NaCl) or CNO (CarboSynth; UK) intraperitoneally (see below).

Rats were trained once per day, 6 days per week. Rats were placed in a trapezoid operant conditioning chamber (Campden Instruments; 25cm wide at touchscreen, 13cm wide at

magazine, 33cm long between screen and magazine, 30cm high) with the touchscreen (15in diagonal, rotated) covered by a black plastic “mask” with two 10x10cm openings (bottom edge of opening is 16cm from floor) through to the touchscreen that differentiated the image locations from the background and helped prevent accidental screen touches. Rewards consisted of 45mg sucrose pellets (Able Scientific; WA, Australia) delivered from a pellet dispenser into the magazine, in which a light and infrared (IR) beam detected entry of the rat’s nose. A house light, tone generator and click generator all acted as conditioned reinforcers (as described below).

4.2.1.3 Modulation of mPFC-AcbSh pathway activity during reversal learning

Saline was administered 30 minutes before pairwise discrimination (PD) sessions when rats advanced from attempting 50 to 100 trials in a session. Upon progression to first reversal (R1), CNO (0.3-3.0mg/kg) was administered 30 minutes before each session to modulate the mPFC-AcbSh projecting neurons during this reversal learning phase. During the second reversal (R2) saline was administered instead of CNO to control for effects of handling and restraint. Performance criteria and progression specifics for each phase are detailed in **Table 4.1** in Results below.

4.2.1.4 Exclusions

Two rats that had hM3Dq injected had unexpected complications arising from surgery and were euthanized. Additionally, 3 of the rats that reached the PD progression criterion were found at post mortem to have off target or no DREADD expression were excluded from analyses, resulting in a final cohort size of *n*=19 (50% of initial cohort) evenly spread across the treatment groups (mCherry *n*=6, black; hM3Dq *n*=6, purple; hM4Di *n*=7, orange).

4.2.2 Experiment 2: High-throughput touchscreen testing using the PhenoSys

4.2.2.1 Animals

Female Sprague-Dawley rats (*n*=10; 7 weeks old, 160-180g) were briefly anaesthetized (<5 min) under 5% isoflurane in oxygen and implanted with subcutaneous RFID transponders (2.1 x

12mm; PhenoSys, Berlin) into the left flank using a purpose-built syringe and needle (**Figure 4.1 A**). Tissue adhesive (Vetbond 3M; NSW, Australia) was used to seal the incision site.

4.2.2.2 Housing and testing conditions

Rats were housed in two living, activity and touchscreen systems (PhenoSys; GmbH, Berlin) in a temperature (20-23°) and humidity (35-65%) controlled room under a reverse 12h light/dark cycle (lights off 12:00h). Rats had *ad libitum* access to water in the home cage and food provided daily in the hour before onset of the dark cycle to maintain body weight at ~85-90% of free feeding weight. The PhenoSys consists of a home cage (34cm wide x 20-26cm high x 55cm deep) connected to a sorter cage (19cm wide x 10cm high x 25cm deep**)** by a plastic tube. After entry into the sorter cage rats were required to trace out a U shape around the central dividing wall to exit the sorter via a second plastic tube to access the touchscreen chamber. Two RFID readers were positioned under the sorter which was mounted directly upon a scale to measure body weight (this arrangement is shown in **Figure 4.1 B** and **C**). In order to monitor and restrict access to only one known animal at any one time, two 8cm diameter circular metal guillotine gates controlled entry to and from the sorter. Only when an RFID reading from one transponder was recorded in the sorter in combination with a body weight recording within 10%± of the known animal body weight did the custom software (PhenoSoft) allow passage of a single rat from the home cage to the trapezoid touchscreen chamber (**Figure 4.1 D**). Nose touches to the screen were detected by breaking an IR beam grid positioned over the front of the screen behind the mask. The rest of the chamber set-up replicated those used for Experiment 1, except there was no house light or click generator. Illumination of the entire touchscreen with bright white was used in place of the house light with no substitute for the trial initiation click signal. Upon completion of the touchscreen session the rat was allowed to reenter the sorter, and subsequently return to the home cage.

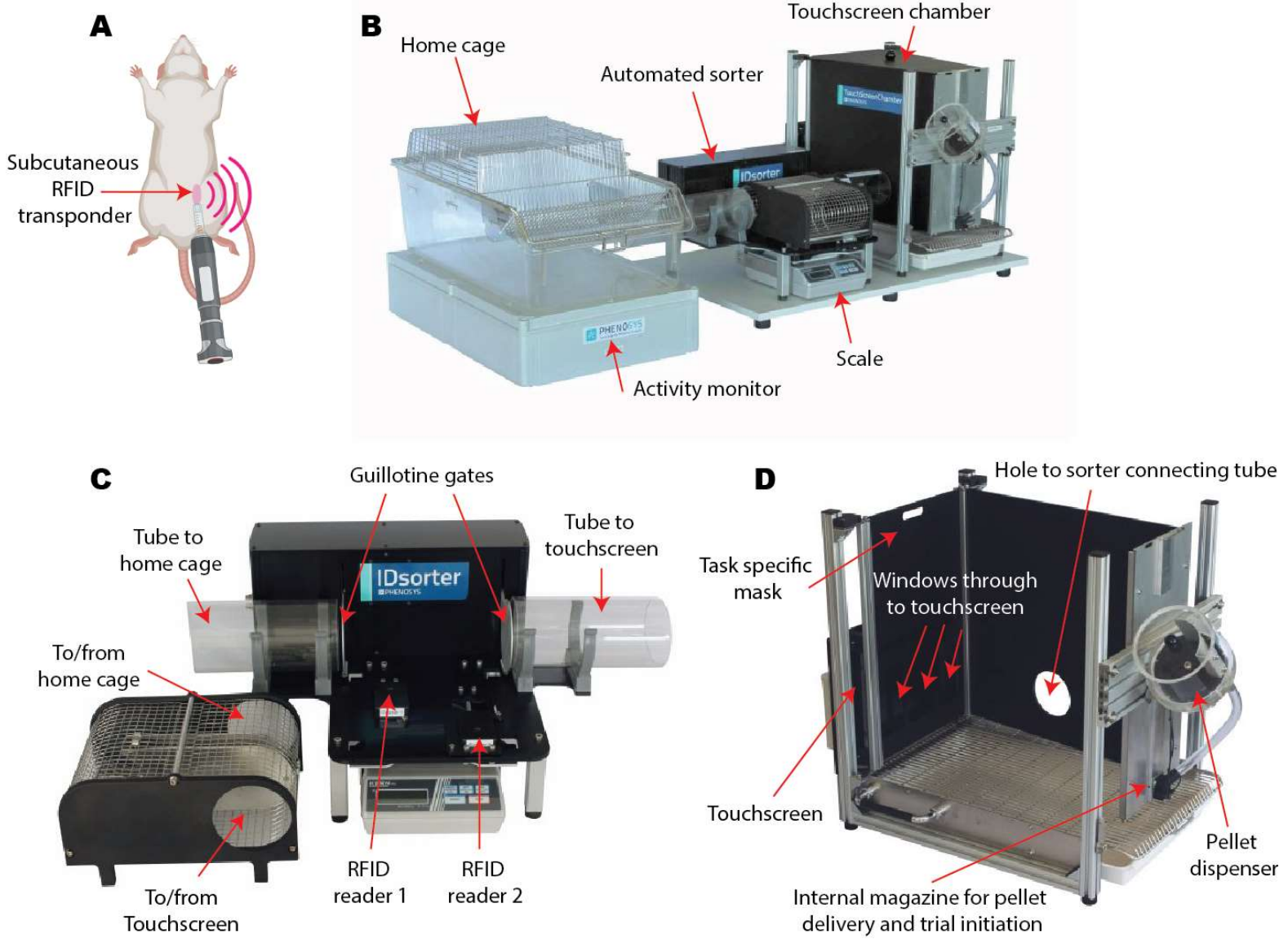

A
Subcutaneous
RFID
transponder
B
Home cage
Automated sorter
Touchscreen chamber
Scale
Activity monitor
C
Guillotine gates
Tube to
home cage
Tube to
touchscreen
To/from
home cage
To/from
Touchscreen
RFID
reader 1
RFID
reader 2
D
Hole to sorter connecting tube
Task specific
mask
Windows through
to touchscreen
Touchscreen
Pellet
dispenser
Internal magazine for pellet
delivery and trial initiation

Figure 4.1 The PhenoSys is an automated home cage and touchscreen testing system. **A)** Rats were implanted with unique radiofrequency identification (RFID) transponders. **B)** Overview of the entire PhenoSys system. The home cage is 34cm wide x 20cm high (26cm at the top of the wire cage lid) x 55cm deep with an 8cm diameter hole 20cm from the front on the right-hand side which connects to a 28cm long plastic tube of 8.5cm diameter that runs to the sorter cage which is mounted directly upon a scale. **C)** The sorter cage is 19cm wide x 10cm high and 25cm deep, divided lengthways for 2/3 by an internal metal wall. The sorter is connected to the Touchscreen chamber via a 19cm long plastic tube of 8.5cm diameter. Two RFID readers are positioned under the sorter, the first at the entrance the second diagonally opposite just past the U bend. Two 8cm diameter metal guillotine gates controlled entry to and from the sorter by opening upwards and were fitted at the bottom with magnetic strips to protect from closing on any part of an animal. **D)** The touchscreen is 23cm wide at the touchscreen tapering to 14cm wide at the pellet magazine end, 39cm deep and 37cm high; touchscreen height 20 cm. Removable black plastic masks sit in front of the touchscreen with appropriate windows for touchscreen interaction. The pellet dispenser is externally mounted and delivers pellets via a plastic tube to an internal magazine tray fitted with an IR beam to detect beam breaks from entry and exit of the rat's nose. Note that the hole in the side wall for touchscreen entry/exit was on the facing wall in our setup (as per **B**) rather than that shown in **D**. Images from (65).

4.2.3 Pairwise discrimination and serial reversal learning

To examine cognitive flexibility, specifically the ability to change reward-related responding to accommodate a change in reward contingency, a serial visual reversal learning task was utilized (adapted from 43). Similar protocols for this task were implemented for both Experiment 1 and 2, with some notable differences as follows.

Before the reversal learning task could be initiated, multiple pre-training stages were required to progressively shape the behaviour of rats and prepare them for the task; for details see (43). Briefly (**Figure 4.2**), rats **1)** are habituated to the sucrose pellets (~20 pellets per rat) spread throughout the PhenoSys apparatus and touchscreen chambers (Habituation), **2)** learn to associate the touchscreen images with sucrose rewards (Initial Touch; **A**), **3)** learn to touch the image to receive a reward (Must Touch; **B**), **4)** learn to initiate the presentation of the image/s (Must Initiate; **C**), and **5)** learn the 'punishment' for making an incorrect response (touching the blank window; Punish Incorrect; **D**). These steps were consistent across both experiments with some changes to specific details (See **Supplementary Tables S4.1** and **S4.2**). For the entirety of testing the maximum session duration in Experiment 1 was 60 minutes/100 trials whereas this was reduced to 30 minutes/30 trials in Experiment 2, considering that rats in the PhenoSys were permitted multiple sessions per day. For the same reason, smaller sucrose pellets were used in Experiment 2 (20mg dustless precision pellets; Abel Scientific), to prevent reward devaluation or saturation of sucrose leading to reduced motivation.

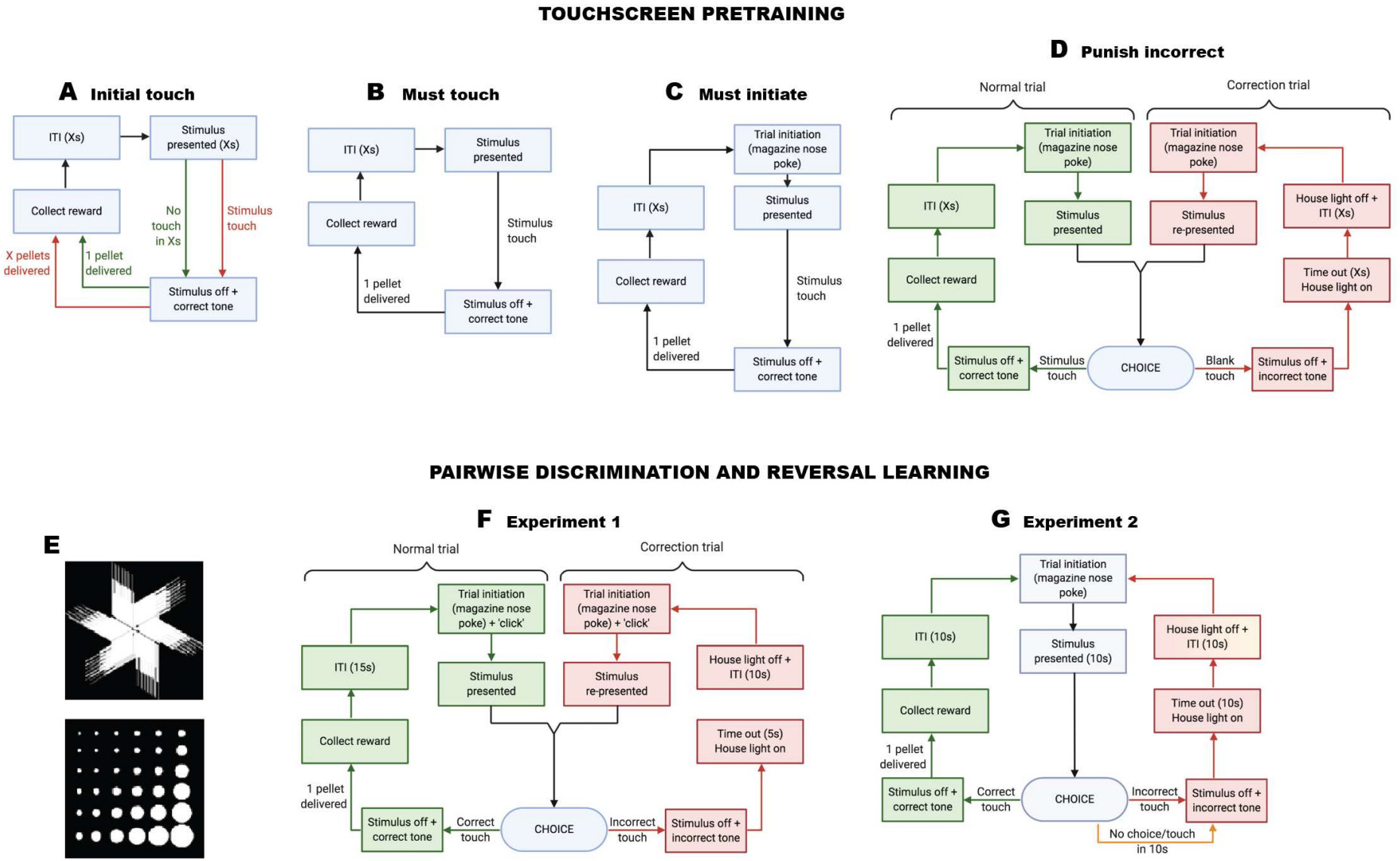


Figure 4.2 Schematic overview of touchscreen pre-training and serial reversal learning protocol. Image based on (43) and adapted for our protocol. See text for brief overview and (43) for detailed description of each stage. See **Supplementary Tables S4.1** and **S4.2** for specific parameters in each stage for each experiment. **E)** The marble (top) and fan/pinwheel (bottom) images used for all stages of reversal learning in both experiments.

