

EFFECTS OF GPR55 RECEPTOR BLOCKADE ON SCHEDULE-INDUCED POLYSIPDIA
IN RATS

By

Lindsay Lo

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Abstract

Compulsivity, a tendency toward repetitive, habitual actions that are repeated despite adverse consequences, is a core trait of many psychiatric pathologies. Despite its prevalence and severity, there are few effective pharmacological interventions for compulsive symptoms. The schedule-induced polydipsia (SIP) rodent model, in which chronically food-restricted animals develop adjunct compulsive drinking behaviours, has been used to investigate neurochemical substrates of compulsion. Recently, drinking behaviour was associated with bi-directional gamma-aminobutyric acid (GABA) plasticity signalling between G-protein-coupled receptor 55 (GPR55) mediated GABA potentiation and cannabinoid type I receptor (CB1) mediated GABA depression, in the bed nucleus of the stria terminalis (BNST). Compulsive, high drinking was correlated with deficient GPR55 GABA signalling. This project investigated a potential causal link between deficient GPR55 signaling and the development of compulsive drinking. We hypothesized that antagonism of GPR55, using CID 16020046 (CID), would turn low-drinking rats into high drinkers. However, administration of CID did not significantly increase water intake in low-drinking rats. Further, there was no difference in CID-induced water intake between animals displaying low vs high drinking behaviour. Animals receiving the higher CID dose elicited a greater increase in CID-induced water intake, although marginal, compared to the lower dose, irrespective of drinking classification. This suggests dosing may have been too low for a sufficient amount of CID to reach its target in the brain. In addition, the targeting of one receptor may not have been sufficient to evoke detectable behavioural changes, as a wide range of receptors and signalling systems have been implicated in the development of compulsive phenotypes. Regardless, this project served as a useful starting point to investigate a causal link between GPR55/CB1 signalling and the development of compulsive behaviour.

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List of Abbreviations and Symbols

2-AG – 2-arachidonylglycerol

ACC – Anterior cingulate cortex

ANOVA – Analysis of variance

ANCOVA – Analysis of covariance

BNST – Bed nucleus of the stria terminalis

CB1 – Cannabinoid receptor type I

CID – GPR55 antagonist CID 16020046

CSTC – Cortico-striato-thalamocortical circuit

DMSO – Dimethyl sulfoxide

DSM-5 – Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

ECS – Endocannabinoid system

GABA – Gamma-aminobutyric acid

GPR55 – G-protein-coupled receptor 55

HD – High drinking

HPA – Hypothalamic–pituitary–adrenal

IP – intraperitoneally

KO – Knock out

LD – Low drinking

LH – Lateral hypothalamus

LPI – Lysophosphatidyl inositol

LTD^{GABA} – Long term depression of gamma-aminobutyric acid

LTP^{GABA} – Long term potentiation of gamma-aminobutyric acid

OCD – Obsessive Compulsive Disorder

OFC – Orbitofrontal cortex

ovBNST – Oval Bed nucleus of the stria terminalis

PFC – Prefrontal cortex

PLA – Phospholipase A

PP – Primary polydipsia

PVS – Positive Valence Systems

RDoC – Research Domain Criteria

SIP – Schedule induced polydipsia

SRI – Serotonin reuptake inhibitors

SSRI – Selective serotonin reuptake inhibitors

vIPAG – Anterior ventrolateral periaqueductal gray

Chapter 1

Introduction

1.1 Compulsion

The DSM-5 defines compulsions as “repetitive behaviours or mental acts that an individual feels driven to perform in response to an obsession or according to rules that must be applied rigidly” (American Psychiatric Association, 2013). They are characterized as uncontrollable and unwanted thoughts or repetitive behaviours which present in various forms such as handwashing, ordering, checking, counting, or word repeating (American Psychiatric Association, 2013). Such acts are aimed to reduce distress felt by an individual in response to certain situations or thoughts. (Björgvinsson et al., 2007; Hollander et al., 2007; Milad & Rauch, 2012). The persistent, time-consuming nature of compulsions cause those that suffer from them to experience significant distress and daily life impairment (American Psychiatric Association, 2013). Further, patients experiencing compulsive disorders are at a higher risk for co-morbidities such as depression and anxiety (Fineberg et al., 2011; Hollander et al., 2007; Menzies et al., 2008; Pallanti et al., 2011; Peris et al., 2010). Obsessive compulsive disorder (OCD) is the primary disorder of compulsions, however, compulsivity is a defining clinical feature in a variety of psychiatric disorders including eating disorders, schizophrenia, substance use disorder, and pathological gambling (American Psychiatric Association, 2013; Blanco et al., 2001; Everitt & Robbins, 2005; Fairburn & Brownell, 2002; Koob, 2009). Despite an increasing body of research identifying etiological commonalities across disorders, the exact neurobiological underpinnings remain elusive. This, in combination with the complex nature of these disorders, has resulted in a lack of efficacious treatment options for those suffering from compulsions.

1.1.1 Neurocircuitry of Compulsions

Compulsivity is strongly linked to poor behavioural inhibition and self-regulation (Dalley et al., 2011; Menzies et al., 2008; Milad & Rauch, 2012; Moreno & Flores, 2012; Pallanti et al., 2011). Inhibitory control is exerted by top-down connectivity from the frontal cortex to the striatum (Dalley et al., 2011; Hwang et al., 2010; Morris, Kundu, Dowell, et al., 2016). Fronto-striatal loops link the frontal cortex to the basal ganglia, bridging decision making, habit learning, cognition, and emotion (Balleine & O’Doherty, 2010; Gläscher et al., 2009; Morris, Kundu, Baek, et al., 2016; Morris, Kundu, Dowell, et al., 2016). As such, maladapted fronto-striatal neurocircuits are often considered to be the neural basis of compulsivity (Dalley et al., 2011; Di Filippo et al., 2009; Fineberg et al., 2010; Milad & Rauch, 2012; Moreno & Flores, 2012). Specifically, the cortico-striato-thalamocortical circuit (CSTC) is strongly implicated in compulsive disorders such as OCD (Menzies et al., 2008; Milad & Rauch, 2012; Saxena & Rauch, 2000). Within the CSTC, neurons from frontal lobe areas such as the prefrontal cortex (PFC), orbitofrontal cortex (OFC), and anterior cingulate cortex (ACC) project to the targets in the striatum and thalamus (Alexander et al., 1986; Milad & Rauch, 2012). Neurons from the thalamus then project back to the frontal lobe completing the loop (Alexander et al., 1986; Milad & Rauch, 2012). Connections within the CSTC loop are implicated in affective and reward processing, working memory and executive function, and motor and response inhibition (Alexander et al., 1986; Milad & Rauch, 2012). In OCD patients, abnormal activity within and between nodes of the CSTC pathway is observed (Benkelfat et al., 1990; Chamberlain et al., 2008; Saxena & Rauch, 2000). Following successful treatment, abnormal activity ceases and connectivity patterns become similar to those of healthy individuals (Baxter et al., 1992;

Benkelfat et al., 1990; Saxena & Rauch, 2000). However, evidence is inconsistent on the exact CSTC components involved in compulsions and what constitutes abnormal activity. Both hyper- and hypoactivity in the caudate, OFC, and ACC have been reported in OCD patients (Aylward et al., 2019; Menzies et al., 2008). Cognitive studies of OCD also show results inconsistent with what would be expected from the proposed cortico-striato-thalamocortical neurocircuitry (Menzies et al., 2008; Rauch et al., 2007; Vanderwee et al., 2003). For example, while response inhibition and attentional set-shifting are consistently shown to be impaired, reports on decision making are not (Van Der Wee et al., 2003). This suggests that the CSTC model may not be entirely sufficient to explain compulsivity (Mataix-Cols et al., 2004; Milad & Rauch, 2012; Sun et al., 2019).

Recently, compulsions have been related to the Research Domain Criteria (RDoC) domain of Positive Valence Systems (PVS) (Gerdeman et al., 2003; Graybiel, 2008; Olino, 2016), which dictates responses to positive motivational situations such as reward seeking and reward/habit learning (National Institute of Mental Health, n.d.-a). Within the PVS domain, habit is a subconstruct. Maladaptive habit-related behaviour, such as compulsivity, is suggested to be a pathological expression of processes related to adaptive goals (National Institute of Mental Health, n.d.-b). As such, other neural areas involved in adaptive behaviour and reward seeking may be the missing components of a more complete model of compulsivity. One area that spans both these domains, yet is often overlooked, is the bed nucleus of the stria terminalis (BNST). With extensive connections to the PFC, OFC, amygdala, hypothalamus, and some motor regions, the BNST has been hypothesized to coordinate behavioural and physiological adaptive responses (Dong & Swanson, 2006; Dumont, 2009; Fendt et al., 2003; Hao et al., 2019; Kim & Kim, 2021). Further, the BNST is implicated in a range of functions related to compulsivity, such as

anxiety, fear, threat, and reward, suggesting dysregulation of this area may lead to the development of compulsions (Dumont et al., 2005; Dumont, 2009; Fendt et al., 2003; Kohl et al., 2016; Sajdyk et al., 2008; Stein et al., 2010).