Following successful completion of pretraining steps, rats in both experiments progressed to PD, the first phase of the serial visual reversal learning task, where they learned to discriminate between two simultaneously displayed images (**Figure 4.2 E**) one of which was the rewarded stimulus (CS+) and the other the non-rewarded or 'punished' stimulus (CS-). The initial reward contingencies of the two images were balanced between DREADD groups in Experiment 1 and counterbalanced across the two systems of the PhenoSys in Experiment 2, with all rats in each system necessarily allocated the same contingencies. When PD was successfully acquired (Experiment 1 see **Table 4.1**; Experiment 2, 30 trials completed with ≥80% response accuracy) the reward contingencies of the two images were swapped (first reversal [R1]) until the new stimuli and reward pairing was successfully acquired before being swapped back again (second reversal [R2]); a third reversal (R3) was included in the PhenoSys experiment as permitted by the rapid rate of learning.

4.2.3.1 Adapted protocol for Experiment 2 reversal learning in the PhenoSys home cage paradigm

There were several specific differences between the reversal learning protocols in the two experiments, which were employed to accommodate the different learning environments. These included modifications to the number of sessions, trials within sessions, progression criterion and timing of progression.

The first critical difference between the experimental protocols relates to how stimuli were presented. In Experiment 1, the stimuli remained illuminated until one of the images was touched, whereas in Experiment 2 the stimulus was presented for a limited duration meaning that trial omission was a possible outcome. In both cases, if the correct image was touched the correct tone was played, a sucrose pellet was delivered and the magazine tray light illuminated. Similarly, if the incorrect image was touched, or a trial was omitted in Experiment 2, the incorrect tone was played and the house light illuminated for a time out. "Correction trials" followed an incorrect response in Experiment 1, in which the stimuli were presented on the same side as in the preceding trial and continued until the correct image was touched. Correction trials were used to promote learning but did not contribute towards performance calculations. However, Experiment 2 did not utilize correction trials in this way and, as such, responses to all trials contributed toward session accuracy. Moreover, compared to Experiment

1, in which rats were exposed to the touchscreen operant chamber only once per day (Maximum duration 60 min or 100 correct trials), the maximum number of sessions each rat could participate in per day in Experiment 2 was largely unrestricted and determined by the individual rat's willingness and ability (given competition between rats for priority) to enter the touchscreen (maximum duration 30 min or 30 correct trials).

4.2.3.2 Experiment 2 limitations and exclusions

One limitation of the current PhenoSys set up is the absence of a 'click' or tone to accompany trial initiation via nose poke into the magazine tray. While this did not appear to hamper learning in the first instance, the implications of this became apparent on day 6 of the experiment during which one feeder became jammed and stopped delivering pellets (see **Results section 4.3.8** for details).

A second limitation is that until near the end of this experiment PhenoSoft only allowed allocation of one reward contingency pairing for the entire cage of animals (*n*=5), rather than possessing the capacity to individually allocate (and thus reverse) reward contingencies for each rat. While this was eventually resolved, it led to several instances of "overtraining" while fast learners waited for slower cage mates to progress to the next phase (see for example rat 1.2 in PD **Figure 4.7 C1**). This also necessitated the premature reversal of reward contingencies for one rat (rat 1.4) before PD learning had been successful, in order to progress the other animals in the cage. Although this rat then went on to learn PD and the three subsequent reversals, all data was excluded from analyses. A second rat (rat 2.4) accidentally only underwent 2 reversals and was therefore also excluded from analyses. For interest these data can be found in **Supplementary Figure S4.1**.

4.2.4 Determining estrous cycling throughout Experiment 1 touchscreen testing

Standard practice in the lab is to synchronize the estrous cycles of female rats using the Whitten effect by housing a male rat in the same room for the entirety of experimental duration. The efficiency of this practice was validated during Experiment 1, where the proportion of female rats in the same cycle stage was examined via Cresyl Violet staining of vaginal lavage samples (66, 67) taken after 4 and 12 weeks of the experiment to confirm majority (>80%) of animals were synchronized. See **Supplementary Figure S4.2**.

4.2.5 Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) and significance for all tests was set at $p<.05$. Fisher's exact test was used to analyze the distribution of Experiment 1 PD outcome by image designated CS+. Mixed-effects models were used to analyze number of trials (total, correction and correct) and incorrect:correct ratio to reach criterion, and touchscreen outcomes of the first 100 trials (Experiment 1). Pearson's correlation was used to analyze the relationship between session performance and session start time in Experiment 2. One-way RM ANOVA was used to analyze days, sessions and total trials to criterion in Experiment 2. Two-way RM ANOVA was used to analyze the proportions of trial outcomes to criterion in Experiment 2. Significant primary analyses were followed by Tukey's post-hoc multiple comparisons.

4.3 Results

4.3.1 Experiment 1: Overall learning performance throughout the phases of the serial visual reversal task

4.3.1.1 Pairwise discrimination (PD)

Following successful completion of pretraining, 38 rats started the initial PD phase of the serial visual reversal learning task (**Figure 4.3 A left**). After 20 touchscreen sessions it was evident that 24 rats were on a trajectory indicating task acquisition to criterion (green and grey lines) whereas 14 rats were not (red lines) and were removed from the experiment (**Figure 4.3 B**). By amending the progression criterion from that of Horner (43) and extending the number of sessions to 30, we showed that 22 rats (58%) had acquired the PD task with sufficient accuracy to progress (**Table 4.1**). However, there was large variation in the *rate* of task acquisition (green bordered circles **Figure 4.3 B**) with the number of sessions required to reach criterion ranging from 11 to 28 (Median = 22, IQR = 17.25 to 27; **Figure 4.3 C**), although learning was not impacted by which image was reward-paired (**Figure 4.3 D**; p=.7521).

4.3.1.2 First reversal (R1)

Of the rats that were progressed through the PD phase (n=19), 16 (84%) successfully reached R1 progression criterion (**Figure 4.3 F2**) which was slightly modified for the final 10 sessions (See **Table 4.1**). Compared to PD the median number of sessions to R1 criterion increased to 25 (dashed blue line **Figure 4.3 F3**) while the range (17 to 30) decreased, suggesting the R1 phase was more difficult to acquire for all individuals.

4.3.1.3 Second reversal (R2)

Using the same criterion as in R1, 12/16 (75%) of rats successfully achieved completion i-iv (solid filled bars **Figure 4.3 G2**). Two rats failed to successfully complete R2, with accuracy scarcely improving above chance (red circles **Figure 4.3 G1**). Testing for the two remaining rats had to be prematurely and immediately ended due to unavoidable circumstances (see **footnote[1]** in **Supplementary Information**). However, both rats had reached 78% accuracy and were on target to reach criterion so were subsequently classified as having done so (stacked faded bars **Figure 4.3 G2**). For these 14 rats the median number of sessions to R2

criterion decreased to 18 (dashed blue line **Figure 4.3 G3**) with the range returning to that seen in PD (11to 28 sessions).

Table 4.1: Progression criteria for touchscreen cognitive testing

Phase	**Sessions**	**Removal criterion**
All	After 20th	Accuracy no greater than chance
	After 30th	Failure to reach progression criterion
Phase	**Sessions**	**Progression criteria. The session in which a rat achieves any one of:**
Pairwise discrimination	1-30	**i)** second consecutive session with accuracy ≥ 80% **ii)** accuracy ≥75% in session immediately following one with accuracy ≥80% **iii)** a second session with accuracy ≥80% following accuracy dropping below 75% after previous session with accuracy ≥80%
	26-30	**i-iii)** **iv)** accuracy ≥80% in session immediately following one with accuracy ≥75%
First reversal	1-20	**i-iii)**
	21-25	**i-iv)**
	26-30	**i-iv)** **v)** accuracy ≥80% **vi)** accuracy ≥78%
Second reversal	1-20	**i-iii)**
	21-30	**i-iv)**

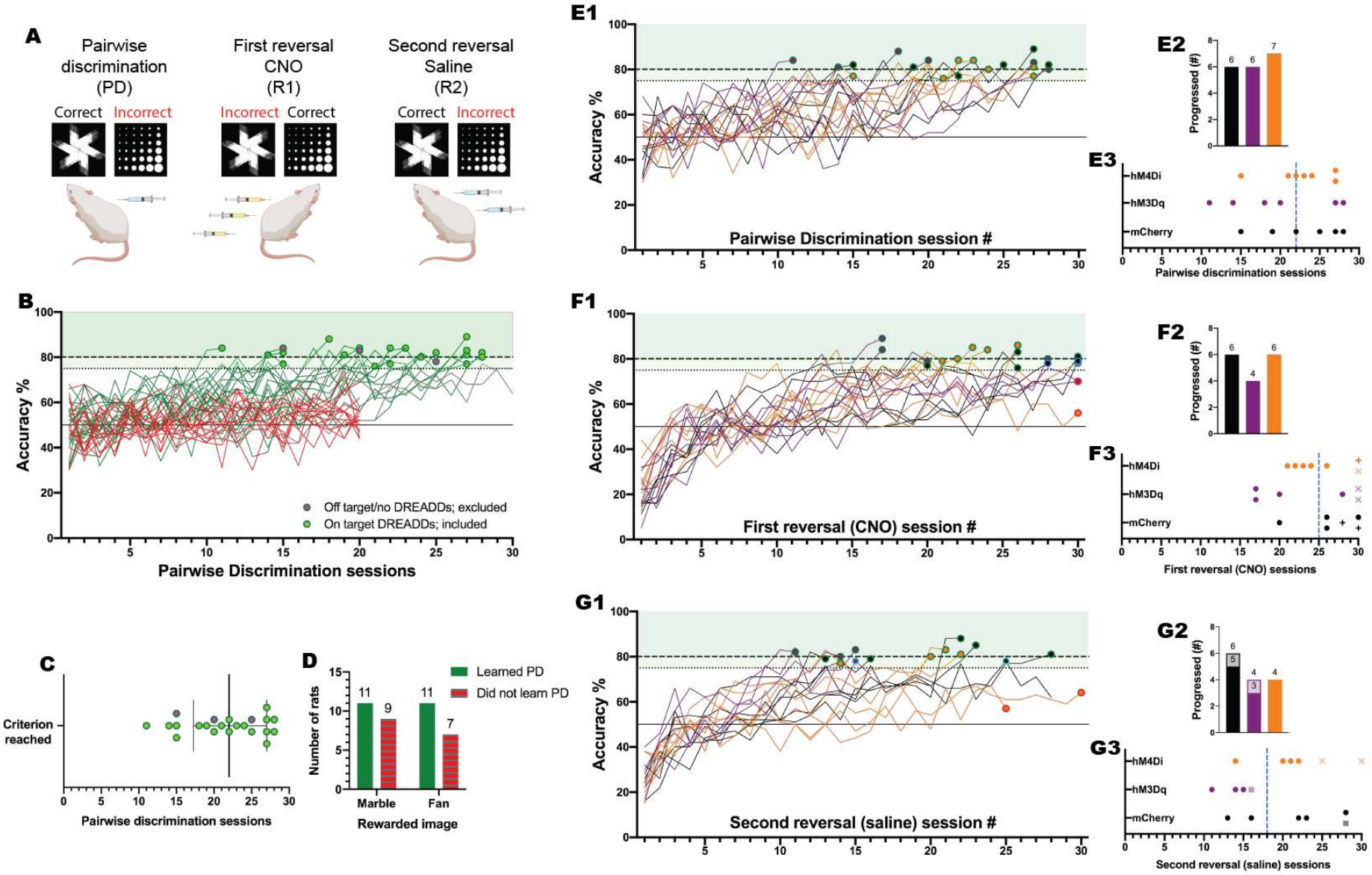

A
Pairwise discrimination (PD)
Correct Incorrect
First reversal CNO (R1)
Incorrect Correct
Second reversal Saline (R2)
Correct Incorrect
B
Accuracy %
Off target/no DREADDs; excluded
On target DREADDs; included
Pairwise Discrimination sessions
C
Criterion reached
Pairwise discrimination sessions
D
Learned PD
Did not learn PD
Number of rats
11
9
11
7
Marble
Fan
Rewarded image
E1
Accuracy %
Pairwise Discrimination session #
E2
Progressed (#)
6
6
7
E3
hM4Di
hM3Dq
mCherry
Pairwise discrimination sessions
F1
Accuracy %
First reversal (CNO) session #
F2
Progressed (#)
6
4
6
F3
hM4Di
hM3Dq
mCherry
First reversal (CNO) sessions
G1
Accuracy %
Second reversal (saline) session #
G2
Progressed (#)
6
5
4
3
4
G3
hM4Di
hM3Dq
mCherry
Second reversal (saline) sessions

Figure 4.3 Serial visual reversal learning accuracy and progression outcomes with chemogenetic modulation of mPFC-AcbSh. A) Schematic showing the touchscreen testing procedure. **B)** Accuracy (%) over time throughout the initial pairwise discrimination (PD) phase for rats that did (green) and did not (red, grey) reach progression criterion (see **Table 4.1**). **C)** The number of PD sessions required for each rat that did learn the task to reach progression criterion with the group median 22 and IQR (17.25 to 27) overlaid. **D)** Contingency graph showing that the ability of rats to learn the task was not dependent on which image was designated as correct. Fisher's exact test *p*=.7521. **E-G 1)** Accuracy (% correct touches in accuracy trials) across a maximum of 30 sessions (1 per day, 6 days a week), **2)** The number of rats that successfully reached progression criterion, and **3)** The number of sessions required to reach progression or removal criterion, in each of pairwise discrimination **(E)**, first reversal **(F)** and second reversal **(G)** for individual rats with either hM3Dq-activating-DREADDs (purple), hM4Di-inhibting-DREADDS (orange). or AAV-mCherry (black) expressed in mPFC-AcbSh projecting neurons. Symbols show the session in which an animal reached progression criterion i-v (green bordered circles **1**, opaque circles **2**), the session in which an animal reached progression criterion vi (blue bordered circles **1**, plus signs **2**), the final session before removal for rats that failed to reach progression criterion (red bordered circles **1**, crosses **2**), and indicate the two rats that reached 78% before a necessary and unavoidable premature end to testing and as such were subsequently considered as having reached criterion (aqua bordered circles **1**, translucent squares **2**). **2)** Solid bars with numbers above indicate the number of rats per group that successfully reached progression criterion; extended faded bars in **G2** indicate inclusion of the two rats that reached 78% in second reversal. **3)** Dashed blue line indicates median number of sessions required to successfully reach progression criterion (i.e. excludes rats that failed to learn and were removed [crosses]). See **Table 4.1** for criterion details.

4.3.2 Experiment 1: mPFC-AcbSh circuit modulation does not alter overall reversal learning ability or accuracy

Consistent with a similar number of animals from each DREADD-manipulation group that reached progression criterion, overall performance was similar across groups (**Figure 4.4 A**). With the exception of the early perseverative phase of R1, fluctuations in and divergence between group means for accuracy reflect the progression of individual animals (green bordered circles, **Figure 4.4 A**) to the next phase and are not indicative of changes in learning between groups or associated with modulation of the mPFC-AcbSh pathway.

However, accuracy, and therefore progression through reversal learning phases, does not consider the outcome for or number of correction trials completed. Therefore, additional performance measures utilized were the number of total, correct and incorrect trials as well as the overall ratio of incorrect to correct trials required to reach criterion within each phase. In order to reliably compare group differences across all three phases of learning, only rats that successfully completed R2 were included in analyses (hM3Dq n=4, hM4Di n=4, mCherry n=6). All animals, regardless of DREADD group, required more correct (**Figure 4.4 C**), incorrect (**D**), and total (**B**) trials to reach progression criterion for R1 than for either PD (all ps $\leq$.0006) or R2 (all ps $\leq$.0085), with no differences between PD and R2 (all ps$\geq$.5378), indicating that R1 was the most difficult phase to learn. Interestingly, there was no corresponding cohort level difference in the overall incorrect:correct ratio between phases (p=.1142, **Figure 4.4 E**). While the circuit activated hM3Dq group required fewer total (p=.0468; **Figure 4.4 B**) and correct (p=.0350; **C**) trials to reach R1 criterion than the control mCherry group, this pattern held during R2 when there was no circuit modulation (total p=.0067; correct p=.0040) and is consistent with the accompanying overall difference between these groups (total p=.0384; correct p=.0364) indicating an underlying difference between these groups *independent* of circuit modulation.