1.1.2 Neurophysiological Basis of Compulsion

While the exact neurochemical basis of compulsions is unknown, several neurotransmitter signalling systems have been implicated. Dysfunctional serotonin receptors are linked to compulsive behaviours in both animal and human studies (Fineberg et al., 2011; Milad & Rauch, 2012; Moreno & Flores, 2012). Evidence from PET studies show reduced serotonin transporter binding in OCD patients (See review Spies et al., 2015). Animal models of OCD also show altered activity of serotonin receptors within the CSTC (Winter et al., 2018). Clinically, serotonin reuptake inhibitors (SRIs) and selective serotonin reuptake inhibitors (SSRIs) are first-line pharmacotherapy for OCD, indicating the serotonergic systems involvement in compulsions (Björgvinsson et al., 2007; Dougherty et al., 2004; Fineberg et al., 2010). However, approximately 30-50% of patients do not show improvement from pharmacotherapy with SRI/SSRIs and full remission is uncommon, making the serotonin hypothesis an incomplete explanation (Fineberg et al., 2011; Pittenger & Bloch, 2014; Walsh & McDougle, 2004).

Neuroanatomical models show that dopamine signalling is involved with top-down control of fronto-striatal neurocircuitry, suggesting that dopamine may partly mediate deficits in inhibition. (Dalley et al., 2011; Fineberg et al., 2010; Moreno & Flores, 2012). Accordingly, hypotheses based on RDoC's PVS domain posit compulsions as maladaptive processes in reward seeking and reward/habit learning. Chronic treatment with the dopamine D₂/D₃ receptor agonist quinpirole causes compulsive checking in rats (Tizabi et al., 2002). Some evidence shows that OCD patients exhibit increased dopamine binding within the basal ganglia (See review Denys &

Westenberg, 2004). However, several studies show a lack of dopamine signalling differences between those exhibiting compulsive behaviours or disorders and healthy controls.

Finally, the lack of inhibitory control central to compulsivity suggests deficits in GABAergic signalling. OCD patients exhibit reductions in GABA inhibition and hyperactivity in several circuits, including the fronto-striatal loop (Greenberg et al., 2000; Richter et al., 2012). Animal models also show lower GABA concentrations in compulsive animals compared to healthy controls, particularly in fronto-striatal circuitry (Fineberg et al., 2011; Richter et al., 2012; Winter et al., 2018). These findings propose a potential reason for the inability to inhibit unwanted and intrusive thoughts or behaviours. Recently, depression of GABAergic signalling within the BNST was found in compulsive rats (Angelis et al., 2019; Hawken et al., 2019), suggesting that GABA may be a key pharmacological substrate for compulsivity.

1.1.3 The Role of the Bed Nucleus of The Stria Terminalis in Compulsivity

With previous explanations inadequate, the BNST may be the missing component to understanding compulsivity. Mounting evidence has implicated the BNST in a variety of psychiatric disorders that include compulsive behaviour such as OCD, addiction and anxiety (Angelis et al., 2019; Avery et al., 2016; Hawken et al., 2019; Kohl et al., 2016; Lebow & Chen, 2016; Luyten et al., 2016). Further, deep brain stimulation of the BNST effectively treated severe, treatment-resistant OCD patients (Luyten et al., 2016).

Located in the basal forebrain, posterior to the nucleus accumbens, the BNST is a cluster of nuclei with extensive connections to the amygdala, hypothalamus, and the prefrontal and orbitofrontal cortices via the caudate and nucleus accumbens (Dumont, 2009; Kohl et al., 2016; Lebow & Chen, 2016). Thus, the BNST has many direct and indirect connections to areas

implicated in compulsivity, such as the OFC and striatum. Anatomical connections within the BNST suggest that it is a center for coordination between autonomic, neuroendocrine, and somatic systems (Dong & Swanson, 2006; Dumont, 2009). As a coordinating structure, the BNST likely plays an important role in the maintenance of homeostasis (Dumont, 2009). BNST projections to motor regions allow coordination of physiological and behavioural responses, supporting its role in adaptation to homeostatic challenges (Dumont, 2009).

Pharmacologically, the BNST is involved in all three proposed signalling systems for compulsions. It is robustly innervated by serotonergic, dopaminergic, and GABAergic inputs (Glangetas & Georges, 2016). A high density of serotonin receptors are contained within the BNST and treatment with SSRIs elicits chronic activation of the area (Lebow & Chen, 2016). Dopamine enhances excitatory glutamatergic inputs onto dorsolateral BNST neurons, while decreasing inhibitory influence on the oval BNST (ovBNST) (Glangetas & Georges, 2016). Finally, the BNST primarily consists of GABAergic neurons, suggesting that it is highly involved with inhibitory signalling (Poulin et al., 2009). Accordingly, BNST mediated GABA signalling has been correlated with anxiety and compulsive behaviour (Angelis et al., 2019; Avery et al., 2016; Dumont, 2009; Hawken et al., 2019; Kim & Kim, 2021). Together, the BNST's functional and neurobiological characteristics strongly suggest a role in compulsivity.

1.1.4 Models of Compulsion

One obstacle in further investigating the neural mechanisms of compulsion is the lack of valid pre-clinical animal models. No one model can account for the entire syndrome, and there is often difficulty in attributing outcomes to compulsion directly. For example, paradigms such as self-administered drug addiction may model some aspects of compulsion. However, the impact

of addiction and drug use on brain circuitry and behaviour cannot be ignored or easily separated when examining outcomes. Appropriate animal models need to replicate clinical conditions and have neurobiological mechanisms relevant to the human condition (Bale et al., 2019). Therefore, it is important to have a model that focuses on impaired inhibitory control and a compulsive outcome that is seen across several disorders.

1.2 Schedule-Induced Polydipsia Paradigm

One model that has emerged as a robust and replicable animal model for compulsion is the schedule-induced polydipsia (SIP) model (Albelda & Joel, 2012; Angelis et al., 2019; Falk, 1966; Flores et al., 2014; Gregory et al., 2015; López-Grancha et al., 2008; Moreno & Flores, 2012; Navarro et al., 2015; Pellón et al., 2011; Platt et al., 2008). Within the SIP paradigm, chronically food-restricted animals develop adjunct excessive drinking behaviours when subjected to a fixed-time feeding schedule (Falk, 1961). Within a subset of animals, the drinking is so excessive that they risk hyponatremia and death, positing a clear maladaptive, non-physiological driven behaviour (Banasikowski & Hawken, 2019; Moreno & Flores, 2012; Platt et al., 2008). SIP has been used to measure compulsivity spanning a variety of disorders, including OCD, schizophrenia, and alcohol abuse (Gilpin et al., 2008; Hawken et al., 2019; Rosenzweig-Lipson et al., 2007). Additionally, SIP evokes compulsive behaviour in the form a primary polydipsia, a condition seen in humans in several psychiatric conditions including OCD, schizophrenia, and ADHD (Dundas et al., 2007; Illowsky & Kirch, 1988; Oades & Daniels, 1999). Primary polydipsia (PP), is excessive, non-physiological drinking that commonly co-occurs with disordered thinking and psychotic states (de Leon, 2003; Dundas et al., 2007; Illowsky & Kirch, 1988). It is commonly reported as an anxiety reliever or to soothe obsessions

and delusions, matching the DSM-5 definition of compulsions (Abramowitz & Jacoby, 2014; American Psychiatric Association, 2013; Tranulis et al., 2005). Similar neurobiological mechanisms are present for both PP and OCD, such as hypometabolism in the basal ganglia and dysregulation of the ACC and OFC (Subramanian et al., 2017). Further, schedule induced behaviours can be evoked in various species, including humans, adding to its ecological validity (Wallace & Singer, 1976). The excessive and persistent nature of SIP has led to its recognition as a useful and reliable paradigm for studying compulsive-based disorders.

Perhaps one of the most interesting aspects of SIP is that only a subset of animals develop excessive drinking to the point of maladaptiveness (>15 mLs/2 hours) (Angelis et al., 2019; Gregory et al., 2015; Hawken et al., 2019; Moreno & Flores, 2012). A clear division between high drinking (HD) (>15 mLs/2 hours) and low drinking (LD) animals was observed following a 21-day SIP protocol consisting of 2 hour/day sessions in which sugar pellets were delivered on a strict 60 second fixed-time schedule (Angelis et al., 2019; Gregory et al., 2015; Hawken et al., 2019). This phenomenon provides an invaluable opportunity to explore genetic and environmental variants causing this split in behavioural phenotypes.