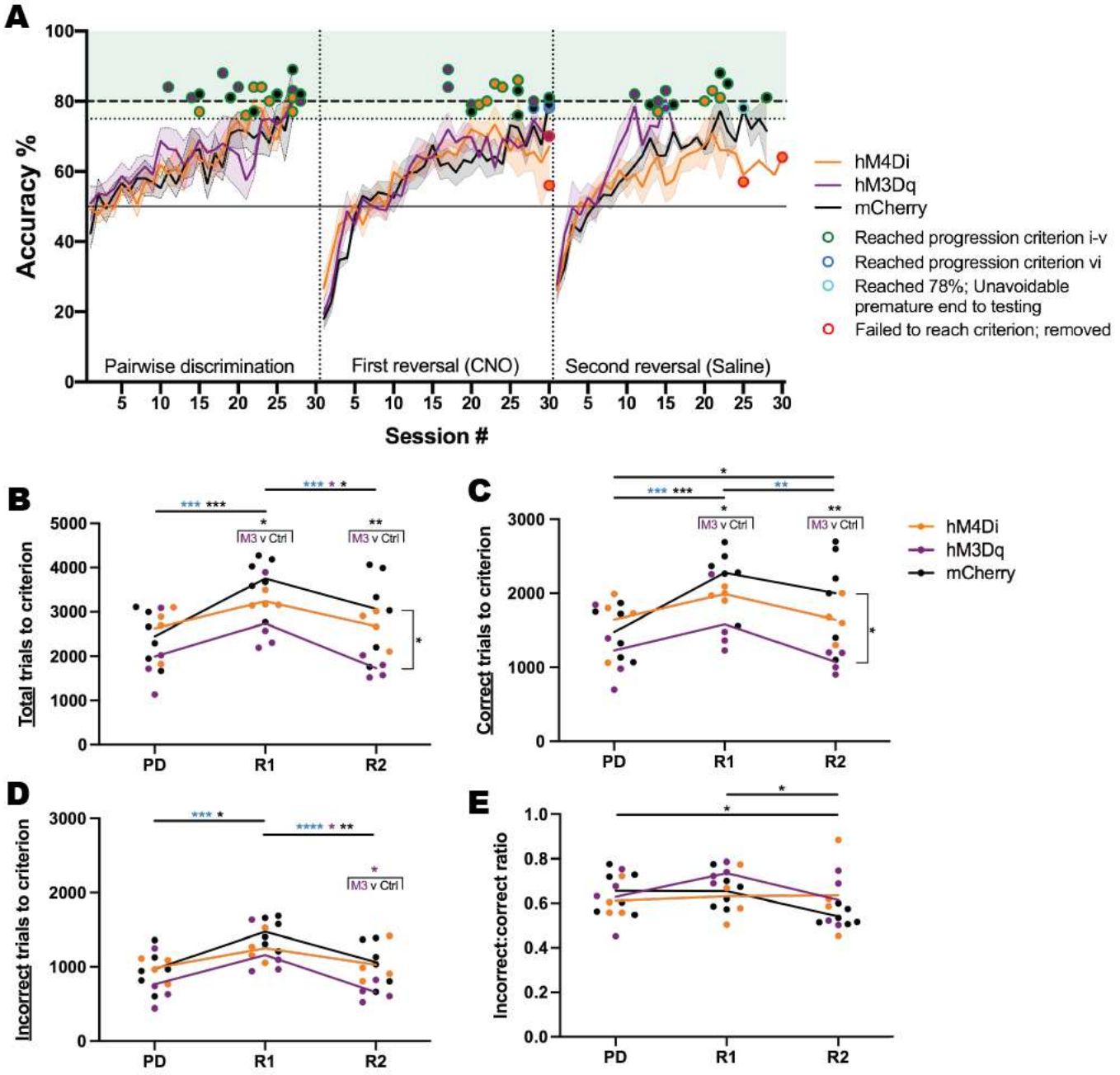


Figure 4.4 Serial reversal learning accuracy and trial outcomes with chemogenetic modulation of mPFC-AcbSh. **A)** Mean accuracy (%) ± SEM (shaded bands) per session across initial pairwise discrimination (PD) acquisition (left panel), first reversal (R1) with CNO (middle panel) and second reversal (R2) with saline (right panel) for rats with hM3Dq-activating-DREADDs (purple; *n*=6), hM4Di-inhibiting-DREADDs (orange; *n*=7), or AAV-mCherry (black; *n*=6) expressed in mPFC-AcbSh projecting neurons. Circles represent the session each rat reached progression or removal criterion. Total **(B)**, correct **(C)**, and incorrect **(D)** trials to reach criterion, and the overall ratio of incorrect:correct trials **(E)** within each phase. Dots show individual rats, lines are group mean. Only rats that successfully achieved progression (completion) criterion in R2 are included; hM3Dq *n*=4, hM4Di *n*=4, mCherry *n*=6. **B-E)** Two-way mixed ANOVA followed by Tukey's post-hoc multiple comparisons. **B)** Sphericity assumed, Geisser-Greenhouse's epsilon =.8253. Phase $F(2, 22)=14.69$, $p<.0001$ (PD<R1 $p=.0001$, R1>R2 $p=.0009$); Group $F(2, 11)=4.176$, $p=.0447$ (hM3Dq < mCherry $p=.0384$); Interaction $F(4, 22)=1.498$, $p=.2372$. Between group: R1 hM3Dq < mCherry $p=.0468$, R2 hM3Dq < mCherry $p=.0067$. Within group: hM3Dq R1>R2 $p=.0135$, mCherry PD<R1 $p=.0002$, R1>R2 $p=.0424$. **C)** Sphericity assumed, Geisser-Greenhouse's epsilon =.8953. Phase $F(2, 22)=10.49$, $p=.0006$ (PD<R1 $p=.0006$, R1>R2 $p=.0085$); Group $F(2, 11)=4.271$, $p=.0424$ (hM3Dq < mCherry $p=.0364$); Interaction $F(4, 22)=2.006$, $p=.1289$. Between group: R1 hM3Dq < mCherry $p=.0350$, R2 hM3Dq < mCherry $p=.0040$. Within group: mCherry PD<R1 $p=.0003$, PD<R2 $p=.0153$. **D)** Phase $F(1.477, 16.24)=16.87$, $p=.0003$ (PD<R1

p=.0003, R1>R2 *p*<.0001); Group *F*(2, 11)=3.142, *p*=.0833; Interaction *F*(4, 22)=0.8521, *p*=.5077. Between group: R2 hM3Dq < mCherry *p*=.0446. Within group: hM3Dq R1>R2 *p*=.0497; mCherry PD<R1 *p*=.0259, R1>R2 *p*=.0041. **E)** Phase *F*(1.891, 20.08)=2.439, *p*=.1142; Group *F*(2, 11)=0.4596, *p*=.6431; Interaction *F*(4, 22)=1.280, *p*=.3080. Within group: mCherry PD>R2 *p*=.0328, R1>R2 *p*=.0426. Black vertical brackets show significant main effect group differences. Black horizontal brackets show significant between group comparisons within the enclosed phase (i.e. simple effects within phase). Black horizontal lines show between phase comparisons of phases underneath line ends: Main effect of phase = blue*; Simple effects within group (hM3Dq = purple*, hM4Di = orange*, mCherry = black*). *p<.05, **p<.01, ***p<.001, ****p<.0001.

4.3.3 Experiment 1: Suppressing activity in mPFC-AcbSh projecting neurons reduces early perseverative responding

To interrogate the effect of modulation of mPFC-AcbSh neuronal projections on acute behavioural flexibility immediately following a switch in reward contingencies, a period in reinforcement learning known to specifically reflect perseverative responding, multiple measures of performance in the first 100 accuracy trials of R1 (with circuit modulation) were compared with performance over the same period in both PD and R2 (no circuit modulation). To maintain consistent group membership across phases to permit valid statistical analysis, only rats that successfully reached R1 progression criterion are included in the following graphs and analyses (hM3Dq *n*=4, hM4Di *n*=6, mCherry *n*=6).

Firstly. trial-by-trial performance during the early perseverative phase of reversal learning was tracked using chronological step graphs (**Figure 4.5**) of the first 100 trial block in each of the three phases. The X-axis tracks incorrect responses and the Y-axis tracks correct responses, where the outcome of each trial progresses the performance line by 1 unit (incorrect response → X+1; correct response → Y+1). Three observations are immediately evident with visual inspection of the step graphs, 1) performance in PD and R2 is relatively similar both between phases and between groups, 2) performance in R1 is worse than performance in both PD and R2, and 3) performance in R1 is different between groups as a result of mPFC-AcbSh DREADD modulation, with CNO-mediated inhibition of the circuit increasing the proportion of initial correct touches in R1 (**Figure 4.5 B$_2$**).

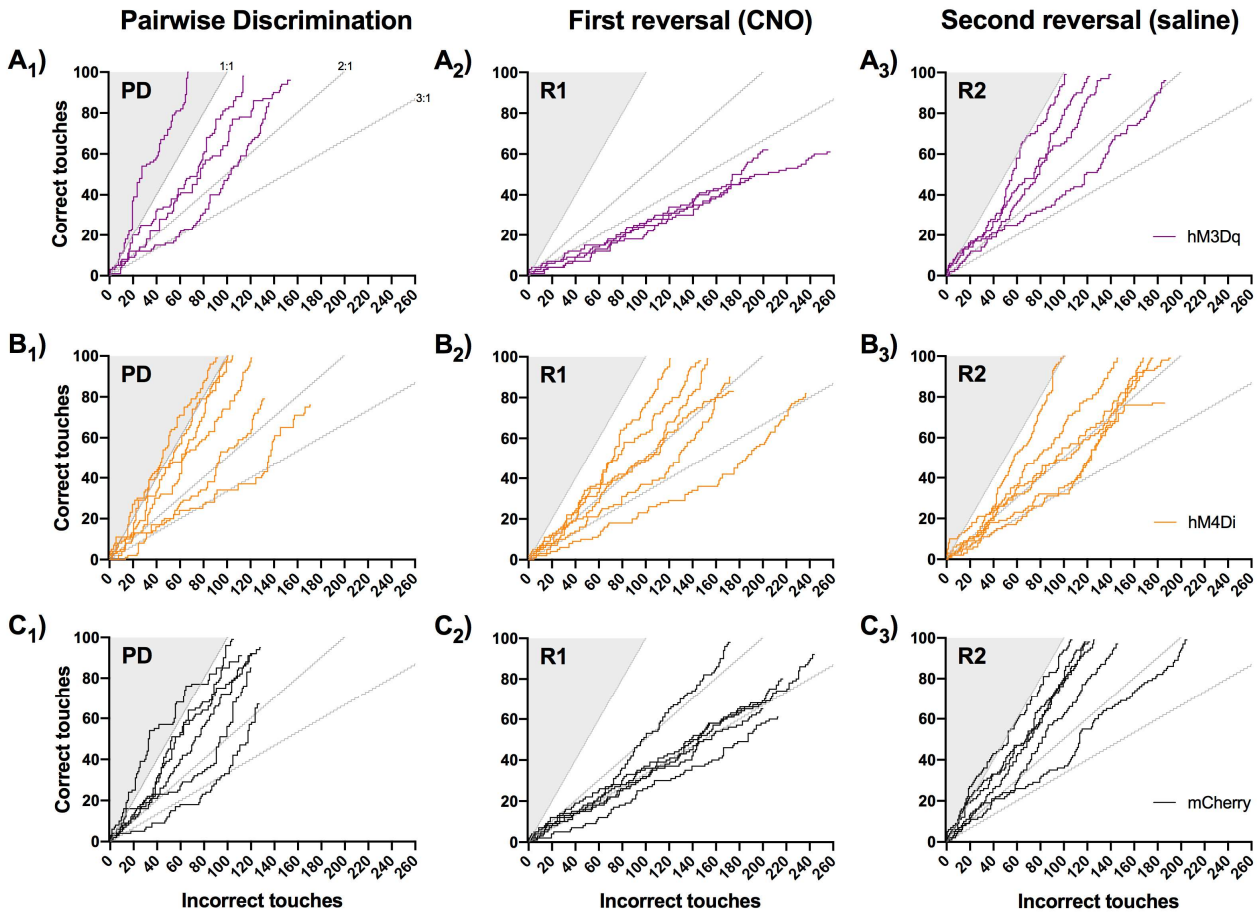


Figure 4.5 Step graphs showing individual rats performance in the first 100 accuracy trial block in each phase of the serial reversal learning task. Each line shows an individual rats' progressive performance in the first 100 accuracy trials of the three phases of the reversal learning protocol (pairwise discrimination, **PD [1]**; first reversal, **R1 [2]**; second reversal, **R2 [3]**), for rats with hM3Dq-activating-DREADDs (**A,** purple, *n*=6), hM4Di-inhibiting-DREADDs (**B,** orange, *n*=7) or AAV-mCherry (**C**, black, *n*=6), in mPFC-AcbSh projecting neurons. Incorrect touch → X+1; Correct touch → Y+1 (max 100). Grey lines radiating out from the origin represent hypothetical performance maintaining an incorrect:correct ratio of 1 (**1:1** left most line bordering the shaded triangle), 2 (**2:1** middle line) and 3 (**3:1** right most line; line labels in **A1** apply to all graphs in figure). Thus, the shaded area left of the 1:1 line represents performance better than chance, i.e. an incorrect:correct ratio of <1.

In addition to those already described above we also analyzed absolute accuracy, which includes performance on correction trials, a perseveration index, which indicates the average number of correction trials required before a correct response is performed, as well as the latencies to respond, and collect rewards. While there was variability in response profiles in PD, there were no significant differences between groups on any performance measure during the initial phase of learning (**Figure 4.6**). Performance across the cohort worsened from PD to R1 (blue asterisks **Figure 4.6;** decreased accuracy $p<.0001$ **A**, and absolute accuracy $p=.0003$ **B**; more total $p<.0001$ **E**, and incorrect $p<.0001$ **D**, trials; increased incorrect:correct ratio $p=.0004$ **F**). Performance decline was most substantial following mPFC-AcbSh circuit activation (purple asterisks**)**, followed by the control mCherry group (black asterisks), whilst inhibiting the circuit

blunted this decrement in performance (orange asterisks; **Figure 4.6 A-G**). Circuit inhibited hM4Di rats demonstrated greater accuracy than both other groups (hM3Dq *p*=.0121, mCherry *p*=.0366; **Figure 4.6 A**) and greater absolute accuracy than circuit activated hM3Dq rats (*p*=.0055; **B**). Further, while there were no significant group differences in incorrect (*p*=.9449; **Figure 4.6 D**) or total (*p*=.8291; **E**) responses made in R1, circuit activated hM3Dq rats made fewer correct responses (**C**) than both circuit inhibited hM4Di (*p*=.0021) and mCherry control (*p*=.0486) groups, leading to a greater incorrect:correct ratio (**F**) and perseveration index (**G**) than both the circuit inhibited hM4Di (*p*=.0008 and *p*=.0025 respectively) and mCherry control (*p*=.0382 and *p*=.0346 respectively) groups.

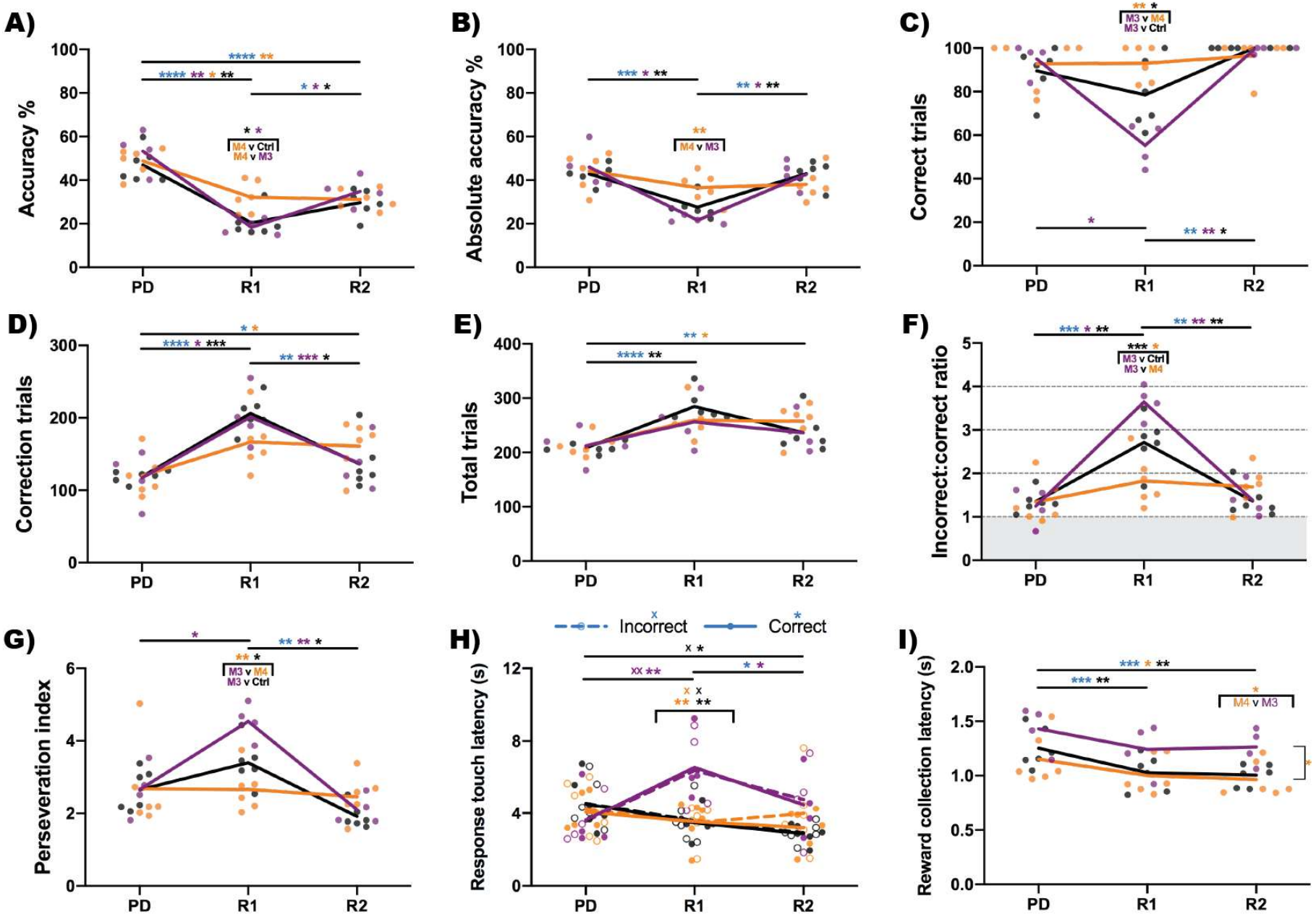

A)
Accuracy %
M4 v Ctrl
M4 v M3
B)
Absolute accuracy %
M4 v M3
C)
Correct trials
M3 v M4
M3 v Ctrl
D)
Correction trials
E)
Total trials
F)
Incorrect:correct ratio
M3 v Ctrl
M3 v M4
G)
Perseveration index
M3 v M4
M3 v Ctrl
H)
Incorrect
Correct
Response touch latency (s)
I)
Reward collection latency (s)
M4 v M3
PD
R1
R2

Figure 4.6. Performance in the first 100 accuracy trial block of each phase of the serial reversal learning task. Individual animals (dots) and group means (lines) for rats with either activating-hM3Dq-DREADDs (purple), inhibiting-hM4Di-DREADDs (orange), or only mCherry (black) in mPFC-AcbSh neuronal projections. **Only data from rats that achieved R1 progression criterion are included** (mCherry *n*=6, hM3Dq *n*=4, hM4Di *n*=6). **A-I)** Mixed-effect analysis followed by Tukey's post hoc multiple comparisons. **A)** Accuracy (% correct touches in accuracy trials). Phase $F(1.422, 18.49)=53.40$, $p<.0001$, (PD>R1 and PD>R2 $p<.0001$, R1<R2 $p=.0161$); Group $F(2, 13)=3.834$, $p=.0491$; Interaction $F(4, 26)=2.586$, $p=.0604$. Between-group: R1 hM4Di > mCherry $p=.0366$, hM4Di > hM3Dq $p=.0121$. Within-group: hM3Dq PD>R1 $p=.0096$, R1<R2 $p=.0346$; hM4Di PD>R1 $p=.0433$, PD>R2 $p=.0095$; mCherry PD>R1 $p=.0065$, R1<R2 $p=.0304$. **B)** Absolute accuracy (accuracy across all completed trials): Phase $F(1.775, 23.08)=30.32$, $p<.0001$ (PD>R1 $p=.0003$, R1<R2 $p=.0013$); Group $F(2, 13)=0.5777$, $p=.5750$; Interaction $F(4, 26)=4.284$, $p=.0085$. Between-group: R1 hM4Di > hM3Dq $p=.0055$. Within-group: hM3Dq PD>R1 $p=.0404$, R1<R2 $p=.0131$; mCherry PD>R1 $p=.0064$, R1<R2 $p=.0054$. **C)** Correct trials: Phase $F(1.701, 22.11)=20.78$, $p<.0001$ (R1<R2 $p=.0030$); Group $F(2, 13)=6.008$, $p=.0142$; Interaction $F(4, 26)=6.377$, $p=.0010$. Between-group: R1 hM3Dq < hM4Di $p=.0021$, hM3Dq < mCherry $p=.0486$. Within-group: hM3Dq PD>R1 $p=.0290$, R1<R2 $p=.0077$; mCherry R1<R2 $p=.0450$. **D)** Number of correction trials. Phase $F(1.956, 25.43)=30.43$, $p<.0001$, (PD<R1 $p<.0001$, PD<R2 $p=.0045$, R1>R2 $p=.0167$); Group $F(2, 13)=0.05693$, $p=.9449$; Interaction $F(4, 26)=2.683$, $p=.0537$. Within-group: hM3Dq PD<R1 $p=.0339$, R1>R2 $p=.0004$; hM4Di PD<R2 $p=.0465$; mCherry PD<R1 $p=.0009$, R1>R2 $p=.0211$. **E)** Total trials: Phase $F(1.849, 24.04)=18.94$, $p<.0001$; Group $F(2, 13)=0.1901$, $p=.8291$; Interaction $F(4, 26)=1.456$, $p=.2440$. Within-group: hM4Di PD<R2 $p=.0121$; mCherry PD<R1 $p=.0079$. **F)** Ratio of incorrect to correct touches. Phase $F(1.771, 23.02)=49.66$, $p<.0001$, (PD<R1 $p=.0004$, R1>R2 $p=.0015$); Group $F(2, 13)=3.117$, $p=.0784$; Interaction $F(4, 26)=9.196$, $p<.0001$. Between-group: R1 hM4Di < hM3Dq $p=.0008$; hM3Dq > mCherry $p=.0382$. Within-group: hM3Dq PD<R1 $p=.0108$, R1>R2 $p=.0032$; mCherry PD<R1 $p=.0089$, R1>R2 $p=.0086$. **G)** Perseveration index of the average number of correction trials per incorrect response to an accuracy trial. Phase $F(1.714, 22.29)=19.73$, $p<.0001$ (R1>R2 $p=.0013$); Group $F(2, 13)=1.715$, $p=.2183$; Interaction $F(4, 26)=4.828$, $p=.0048$. Between-group: R1 hM4Di < hM3Dq $p=.0025$, hM3Dq > mCherry $p=.0346$. Within-group: hM3Dq PD<R1 $p=.0254$, R1>R2 $p=.0054$; mCherry R1>R2 $p=.0106$. **H)** Latency to touch an image following presentation. Correct image (solid circles and lines, asterisks are significance markers); sphericity assumed, Geisser-Greenhouse's epsilon =.8305; Phase $F(2, 26)=3.641$, $p=.0404$ (R1>R2 $p=.0317$); Group $F(2, 13)=2.835$, $p=.0951$; Interaction $F(4, 26)=5.161$, $p=.0034$. Between-group: R1 hM3Dq > hM4Di $p=.0018$, hM3Dq > mCherry $p=.0014$. Within-group: hM3Dq PD<R1 $p=.0013$, R1>R2 $p=.0235$; mCherry PD>R2 $p=.0248$. Incorrect image (unfilled circles and dashed line, crosses are significance markers); Sphericity assumed, Geisser-Greenhouse's epsilon =.9858; Phase $F(2, 26)=1.068$, $p=.3583$; Group $F(2, 13)=1.289$, $p=.3085$; Interaction $F(4, 26)=4.770$, $p=.0051$. Between-group: R1 hM3Dq > hM4Di $p=.0148$, hM3Dq > mCherry $p=.0193$. Within-group: hM3Dq PD<R1 $p=.0040$; mCherry PD>R2 $p=.0443$. **I)** Latency between touching the correct image and collecting sucrose pellet; sphericity assumed, Geisser-Greenhouse's epsilon =.9977; Phase $F(2, 26)=14.19$, $p<.0001$ (PD>R1 $p=.0004$, PD>R2 $p=.0002$); Group $F(2, 13)=4.034$, $p=.0434$ (hM3Dq > hM4Di $p=.0404$); Interaction $F(4, 26)=0.2722$, $p=.8932$. Between-group: R2 hM4Di < hM3Dq $p=.0365$. Within-group: hM4Di PD>R2 $p=.0270$; mCherry PD>R1 $p=.0072$, PD>R2 $p=.0030$. Black horizontal brackets indicate the reported significant between group comparisons within the enclosed phase. Black vertical brackets show significant main effect group differences. Black horizontal lines above the data indicate between phase comparisons of the phases at each end of the line. Between phase comparisons: blue* = collapsed across group (i.e. all animals); purple* hM3Dq; orange* = hM4Di; black* mCherry. *$p<.05$, **$p<.01$, ***$p<.001$, ****$p<.0001$.

Across the entire cohort performance improved from R1 to R2 (blue asterisks **Figure 4.6**; increased accuracy p=.0161 **A,** and absolute accuracy p=.0013 **B**; more correct trials p=.0030 **C**; fewer incorrect trials p=.0045 **D**; decreased incorrect:correct ratio p=.0015 **F**, and perseveration index p=.0013 **G**), such that absolute accuracy, correct trials, incorrect:correct ratio and perseveration index in R2 returned to similar values as in PD (all ps≥.0746), however R2 accuracy remained lower than in PD (p<.0001) with rats completing more incorrect (p=.0167) and total (p=.0012 **E**) trials in R2 than in PD. This pattern of improvement was mirrored by the hM3Dq and mCherry groups (purple and black asterisks respectively **Figure 4.6**), but not by the hM4Di group (all ps≥.7343), in line with the decline in performance from PD to R1 occurring only in the hM3Dq and mCherry groups. Consequently, all measures were similar between PD and R2 for both the hM3Dq (all ps≥.1245) and mCherry groups (all ps≥.0611), whereas hM4Di (orange asterisks **Figure 4.6**) had significantly lower accuracy (p=.0095 **A**) and more incorrect (p=.0465 **D**) and total (p=.0121 **E**) trials in R2 than PD.

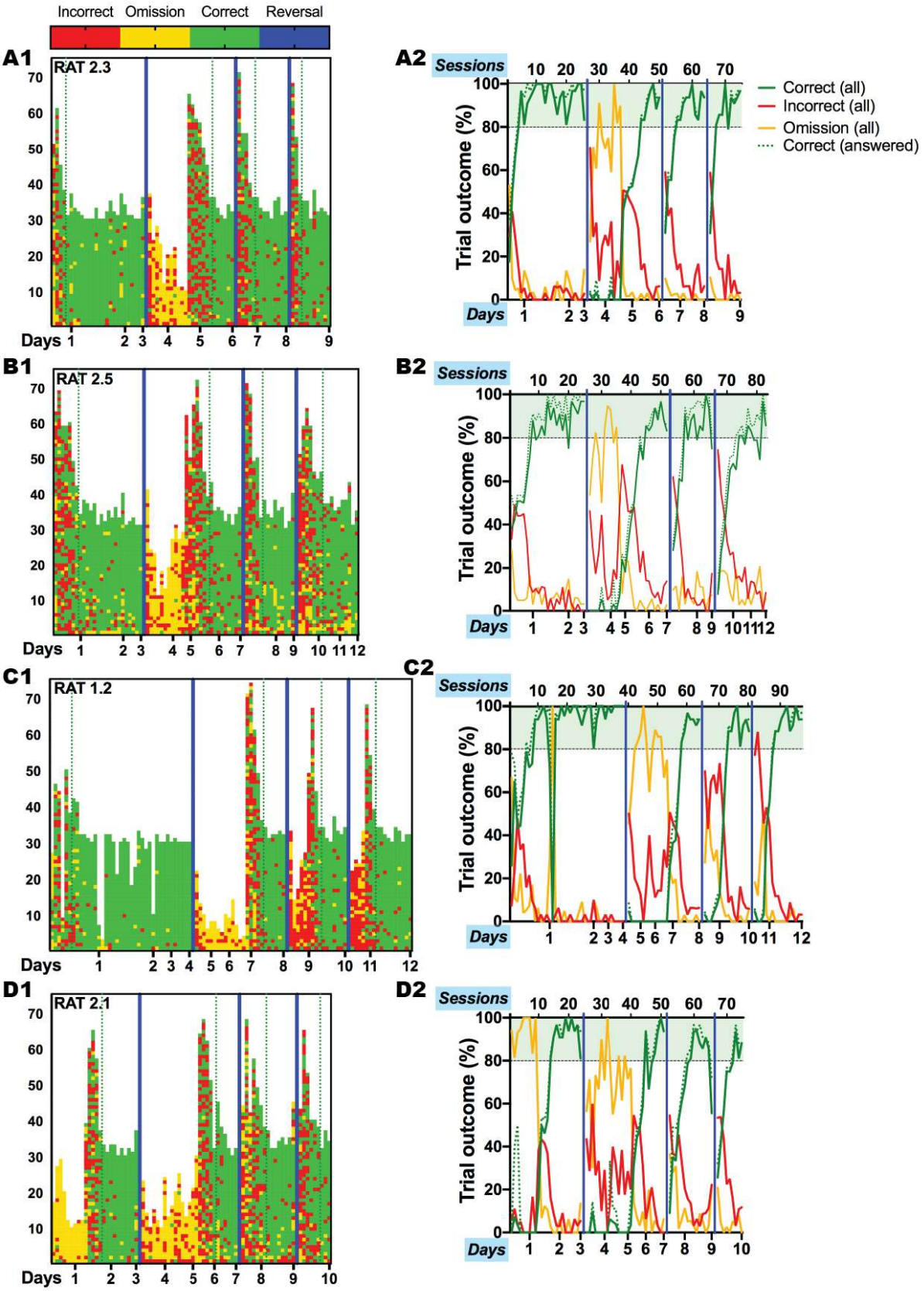
Response latencies to both the correct (purple asterisks p=.0013 **Figure 4.6 H**) and incorrect (purple crosses p=.0040) image in R1 were prolonged following activation of mPFC-AcbSh projecting neurons, which was normalized when DREADD stimulation ceased in R2 (PD vs R2 both ps≥2842). Circuit inhibition did not affect the time taken to touch either the correct or incorrect image (**Figure 4.6 H**), while the latency for the mCherry group to touch the correct (black asterisks p=.0248) and incorrect (black crosses p=.0443) images was significantly shorter in R2 compared to PD. Circuit activation with hM3Dq therefore produced longer response latencies for both correct and incorrect touches than both circuit inhibited hM4Di (p=.0018 and p=.0148 respectively) and control mCherry rats (p=.0014 and p=.0193 respectively).

All rats showed a decrease in the latency to collect rewards across phases of learning (p<.0001), which was evident in the transition from PD to both R1 (p=.0004) and R2 (p=.0002; blue asterisks **Figure 4.6 I**). This was consistent for control animals (PD to R1; p=.0072) (PD to R2; p=.0030, black asterisks **Figure 4.6 I**), however the hM4Di group only showed decreased latency from PD to R2 (orange asterisks, p=.0270). Overall, reward collection latency was different between groups (p=.0434), being longer for the hM3Dq group than the hM4Di group (p=.0404). However, this was not a result of circuit modulation, as there was no significant interaction between circuit modulation and experimental phase (p=.8932) and the only

significant within phase difference between these groups was in R2 (*p*=.0365 **Figure 4.6 l**) when there was no circuit modulation.

4.3.4 Experiment 2: Home-cage PhenoSys paradigm unveils variability in individual learning styles

These data represent the first optimization of the PhenoSys platform for examining cognitive flexibility in rats. As such they are not without flaws but are nevertheless instructive and illuminating and highlight the capacity of the PhenoSys once fully optimized to revolutionize cognitive behavioural testing. One major benefit of the novel home-cage and touchscreen testing system is the ability to tailor test parameters to individual animals as they progress through the serial visual learning task. This enables a deep examination of variability in learning styles between animals and allows clustering of rats into groups based on similarities and differences in the way that they learn this task. Demonstrated below in the style of a heat map is trial by trial data, where every square (pixel) represents the outcome of a single trial (green = correct; red = incorrect; yellow = omission) and every column is one session in chronological order from left to right (**Figure 4.7 1**). The line graphs show the percentage of each trial outcome per session, with the addition of the percent correct of only trials responded to (i.e. omissions are ignored; dotted green line) which is used to determine progression criterion (**Figure 4.7 2**). The session rats reached criterion is indicated on the heat maps (**Figure 4.7 1**) by the dotted dark green line which is immediately to the right of the first session they completed 30 correct (the maximum possible) trials with an accuracy of at least 80%, which is when the dotted dark green line in the line graphs (**Figure 4.7 2**) first reaches or exceeds the upper light green shaded area.


Incorrect
Omission
Correct
Reversal
A1
RAT 2.3
Days
A2
Sessions
Trial outcome (%)
Days
Correct (all)
Incorrect (all)
Omission (all)
Correct (answered)
B1
RAT 2.5
B2
C1
RAT 1.2
C2
D1
RAT 2.1
D2

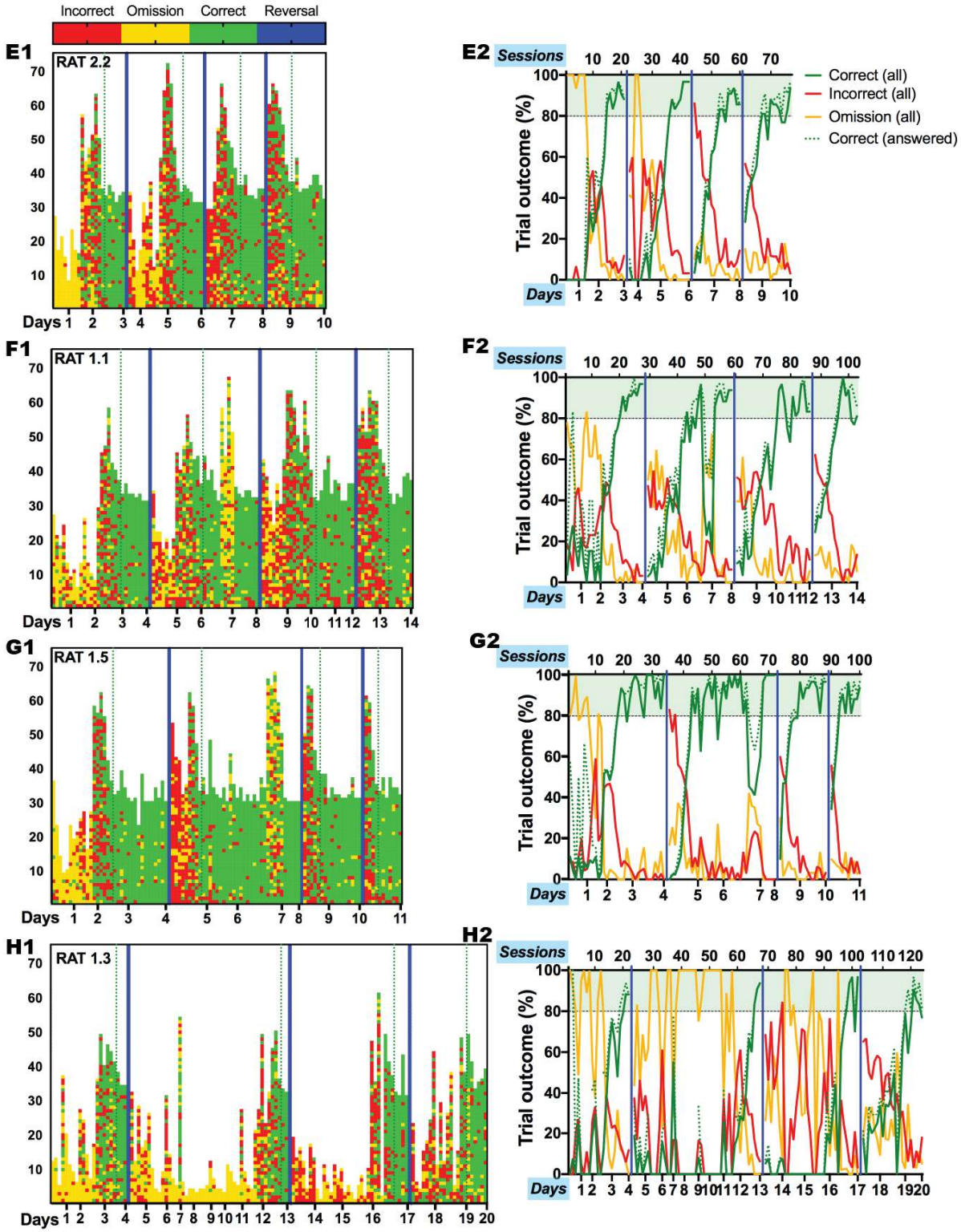


Figure 4.7 Chronological touchscreen reversal learning performance for individual rats in the PhenoSys. **Left)** Trial by trial data with every square representing a trial outcome (green = correct, red = incorrect, yellow = omission) and every column representing a single session in chronological order from left to right. Blue columns represent the point of reversal of reward contingencies. **Right)** Corresponding session outcome showing proportion of all trials that were correct (solid green line), incorrect (red line) or omitted (yellow line) and proportion of trials answered that were correct (dotted green line).

A summary of performance in each session (each column) was determined by calculating the mean outcome of all *trials* in a session (correct = +1, omission = 0, incorrect = -1; **Figure 4.8 A**) and the mean outcome of trial *responses* in a session (i.e. percentage correct; correct = +1, incorrect = -1; **Figure 4.8 B**) illustrated again with heat maps that are aligned by reversals to aid comparison across each phase. The start time of each session is also shown (**Figure 4.8 C**) but was found to be inconsequential as there was no relationship between session time and response accuracy (r=.05162, p=.1766). While these summary data are reflective of performance and informative, they necessarily lack the detail and nuances of individual behaviour that is readily apparent in the raw heat maps. Given that omissions are a key component of the different response patterns (discussed in detail below), inspecting the raw data is critical because omissions fall intermediately between correct and incorrect and cannot be differentiated from 50% (chance) accuracy. Additionally, comparing session and response means only provides insight into the contribution of omissions if responses are predominantly correct or incorrect. Furthermore, the number of trials per session, which is a key variable, is not coded for in these summary data.

4.3.5 Experiment 2: Comparison of learning rates between individuals

The number of days, sessions, and trials (including outcome) required to reach criterion in each phase was compared to statistically analyze performance over time (**Figure 4.8 D-H**). The broad behavioural patterns that emerge are described for each phase of learning.

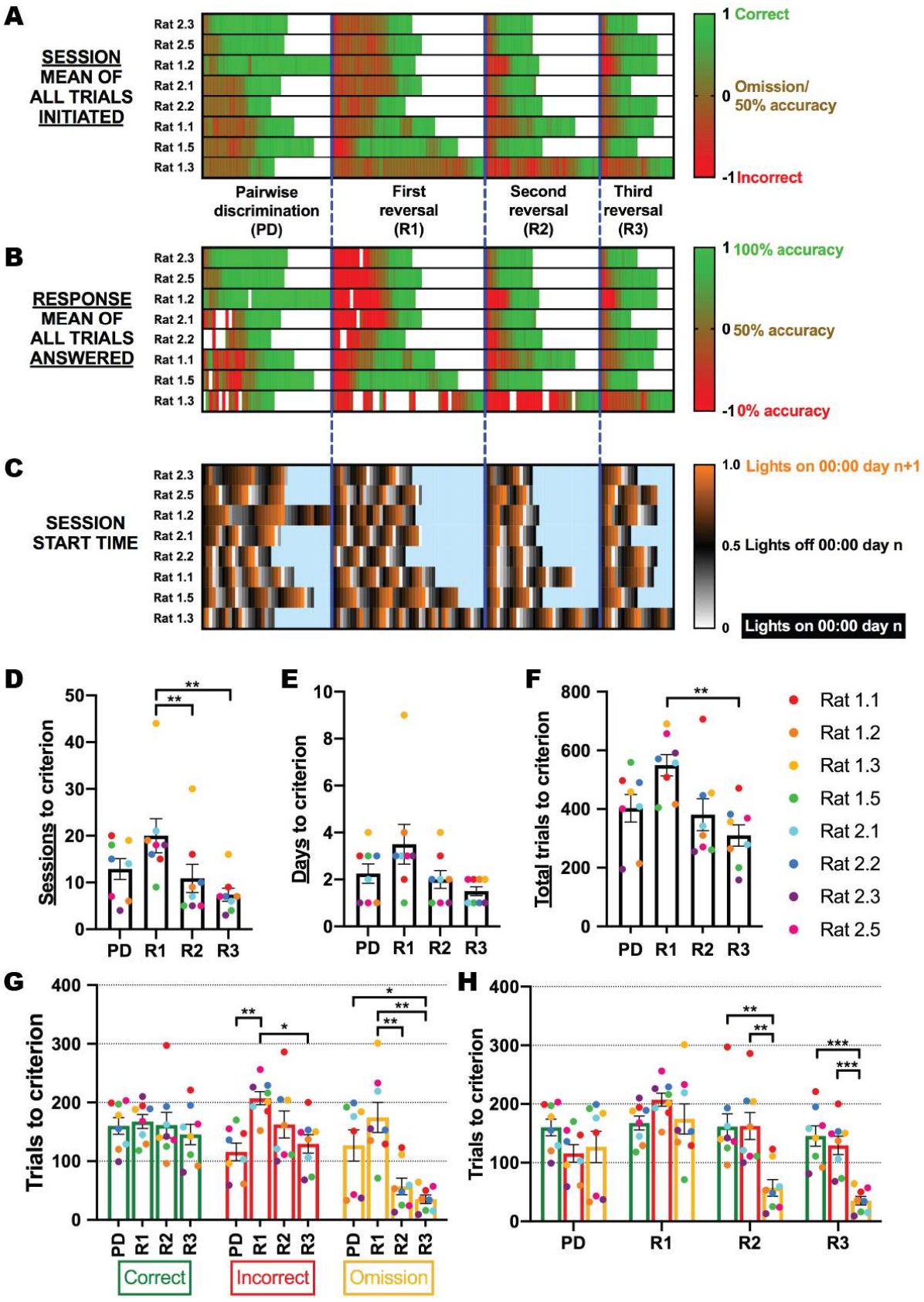


Figure 4.8 PhenoSys touchscreen reversal learning performance summary data. Average outcome of all trials initiated **(A)** and all trials responded to **(B)** in each session, producing session and response means respectively. **C)** Start time of each session. **D-F)** RM One-way ANOVA followed by Tukey's post-hoc multiple comparisons. **D)** Number of sessions to criterion. $F(1.465, 10.25)=8.694$, $p=.0092$; R1>R2 $p=.0099$, R1>R3 $p=.0070$. **E)** Number of

days to criterion. $F(1.620, 11.34)=4.249$, $p=.0484$. **F)** Number of total trials to criterion. $F(2.207, 15.45)=7.994$, $p=.0034$; R1>R3 $p=.0035$. Outcome of trials to criterion grouped by outcome **(G)** and by phase **(H)**. RM Two-way ANOVA followed by Tukey's post-hoc multiple comparisons: Phase $F(2.351, 49.37)=14.35$, $p<.0001$; Outcome $F(2, 21)=7.666$, $p=.0032$; Interaction $F(6, 63)=5.277$, $p=.0002$. **G)** R2 correct > omissions $p=.0045$, incorrect > omissions $p=.0059$; R3 correct > omissions $p=.0005$, incorrect > omissions $p=.0008$. **H)** Incorrect R1>PD $p=.0014$, R1>R3 $p=.0309$; Omissions PD>R3 $p=.0484$, R1>R2 $p=.0092$, R1>R3 $p=.0018$. A-C Data show individual animals and sessions. D-H) Data show individual animals (circles) and cohort mean (bars) ± SEM. *$p<.01$, **$p<.01$, ***$p<.001$.

4.3.6 Experiment 2: Two behavioural patterns of initial pairwise discrimination learning

Within the first day of PD the rats clustered into two behavioural patterns of responding, those that were engaged and motivated to learn the task and those that repeatedly entered the touchscreen but did not engage with the task.

4.3.6.1 Engaged and motivated to learn the task

Three rats (2.3, 2.5, and 1.2; **Figure 4.7 A-C**) immediately engaged with and acquired the PD task requirements, completing the maximum number of trials (30 correct) in multiple sessions and steadily improving accuracy such that they each reached the 80% criterion *on the first day within 7 sessions*. These rats fill the top three rows in the summary data and their performance very quickly transitions from brown (reflective of ~50% accuracy rather than omissions) to bright green (reflecting predominantly correct responses; **Figure 4.8 A** and **B**).

4.3.6.2 Enter the touchscreen chamber but do not engage in the task

Conversely, while the 5 remaining rats voluntarily entered the touchscreen chamber on numerous occasions they failed to engage with the task, largely omitting (yellow in individual heat maps) rather than responding to the limited trials they initiated (**Figure 4.7 D-H**). This translates to predominantly brown columns (**Figure 4.8 A**) and the interspersed white columns highlight multiple sessions in which no responses were made (**Figure 4.8 B**). Omissions may result from a desire to explore the touchscreen chamber even at times when an animal lacks the motivation necessary to engage with the PD task. This 'nonchalant' behaviour resolved promptly and all rats reached progression criterion within 20 sessions (by the end of Day 4) and is demonstrated by the delayed transition from red/brown to green in both the session and response columns (bottom 5 rows **Figure 4.8 A** and **B**). There was no significant difference between the number of correct (green bars), incorrect (red bars) or omitted (yellow bars) trials to criterion with the PD phase (all *ps*≥.1194, **Figure 4.8 H**).

4.3.7 Experiment 2: Adapting to reward contingency reversals becomes faster over time

4.3.7.1 First reversal

There are three general patterns of behavior following the first reversal of reward contingencies. The first is 'give up' then reset, the second is mild perseverative responding that progresses to a "gradual switch" and suggests progressively improved recognition of feedback, while the third is strong perseveration that transitions to a "sudden switch" seemingly akin to a rapid insight or "a-ha" moment. Interestingly, there was no significant difference between the number of correct, incorrect or omitted trials to R1 criterion (all *ps*≥.0706, **Figure 4.8 H**), however the number of incorrect trials to criterion increased from PD to R1 (*p*=.0014, **Figure 4.8 G**) in line with the predominating perseverative response patterns.

Pattern 1, the tendency to initially 'give up' responding when the learned response is suddenly unrewarded followed by a delayed 'reset', is characterized by predominantly omitting trials indicating an unwillingness to engage in the task and is clearly demonstrated in R1 by rats 2.3, 2.5, 2.1 and 1.2 (**Figure 4.7 A-D**). For these individuals, trial initiation and responding decreases over time interspersed with an increasing proportion of trial omissions. These rats spontaneously 'reset' in a day or two and return to initiating and responding to the maximum allowed trials to complete sessions. Importantly, responding occurs *without* a perseverative bias towards the previously rewarded image, with behaviour reminiscent of "trial and error learning" and response accuracy approximating chance before quickly increasing to reach 80% accuracy criterion. Represented as mean session values (**Figure 4.8 A** and **B**), this pattern shows a broad brown band in R1 (predominantly due to omissions) inferred from the corresponding bright red columns (scarce incorrect responses) followed by a much narrower brown band reflecting chance accuracy as correct responses are incorporated and then become predominant with response means subsequently transitioning to bright green (top four rows **Figure 4.8 A** and **B**).

Pattern 2, characterized by mild perseverative responding before a "gradual switch" to the reversed contingency, is demonstrated by rats 2.2 and 1.1 in R1 (**Figure 4.7 E** and **F**). These rats consistently interweave perseverative incorrect responses with trial omissions over the first day or two before touches to the previously unrewarded (now correct and rewarded) image

steadily replace trial omissions. Learning progresses in trial and error fashion such that accuracy rebounds to chance before increasing to reach criterion. The response means have similar lengths of red and brown sections with a gradual transition from bright red through brown whereas the entire length of corresponding sessions means are predominantly brown due to trial omissions early after reversal (rats 2.2 and 1.1 in **Figure 4.8 A** and **B**). Session and response means *gradually* transition from brown to green as accuracy improves from chance to reach criterion.

This starkly contrasts with response Pattern 3, characterized by strong perseverative responding followed by an 'ah ha!' moment prompting a "sudden switch" to touching the previously unrewarded image. Here, accuracy quickly reaches criterion for the reversed contingencies, demonstrated most notably by rat 1.5 in R1 (**Figure 4.7 G**). Many trials are initiated with persistent perseverative responding to the previously rewarded image (i.e. incorrect touches) despite it no longer producing reward with only intermittent omitted trials, which is evident as bright red columns for responses and only slightly more brown columns for corresponding sessions (rat 1.5 **Figure 4.7 A** and **B**). Then, instead of reverting to "trial and error"-style responding as demonstrated by rats that follow the other behavioural response patterns, Pattern 3 is typified by a *rapid* switch to touching the new rewarded image with a simultaneous cessation of perseverative responses. This results in sharply increasing accuracy without an enduring intermediate stretch of sessions at chance performance. This sudden switch is illustrated by a fast transition from bright red through to bright green with only a couple of intermittent brown sessions (rat 1.5 in **Figure 4.8 A** and **B**).

4.3.7.2 Second and third reversals

The three response patterns described above were largely maintained following subsequent reversals besides a substantial reduction of omitted trials to criterion from R1 to both R2 (p=.0092) and R3 (p=.0018), and from PD to R3 (p=.0484; **Figure 4.8 G**). This eliminated the 'give up' component of Pattern 1, excepting the outlier case of rat 1.3, leaving a simple reset approach that became the predominant response pattern for all rats.

Reduced trial omissions also altered the perseverative response patterns as touches to the newly correct image were incorporated sooner in lieu of omitting trials. Consequently, the

switch to newly correct responses occurred earlier than in R1. Pattern 2, mild perseveration to gradual switch, demonstrated by rat 1.1 in R2 (**Figure 4.7 F**), exhibited a switch to correct responding that was relatively faster than in R1 but still slower than the R2+3 “sudden switch” pattern. Pattern 3, strong perseveration to sudden switch, was still exhibited following serial reversals, demonstrated by rat 2.2 in R2 (**Figure 4.7 E**) and rat 1.2 in R2 and R3 (**Figure 4.7 C**), and despite fewer overall responses than in R1 the proportion of incorrect responses remained high. Pattern 2 is characterized by the elongated brown portion in the red to green transition in response means (rat 1.1 R2 **Figure 4.8 B**), whereas Pattern 3 is characterized by a rapid transition from red to green (rat 2.2 R2, rat 1.2 R2 and R3 **Figure 4.8 B**), with the relatively similar colour of corresponding session means highlighting the diminished contribution of trial omissions (**Figure 4.8 A**). This decrease in perseveration over subsequent reversals is corroborated by a significant decrease in incorrect trials to criterion from R1 to R3 (p=.0309; **Figure 4.8 G**). Conversely, the number of correct trials to criterion did not change across phases (all $ps \geq .5880$ **Figure 4.8 G**) indicating that learning was not dependent upon the number of rewards received (i.e. positive feedback).

The importance of trial omissions to the behavioural patterns and how these shifted across serial reversals cannot be understated. The most consistent finding across all rats was the decrease in trial omissions following the second and third reversals indicative of memory of and learning from experience of the first reversal. Comparison of trial outcome to criterion within each phase showed there was no significant differences between correct and incorrect trials to criterion in any phase (all $ps \geq .0706$), but trial omissions before criterion were significantly lower than both correct (R2 p=.0045; R3 p=.0005) and incorrect (R2 p=.0059; R3 p=.0008) trials in both R2 and R3 (**Figure 4.8 H**). Together with the substantial decrease in perseverative responding, these data reveal that rats improve their adaptive capacity to subsequent reversals and most adopt the ‘reset’ approach from the second reversal onwards. These improvements are reflected by the quantitative and qualitative fluctuations in performance to reach criterion across the different phases. Firstly, the total number of sessions (p=.0092; **Figure 4.8 D**), days (p=.0484; **E**), and total trials (p=.0034; **F**) required to reach criterion significantly varied across phases, being greatest in all cases for R1. Sessions (**Figure 4.8 D**) and total trials (**E**) to criterion significantly decreased from R1 to both R2 (p=.0099) and R3 (p=.0070), and from R1 to R3 (p=.0035) respectively, with no significant differences between any phases for days to criterion (all $ps \geq .1322$, **F**).

4.3.8 Experiment 2: Optimization of testing protocol for future experiments

The future utilization of the PhenoSys platform would benefit from a number of methodological changes in order to further optimize functioning. For example, the timing of progression/reversals, the number of sessions per day, incorporation of a trial initiation signal, and identification and removal of outliers during testing could be modified to better accommodate individual differences in learning. Considering that performance was always consistent once rats had reached criterion, testing time in future experiments would be further reduced by reversing reward contingencies at the first available opportunity after a rat has "learned", unless consistency in the duration of particular phases of learning between rats is required (i.e. in tests utilizing a time-sensitive pharmacological intervention). In these cases, rats should be rested and/or have limits imposed on their ability to engage in test sessions so they are not "overtrained".

In the present study, the number of sessions per day was left to the animal's discretion, therefore these were not consistent between or within rats over time. With respect to how number of sessions completed per day influenced learning, it was evident that the fastest learners completed the most sessions per day and the slowest learners completed the fewest. Therefore, future experiments could employ a balancing strategy that individually catered the number of sessions per day to each rat based on their performance. This could be coupled with a minimum and/or maximum number of sessions criteria to generate a data set of consistent length within each phase to allow for longitudinal analysis of learning curves. There are some notable outliers in the data obtained from rats 1.1 and 1.5 during the sixth day of testing which was caused by a jammed pellet dispenser. Because the automated system does not include a trial initiation 'click' sound, incorporated into conventional touchscreens, rats were repeatedly initiating trials expecting pellet delivery and had no auditory feedback from the 'click' to suggest that a new trial had begun. A modification to include this click for trial initiation will be an important consideration for future experiments. Future experiments that require a consistent length data set should also strive to identify outliers early and employ cut offs to eliminate such animals (e.g. rat 1.3) so that they do not retard group progression and promote overtraining in rats that have already reached criterion.

4.4 Discussion

Cognitive inflexibility is a hallmark feature of AN, with patients displaying deeply ingrained patterns of thought and behavior that are highly resistant to change, contributing to the high morbidity and chronicity of the illness (30, 31, 37, 39, 68). The control of cognitive flexibility is largely regulated by neural activity within the PFC, and the transfer of this information via projection pathways between the PFC and other cortical, subcortical and midbrain nuclei (12, 17). While impairments in cognitive flexibility are consistently shown in patients with AN, both while acutely ill and following body weight recovery, it is difficult if not impossible to determine the causal role of this phenotype in the development of AN in human studies. Moreover, the role of PFC dysfunction in cognitive inflexibility can only be assessed in patients with AN *after* illness onset and is therefore likely to be influenced by acute or enduring effects of extreme weight loss. The only feasible way to interrogate the causal relationship between PFC activity and cognitive flexibility in conditions of pathological weight loss is by using animal models. In the previous Chapter, it was shown that suppressing activity in the mPFC-AcbSh projection pathway prevents the development of the ABA phenotype in rats. The experiments detailed here demonstrate that this same pathway-specific inhibition *also* improves cognitive flexibility in a touchscreen deterministic reversal learning task, identifying, for the first time, a neurobiological link between cognitive flexibility and pathological weight loss. Moreover, the optimization of a rat-directed touchscreen testing apparatus for group-housed rats known as the PhenoSys is described (in Experiment 2) and highlights two key benefits of automated testing over traditional touchscreen testing methods. Remarkably, the proportion of animals that were able to acquire the pairwise discrimination task rose from 58% to 100% using the automated system and the rate of learning was ~5 times faster than using conventional touchscreens (with ~10 times higher throughput). While the full spectrum of possibilities arising from the use of the PhenoSys are still being realized, these two benefits alone substantially improve the reliability of touchscreen cognitive testing in rats. The increased throughput, requirement for fewer animals and reduced labor time for experimenters represents a major shift in the way these experiments are conducted and analyzed.

The opposing effects of chemogenetic inhibition or excitation of the mPFC-AcbSh pathway on flexible adaptation to the reversal of reward contingencies, described here, is in keeping with the effects of suppression and stimulation of this pathway on the development of the ABA phenotype. Taken together, the data from **Chapter 3** and in the first experiment of the present

Chapter illustrate that inhibition of the mPFC-AcbSh pathway during ABA exposure allowed animals to better adapt to the change in food availability in order to maintain body weight, whereas animals with this pathway stimulated fail to adapt to the new energy requirements that time-limited food access presents (a consequence of increased perseverative or compulsive wheel running). Performance within each reversal phase can be compartmentalized into the early *perseverative* phase and the late *learning* phase (47, 48, 69, 70), allowing the detection of specific impairments. Dissociating these two phases of learning reveals first, an ability, or lack thereof, to inhibit previously learned responses (i.e. perseveration) and second, an ability to learn a new positively conditioned stimulus-response relationship (47, 48, 69, 70). The effects of stimulation and suppression on reversal learning shown here were *specific* to the early perseverative phase, when cognitive flexibility is the process that is taxed, whereas mPFC-AcbSh circuit modulation did not result in overall differences in the ability to learn new stimulus-response relationships.

There are several challenges involved in better defining the role of the PFC in the determination of cognitive flexibility. These relate to the heterogeneity of outcomes depending on the exact region of the PFC being considered and the nature of the task in the test. For example, improvements in serial reversal learning after inactivation of components of the PFC, namely the PrL and IL, seem to be dependent on the modality of the task employed (visual, lever pressing, nose poke) as well as the reward-based outcomes (deterministic vs probabilistic) (4, 47, 48). While pharmacological inactivation or chemical ablation of PrL neurons is shown to improve reversal learning in both visual (48) and spatial (47) tasks, there is also evidence that PrL inactivation has no effect on these same behaviours (4, 69). Similarly, inactivation of IL has been found to either improve (48), have no effect on (47), or impair (4, 70), serial reversal learning, highlighting a potential issue with test standardization and neuronal modulation techniques. Considering the two mPFC subregions together has not clarified their role in cognitive flexibility, with lesions of both PrL and IL having no effect on probabilistic (45, 50) or deterministic (71) reversal learning, but rather a selective deficit in learning the ASST extradimensional shift (from one cue modality to another) (45). A crucial advantage of the neuronal modulation strategy employed in the present studies is the ability to specifically target neurons in PrL/IL with direct projections to the AcbSh, a hub for reward processing, in contrast to global inactivation of these prefrontal regions. Further, the inconsistency between the reports outlined above may largely be due to a wide variability in methodology employed in these studies, specifically deterministic versus probabilistic reversal learning, spatial versus visual

stimuli, and what constitutes criterion to trigger a reversal of reward contingencies. These discrepancies highlight a growing concern in behavioural neuroscience, which relates to the lack of reproducibility between and within laboratories (72). A desire to overcome this directed the next study in this Chapter, in which the same serial visual reversal task was conducted in a fully automated touchscreen and housing apparatus, the PhenoSys, eliminating the need for experimenter intervention and increasing experimental throughput.

The ability of animal models to provide mechanistic insight into the neurobiology underlying cognitive flexibility depends on the accuracy with which tasks used to test it reflect those used in human cognitive testing (58, 59). While there are clear and substantiated differences in brain structure and morphology between species, particularly in the PFC (14), there are nevertheless many neurobiological, cognitive and behavioural domains that are evolutionarily conserved (12, 17, 58). The best way to facilitate direct comparison between rodents, non-human primates and humans is to utilize standardized tests delivered as similarly as possible across species (44, 58, 59). Touchscreen cognitive testing allows for the delivery of standardized test batteries (43, 44, 59) that can be either (demonstrably) identical across species, or “conceptually identical” with task complexity adjusted for species level differences in capability (59). Crucially, this also allows for replication and standardization of tests between and within laboratories (58, 59). Even though there have been questions raised as to the ethological validity of the use of touchscreens with rodents over traditional measures that measure less anthropomorphic behaviours, such as digging behaviour in the ASST, touchscreens exploit the inherent exploratory nature of rodents as well as their Pavlovian approach behaviour to rewarding stimuli (43, 58). There is a necessary compromise between the increased speed of reversal learning acquisition via behaviours that are ethologically relevant and the slower acquisition but greater cross-species translational capacity of using touchscreens (25). The preliminary findings described here suggest that automation of touchscreen testing not only overcomes this limitation but accelerates the rate of learning such that throughput far exceeded that of behavioural tests traditionally considered more appropriate for rodents.

There are a number of factors that should be addressed in the interpretation of data obtained through this animal-directed testing system. Incorporating trial omission as a possible outcome in the automated touchscreen testing parameters of the PhenoSys proved insightful in differentiating response patterns following reversal of reward contingencies. In combination with increased throughput, inclusion of trial omissions highlighted how previous exposure to

reversal/s was incorporated into decision-making following subsequent reversal/s to improve performance. As touchscreen trial responses, whether correct or incorrect, require the same motor effort to enact, variance in accuracy cannot be a result of discrepancies in motivation or motor deficits (17) and thus response accuracy reliably tracks learning. However, decreased motivation is a likely cause of trial omissions, which may be considered as a failure to overcome learned non-rewarded experiences (73), where animals 'give up' responding instead of attempting responding to the previously unrewarded stimulus, characteristic of response Pattern 1. In contrast, Patterns 2 and 3 were characterized by perseverative responding which reflects something akin to resistance to extinction training, where animals will continue performing a previously rewarded action even when the reward is no longer delivered (e.g. (74)). This type of perseverative responding differs from "learned non-reward" seen in Pattern 1, in which responding abruptly stops in addition to an unwillingness to initiate trials when reward contingencies are initially reversed. In the current study, "learned non-reward" appears to be very sensitive to prior reversal experience, at least when the stimuli remain constant, as it largely disappeared in subsequent reversals. With this overcome, learning over subsequent reversals appeared to be driven by increased sensitivity to and incorporation of negative feedback into decision-making, i.e. facilitated extinction, evidenced by the significant decrease in incorrect trials to criterion across phases and coincident decrease in perseverative responding, rather than a change related to positive feedback. This is likely to be the case as the number of correct trials (i.e. rewards) to criterion did not change across phases.

Another major consideration regarding Experiment 2 is whether the instrumental responding exhibited was goal-directed or habitual. Goal-directed behaviour is flexible, acquired rapidly and occurs where the action performed is in direct pursuit of the desired outcome; thus, goal-directed behaviour ceases when the desired outcome no longer follows the action (10, 75). In contrast, habitual behaviours are inflexible, automatic, and continue in response to a reinforced stimulus even when the outcome ceases or changes (10, 75-77). The behavior exhibited in Pattern 1 ('give up' and reset) following the first reversal suggests a goal-directed approach, considering that responding ceased when the action went unrewarded. Conversely, the perseverative behaviour that characterized Patterns 2 and 3 indicate that responding had become habitual because responding continued when it was no longer rewarded. Historically, it has been thought that goal-directed behaviour is under the control of PrL while habitual behaviour is controlled by IL (75), although recent studies suggest that these relationships are not quite so distinct (78). Further, these mPFC subregions have also been thought to have

dissociable roles in responses to positive and negative feedback, although recent evidence suggests they are not mutually exclusive (4). What is clear, however, is that the mPFC is responsible for controlling instrumental responding, whatever form it may take, and disruptions to mPFC functioning can produce maladaptive behaviours (79). Indeed, the results of Experiment 1 show a clear role for activity in the mPFC-AcbSh pathway on perseverative responding, which may be underpinned by a shift between goal-directed and habitual behaviour.

The transition of behavioural repertoires from goal-directed to habitual occurs with familiarity in order to relieve cognitive load (1, 80). This process is by and large of great benefit for the organism but becomes maladaptive if the environment or context changes to produce negative outcomes (1, 80). An inappropriate transition from goal-directed to habitual behaviour has been implicated in OCD (1, 81, 82), substance use disorders (10), and in the development and/or maintenance of AN (7, 77, 83-85). For patients with AN, dieting and exercise behaviour is initially goal-directed in pursuit of weight loss or fitness ideals but becomes habitual and is not remediated by body weight restoration (77, 83, 84, 86). Recently, evidence has emerged for two distinct subgroups of patients with AN that are either more goal-directed in their approach to food and feeding or more habit-driven (76), with habitual tendencies associated with reduced activity in the orbitofrontal cortex. It is plausible that susceptibility to pathological weight loss in the ABA rodent model is driven by the transition from goal-directed running to habitual running by the same underlying mechanisms as in the human condition. Indeed, the idea that rodent wheel running becomes compulsive in the absence of food is gaining traction in recent studies of ABA and, much like compulsive or habitual behavior in animal models of OCD and addiction, is insensitive to changes in stimulus-response relationships or, in the case of ABA, the metabolic and homeostatic demand for survival. The identification of a neural circuit in rats that controls both perseverative responding (as described here in Experiment 1) *and* susceptibility to pathological weight loss in ABA (as described in **Chapter 3**), indicates that activity in the mPFC-AcbSh pathway is one neurobiological mechanism through which ABA-associated habit formation may occur.

4.5 References

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4.6 Supplementary Material

Supplementary Table S4.1 Experimental 1 touchscreen protocol details

Stage	Image/s*	Response	Tone	Magazine light	45mg Sucrose pellet/s	House light	Magazine click	Next trial?	Trials completed count	Progression criterion
Habituation	N/A	N/A	N/A	N/A	~20 in magazine before session	Off	N/A	N/A	N/A	Consume all pellets in 60 minutes on two consecutive sessions
Initial Touch	One image, one blank window Image displayed until touched or for max of 30 seconds	Touch image	Yes –when image touched	Turns on with pellet delivery – turns off when reward collected	3, collection starts 20s ITI	Off	N/A	Normal – Automatic start after 20s ITI	+1	Complete 100 trials in 60 minutes once or max 3 sessions
		Image not touched after 30s	Yes –when image disappears after 30s		1, collection starts 20s ITI	Off	N/A		+1	
		Touch to blank window during trial	No effect							
Must Touch	One image, one blank window Image displayed until touched	Touch image	Yes –when image touched	Yes – turns off when reward collected	1, collection starts 20s ITI	Off	N/A	Normal – Automatic start after 20s ITI	+1	Complete 100 trials in 60 minutes once
		Touch to blank window during trial	No effect							

Must Initiate	One image, one blank window Image displayed until touched	Image touch	Yes –when image touched	Yes – turns off when reward collected	1, collection starts 20s ITI	Off	Yes – with trial initiation	Normal - Must be initiated with magazine nose poke after 20s ITI	+1	Complete 100 trials in 60 minutes once in first 3 sessions; Complete >90 trials in 60 minutes in 4th session
		Blank touch	No effect							
Punish Incorrect	One image, one blank window Image displayed until image touch or blank touch	Touch image	Yes –when image touched	Yes – turns off when reward collected	1, collection starts 15s ITI	Off	Yes – with trial initiation	Accuracy trial - Must be initiated after 15s ITI	+1	Complete 100 trials in 60 minutes, with accuracy ≥80%for accuracy trials in two sessions
		Blank touch	No	No	0	On for 5s time out; turns off after timeout and 15s ITI begins	Yes – with trial initiation following 5s time-out and 15s ITI	Correction trial - Must be initiated after 15s ITI	+0	
Pairwise discrimination + reversals	Fan/pinwheel in one window and marble array in other window pseudorandom side allocation**	Correct image touched	Yes –when image touched	Yes – turns off when reward collected	1, collection starts 15s ITI	Off	Yes – with trial initiation	Accuracy trial - Must be initiated after 15s ITI	+1	**See Table 4.1 in Results**
		Incorrect image touched	No	No	0	On – 5s time out; turns off after timeout and 10s ITI begins	Yes – with trial initiation following 5s time-out and 10s ITI	Correction trial - Must be initiated after 10s ITI	+0	

* Images used in pre-training were randomly selected each trial from 40 different black and white images (the marble and fan/pinwheel images used in the main task were not displayed in pre-training).
** Image will not be displayed on the same side more than 3 consecutive trials (excluding correction trials, where image+side is repeated until the correct response is made)

NOTE: an incorrect response does not add to the count of completed trials. **NOTE**: ONLY the response to the first image presentation within each trial (accuracy trial, AT) contributes towards the accuracy calculation, i.e. correct responses to correction trials are rewarded to facilitate learning but do not contribute towards accuracy. **NOTE**: Initially rats are very slow to complete the required number of trials in punish incorrect and all three phases of the reversal learning task as they require a lot of correction trials. Therefore, pairs of 50 trial sessions are given until rats are quick enough to complete the required 100 trials in 60 minutes. Additionally, to facilitate the completion of the required number of trials we shortened the inter-trial interval (ITI) from 20s to 15s following a correct response and following an incorrect response to 15s in punish incorrect and 10s in the reversal learning task.

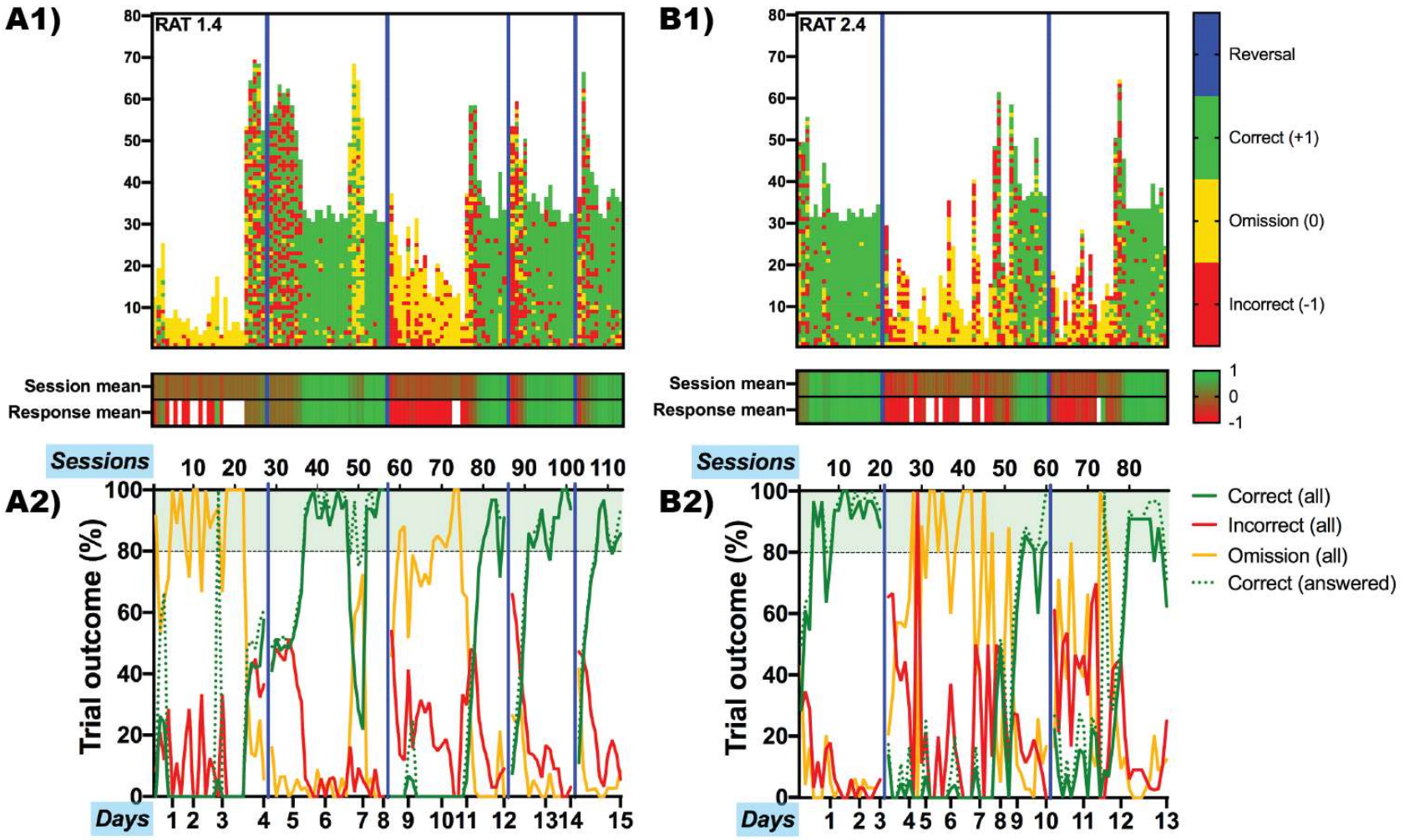
Supplementary Table S4.2 Experiment 2 touchscreen protocol details

Stage	Image/s*	Response	Tone	Magazine light	20mg Sucrose pellet/s	House light (screen)	Next trial?	Trials completed count	Progression criterion
Habituation	N/A	N/A	N/A	N/A	~20 per animal spread throughout PhenoSys	Off	N/A	N/A	Consume all pellets in 60 minutes on two consecutive sessions
Initial Touch	One white rectangle image, one blank window Image displayed until touched or for max of 20 seconds	Touch image	Correct tone when image touched	Turns on with pellet delivery – turns off when reward collected	2, collection starts 20s ITI	Off	Normal – Automatic start after 20s ITI	+1	Complete at least 2 sessions
		Image not touched after 30s	Correct tone when image disappears after 20s		1, collection starts 20s ITI	Off		+1	
		Touch to blank window during trial	No effect						
Must Touch	One white rectangle image, one blank window Image displayed until touched	Touch image	Correct tone when image touched	Yes – turns off when reward collected	1, collection starts 10s ITI	Off	Normal – Automatic start after 10s ITI	+1	Complete 30 trials in 30 minutes twice
		Touch to blank window during trial	No effect						
Must Initiate	One white rectangle image, one blank window	Image touch	Correct tone when image touched	Yes – turns off when reward collected	1, collection starts 10s ITI	Off	Normal - Must be initiated with magazine nose poke after 10s ITI	+1	Complete 30 trials in 30 minutes twice

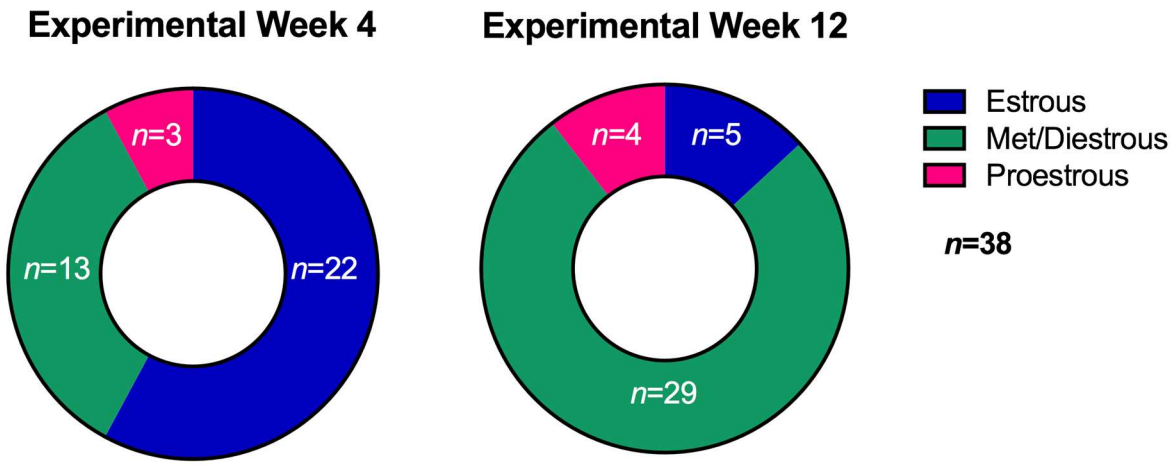
	Image displayed until touched	Blank touch	No effect						
Punish Incorrect	One white rectangle image, one blank window Image displayed until image touch or blank touch	Touch image	Correct tone when image touched	Yes – turns off when reward collected	1, collection starts 10s ITI	Off	Accuracy trial - Must be initiated after 10s ITI	+1	Complete 30 trials in 30 minutes, with accuracy ≥80%for accuracy trials in two sessions
		Blank touch	Incorrect tone when blank window touched	No	0	On for 5s time out; turns off after timeout and 20s ITI begins	Correction trial - Must be initiated after 20s ITI	+0	
Pairwise discrimination + reversals	Fan/pinwheel in one window and marble array in other window for 10s pseudorandom side allocation	Correct image touched	Correct tone when image touched	Yes – turns off when reward collected	1, collection starts 10s ITI	Off	Normal trial - Must be initiated after 10s ITI	+1	Complete 30 trials in 30 minutes with ≥80% accuracy
		Incorrect image touched	Incorrect tone when image touched	No	0	On – 10s time out; turns off after timeout and 10s ITI begins	Normal trial - Must be initiated after 10s ITI	+0	
		No image touched after 10s	Incorrect tone when images removed after 10s	No	0	On – 10s time out; turns off after timeout and 10s ITI begins	Normal trial - Must be initiated after 10s ITI	+0	

* The stimulus image for all pre-training trials was a solid white square

NOTE: In contrast to Experiment 1, there was no Magazine 'click' accompanying trial initiation in Experiment 2. Illumination of the entire screen with white light served as the house light.



Supplementary Figure S4.1 Chronological touchscreen reversal learning performance and summary for excluded rats in the PhenoSys. **1 top)** Trial by trial data with every square representing a trial outcome (green = correct, red = incorrect, yellow = omission) and every column representing a single session in chronological order from left to right. Blue columns represent the point of reversal of reward contingencies. **1 bottom)** Average outcome of all trials initiated (session mean) and all trials responded to (response mean) in each session, producing session and response means respectively. **2)** Corresponding session outcome showing proportion of all trials that were correct (solid green line), incorrect (red line) or omitted (yellow line) and proportion of trials answered that were correct (dotted green line).



Supplementary Figure S4.2 Stage of estrous cycling determined by vaginal smear cytology. Supplementary Methods: **1)** Vaginal lavage was performed with saline in a transfer pipette with one drop of collected sample immediately placed onto glass slides and air dried **2)** Cresyl Violet (CV) powder (1%) was dissolved in 5% EtOH (in double distilled water; ddH_20) **2)** This stock solution was diluted to 0.3% in ddH_20 for staining **3)** Dried slides were stained by 15 second submersion in 0.3% CV solution, followed by 2 x 15 second submersions in clean ddH_20**4)** Glycerol was pipetted onto slides, which were coverslipped and visualised immediately at 10X on a light microscope.

[1]Note: The unavoidable circumstances that necessitated the immediate end to touchscreen testing in Experiment 1 pertained to ACL injury (not work related) that required full knee reconstruction surgery.

CHAPTER FIVE

General Discussion: Motivation for Starvation and Flexible Adaptation

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5.1 Anorexia nervosa: A brain "off balance"

A prevailing neurobiological hypothesis that may underpin the extreme behaviours seen in anorexia nervosa (AN) involves an *imbalance* between decreased reward signalling on the one hand and increased cognitive control on the other. This hypothesis is supported both by functional brain imaging studies in patients (1-3) and experiments in our laboratory using the activity-based anorexia (ABA) rat model (4, 5). The activity-based anorexia (ABA) rodent model reproduces the defining features of AN, namely a *voluntary* reduction of food intake in concert with paradoxical hyperactivity that results in rapid weight loss (4, 6-8). ABA also generates many of the endocrine, physiological and neurological abnormalities that occur in AN, which do not result from starvation or excessive exercise in isolation, generating confidence in the translatability of experimental findings in ABA to increase our understanding of the causes of AN (6, 7, 9). While it is not possible to replicate the psychological symptoms of AN (e.g. drive for thinness, fear of weight gain, perfectionism (10)), deficits in both reward function and the higher-order process of cognitive flexibility arise in AN and ABA alike (6, 7, 9, 11, 12). Thus, the ABA model provides a unique lens through which the underlying neurobiological drivers of AN can be sensibly dissected. Both reward and cognition are multifaceted neurobiological domains comprised of overlapping and dissociable components that can be individually assessed and manipulated in both humans and rodents. The term "reward" encompasses hedonic pleasure, the prototypical 'liking' response to natural rewards such as food, as well as motivation or 'wanting' considered as the incentive salience or drive to obtain reward, in addition to aspects of learning involving trial-and-error experimentation and incorporation of positive and negative feedback to guide future decision-making (13-16). Cognitive control includes aspects of attention, memory and impulsive behaviour, as well as cognitive flexibility, i.e. the ability to adapt behaviour in response to changing environments and uncertain contexts to successfully execute goal-directed actions (17, 18). What the results presented in this thesis demonstrate is that the specifics of the imbalance between reward and control in the ABA model involve *motivation* rather than hedonic processing and *cognitive flexibility.* In regard to the latter, it

should be acknowledged that the extent of the involvement of aspects of cognitive control beyond inflexibility were not evaluated in the present work and remain largely unknown.

5.2 Motivation for starvation

The most successful interventions to prevent ABA involve manipulating the reward context of food to promote feeding, either directly via provision of high-fat diet (19, 20), indirectly via administration of cannabinoid receptor agonists (20, 21), or specifically by increasing activity in the major reward-related neural circuit which coincidentally increases running in anticipation of food (food anticipatory activity [FAA]) (22). Whether these interventions were successful because of aberrant hedonic processing in ABA was examined in **Chapter 2**, in which the development of ABA was not dependent on a transient anhedonia that accompanied weight loss. Instead, findings from each of the three ABA studies reported in this thesis suggest that ABA arises from a reduction in the motivation to eat. This is in contrast to the altered experience of reward in patients with AN who display tactile (23) and social (24) anhedonia, and implement behaviours reminiscent of anhedonia for food (12). However, it has been argued that in fact patients with AN *do not* exhibit a primary anhedonia or inability to experience pleasure (*including* from food) but instead demonstrate a recalibration of reward valence (12) away from illness-aversive stimuli such as high-calorie food (25) toward illness-confirming stimuli such as low-calorie food (26), underweight body images (27-29) and stimuli associated with physical activity (29, 30). Patients with AN also show an increased capacity to *delay* reward (31, 32), and neuronal activity in reward and control regions during reward-related decision-making is insensitive to hunger/satiety in patients, even following weight recovery (33). In other words, hunger does not *motivate* patients with AN who instead demonstrate a strong motivational drive to exercise and avoid food – activities which, while helpful in alleviating anxiety, perpetuate the ill state and hamper treatment efforts (34-38). Therefore, it appears that a motivational deficit specific to feeding rather than diminished hedonic processing underlies the reduced food intake in both ABA and AN.

5.3 The drive to survive

Running in anticipation of food has previously been associated with both motivated behaviour and dopamine signalling (39), is consistently shown to prevent the development of ABA in our hands, and has also recently been associated with resistance to ABA mice (40). Considering that the *specific* development of running in anticipation of food was associated with resistance to weight loss in ABA in *every cohort of animals tested*, a detailed investigation into the precise

nature of the relationship between dopamine, FAA and motivation in ABA is warranted. Future studies should *directly* examine motivation using the classical operant response schedule of progressive ratio (PR) before, during and after exposure to ABA conditions. This could be achieved using home-cage operant testing devices such as the Feeding Experiment Devices (FED3; Open Behaviour) to directly assess motivated responding and PR "breakpoint" across all phases of ABA. Crucially, this will provide an "online" measure of motivation that will determine whether underlying levels of motivation *predict* future susceptibility to ABA, if there is a systematic change in motivational drive throughout development of ABA that aligns with survival, and what effect subsequent recovery and weight regain has on any such changes. Regular operant testing as ABA progresses could be aligned with FAA to validate the explicit role of FAA in motivated behaviour and be combined with recording of dopaminergic signalling, for example using fibre photometry. This would allow the dynamic changes in dopamine in specific brain regions to be aligned with indices of motivation over the time course of ABA and potentially solve some of the controversy regarding the role of dopamine function in ABA (and possibly AN).

5.4 Out with the old, in with the new

Susceptibility to ABA varies for many reasons, including sex, age, and genetic strain (7, 41); however, even when these are held constant within an experimental group, body weight loss occurs along a continuum from Susceptible to Resistant (40, 42). Therefore, other inherent biological variability within groups of animals must influence the development of ABA. Although our battery of behavioural tests did not identify a predictor of ABA susceptibility, there are many caveats to consider when interpreting this, including the fact that repeated animal handling not only influences susceptibility to ABA (43), but also performance on other behavioural tests (44, 45). This likely underpins the difference observed in the proportion of rats that were resistant to developing ABA in **Chapter 2**, which increased following behavioural testing. As such, it may not be appropriate to perform more comprehensive behavioural test batteries that require repeated experimenter intervention prior to examination of susceptibility to ABA. Based on the consistent and predictive association of baseline running activity and ABA outcome, not only in each of the studies presented in this thesis but also in other studies using rats and mice (41), it seems implausible that no other behavioural phenotype predicts susceptibility to ABA. Wheel running in rats is inherently rewarding (46-50), is therefore engaged in voluntarily (49) and in fact rats will "work" for the opportunity to run on wheels (48, 51, 52). This suggests that differences in baseline wheel running constitute one example of a "reward profile" that

determines susceptibility to ABA. Perhaps the conventional behavioural tests used in the present studies were unable to capture subtle (or complex) reward-related differences between rats that went on to become susceptible or resistant to ABA. The broader goal for future studies should therefore be to not only reduce experimenter intervention, but also to refine behavioural examination in order to improve detection sensitivity.

Using touchscreens to examine cognitive behaviour is a step towards this goal of refinement and, indeed, allowed us to identify a discrete neuronal circuit involved in both susceptibility to ABA and cognitive flexibility. Specifically, inhibiting medial prefrontal cortex neurons with direct projections to the shell of the nucleus accumbens (mPFC-AcbSh) *completely* prevented the development of ABA (see **Chapter 3**), a phenomenon which, to the best of our knowledge, has only ever before been achieved in rats with manipulations that involve changing the experimental conditions in which ABA is conducted (19, 20, 53, 54). The same neuronal modulation also decreased perseverative responding following reversal of reward contingencies in a touchscreen reversal learning paradigm to improve adaptive flexibility (see **Chapter 4**). Taken together, these findings indicate that the development of ABA involves a break down in the ability to appropriately adapt behaviour to the change in the environmental reward context. These findings correspond neatly with both prefrontal cortical dysfunction in patients with AN and their alignment with symptoms of cognitive flexibility (1, 55), a confluence that may relate (at least in part) to the improved ability to translate touchscreen-based behavioural outcomes between rodents and humans (56-58). Although the direct role of cognitive flexibility in ABA outcomes was not examined in this thesis, the rapid assessment of touchscreen-based tasks now possible using the PhenoSys (44, 45) (discussed in detail in **Chapter 4**) will allow the temporal dynamics of cognitive flexibility to be examined before, during and after exposure to ABA *in the same animals*. The adoption of automated and experimenter-free systems like the PhenoSys goes further than ever before in the effort to refine and standardise behavioural testing and represents a quantum shift in the way these experiments are conducted and interpreted.

5.5 Mushroom for improvement

While the studies presented in **Chapters 3** and **4** describe a neurobiological link between pathological weight loss and flexible learning styles, it remains unclear precisely how these neural circuits can be modulated in human patients to prevent the development of AN and/or improve recovery. The *interaction* between serotonergic and dopaminergic signalling is a likely neurochemical focus, considering the known roles of prefrontal 5-HT in cognitive flexibility (59)

and ventral striatal DA in reward processing (60, 61) and because disrupted 5-HT and DA signalling are involved in the pathophysiology of both AN and ABA (2, 6, 36). The quest for novel pharmacotherapeutics could therefore focus on compounds that target *both 5-HT and DA* in an anatomically-relevant way. Traditional anti-psychotic (at least partially DA-targeted) or anti-depressant (5-HT targeted) medication have been largely ineffective as therapies for AN, yet the last few decades of research into novel therapeutic treatments for AN has involved only minor alterations to and combinations of existing drugs such as these (62-65). Whether said by Einstein or not, "*the definition of insanity is doing the same thing over and over again and expecting different results*" seems an applicable description of the stagnant status quo of pharmacotherapeutic treatment for AN. However, multiple lines of evidence are emerging to support the potential for psilocybin, derived from magic mushrooms, to both increase prefrontal 5-HT and ventral striatal (nucleus accumbens) DA (66, 67). This represents a regionally specific transmission profile that is the exact inverse of what is seen in patients with AN and in ABA rats, and indicates that psilocybin could "reset" the balance of reward and control that underpins pathological weight loss.

Over the last two years, clinical trials at world class institutes including Imperial College London and Johns Hopkins University have commenced to test the safety and efficacy of psilocybin for patients with AN. These trials have been initiated based on the efficacy of a single dose of psilocybin in combination with specialised psychotherapy in alleviating symptoms of intractable depression for *up to 6 months* (68). Moreover, positive therapeutic effects of psilocybin have been shown for smoking cessation (69), end-of life symptoms of anxiety and depression (70, 71), and obsessive-compulsive disorder (OCD) (72). Given the high comorbidity of AN with mood- and anxiety-disorders and OCD, the positive effects of psilocybin in decreasing these symptoms is promising for its applicability to effectively treat AN. In addition to these comorbid symptoms, other psychedelics improve cognitive flexibility (73), so it seems likely that psilocybin will prove similarly effective at alleviating the rigid adherence to diet and excessive exercise in AN (2, 37, 38, 74-76), features that are underscored by cognitive inflexibility (55, 77-80) that is a key driver of illness maintenance and treatment resistance. Combatting inflexible patterns of thought and behaviour would be a pivotal first step in advancing the treatment of AN beyond that of comorbid psychological pathologies. However, the mechanisms by which psilocybin acts in the brain to promote therapeutic outcomes are incompletely understood.

There has been a recent explosion of research using psilocybin in animal models, with the goal of generating a detailed understanding of the behavioural and neurobiological mechanisms that

underpin its therapeutic effects. The findings are mixed and include effectively reducing marble burying in mice (81), but not alcohol relapse in rats (82), and there are conflicting results in genetic models of depression (83, 84). However, the confounds of behavioural testing discussed above, conflicting interpretation of what specific behaviours measured in these tests represent for human psychopathologies and the inability for psilocybin to completely act in some genetic rodent models may help explain the inconsistent picture (85). To this end, specific care must be taken to ensure appropriate tests, with unambiguous outcome measures that are as close as possible to the equivalent measures in humans, are chosen to determine the efficacy of psilocybin action in animal models of psychiatric illnesses. With this in mind, interrogating the effects of psilocybin on ABA and cognitive flexibility using the PhenoSys is a critical and timely proposal to investigate the therapeutic potential of psilocybin for AN.

5.6 Reinventing the wheel

It is clear that patients with AN do not *want* to eat, despite life-threatening emaciation and the appropriately recognised physiological *need* to eat. Effective treatments need to target this core deficit to promote *wanting* with respect to food intake, even if this does nothing to improve *liking* because “the tempted brain eats” (86) but not if “nothing tastes as good as skinny feels” (2). While the psychosocial symptoms of AN cannot be disregarded, as they exacerbate illness maintenance, these constitute secondary reinforcers of illness-perpetuating behaviour and are therefore inappropriate targets for pharmacotherapeutic treatment aimed at providing long-term benefits for patients. The fact that ABA develops in rodents – *in the absence of any conceivable psychological symptoms constituting a “weight phobia”* – highlights the primary causal role of neurobiological factors in the development of pathological weight loss, which is also likely the case for AN. The detail of these neurobiological contributions is becoming clearer from a rapidly growing body of research utilising the ABA model. Further defining these details with sophisticated tools to modulate and record from neural circuits in combination with advanced behavioural testing will provide specific targets for a new and much needed treatment for one of the most debilitating of all psychiatric disorders, anorexia nervosa.

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