1.2.1 Schedule Induced Polydipsia and Homeostatic Control

Polydipsia, the defining feature of SIP, has no physiological or regulatory purpose for the excessive drinking, suggesting an overall loss of homeostatic control (de Leon, 2003; Dundas et al., 2007; Illowsky & Kirch, 1988; Moreno & Flores, 2012). Drinking is a reward-driven process (Becker et al., 2015; Moreno & Flores, 2012). Thirst involves dopamine pathways and activates brain regions also involved with decision-making, reward, stress, fear, and anxiety (Banasikowski & Hawken, 2019; Egan et al., 2003; Ettenberg & Camp, 1986; Shin & Liberzon,

2010). Increased dopamine transmission worsens compulsivity in animals (Moreno & Flores, 2012; Tizabi et al., 2002). Abnormal GABA signalling has also been correlated with the disruption of typical dopaminergic and reward circuitry pathways (Floresco et al., 2003; Grace & Bunney, 1985; Krawczyk et al., 2013; Moreno & Flores, 2012). Thus, the disregard for normal thirst and satiety signals seen in polydipsia may be linked to loss of homeostatic control within these pathways.

Investigations found homeostatic control of thirst and satiation was mediated by bi-directional GABA plasticity with endocannabinoid constituents G-protein-coupled receptor 55 (GRP55) and cannabinoid receptor type I (CB1), in the ovBNST (Angelis et al., 2019; Hawken et al., 2019). As previously mentioned, the BNST is speculated to be a missing key component in the neurocircuitry of compulsions and plays a prominent role in maintaining homeostasis within the brain (Dumont, 2009; Kohl et al., 2016). Feeding state dictates the polarity of GABA synaptic plasticity (Hawken et al., 2019). When in a sated state, such as during pellet delivery in SIP training, ligand lysophosphatidyl inositol (LPI) with G-protein-coupled receptor 55 (GRP55) promotes long term potentiation of GABA (LTP^{GABA}). When in a food restricted state, such as outside the two-hour/day SIP training, ligand 2-arachidonylglycerol (2-AG) with cannabinoid receptor type I (CB1) promotes long term depression of GABA (LTD^{GABA}). Deficient control of 2-AG/CB1 by LPI/GPR55 was strongly correlated with the subset of rats that developed excessive drinking in the SIP paradigm. *In vitro* brain slice electrophysiology revealed that rats of the high drinking group had chronic depression of GPR55 signaling and, therefore, inhibition of GABA signalling (Angelis et al., 2019; Hawken et al., 2019). When going from a hunger to a sated state, rats with normal drinking behaviours switched from CB1 mediated depression to GPR55 mediated potentiation of GABA signalling. BNST synapses in the high-drinking animals

appeared unable to perform his switch and remained in 2-AG/CB1 mediated GABA depression state (Angelis et al., 2019; Hawken et al., 2019). This posits a potential mechanism for the loss of inhibitory control that is strongly implicated in compulsions.

1.3 The Bed Nucleus Of The Stria Terminalis and Energy Homeostasis

It is proposed that SIP stems from a loss of homeostatic control (Angelis et al., 2019; Banasikowski & Hawken, 2019; Hawken et al., 2019; Moreno & Flores, 2012). A caloric deficit is necessary to invoke SIP, suggesting that energy homeostasis, specifically, is involved. Central to energy homeostasis is the regulation of food intake (Rossi & Stuber, 2018). The BNST is closely involved with integrating information and coordinating responses for both homeostatic control and feeding behaviour (Baldermann et al., 2019; Banasikowski & Hawken, 2019; Dumont, 2009; Hao et al., 2019; Jennings et al., 2013; Kim & Kim, 2021; Luo et al., 2018; Luskin et al., 2021; Rossi & Stuber, 2018). Neurons in the BNST are activated during food consumption (Angeles-Castellanos et al., 2007) and feeding behaviours are regulated by GABAergic outputs from the ovBNST to the lateral hypothalamus (LH) (Jennings et al., 2013). Photoactivation of GABAergic BNST-LH projections to the anterior ventrolateral periaqueductal gray (vIPAG) induces feeding behaviour in sated mice with a corresponding change in body weight (Hao et al., 2019). Interestingly, in humans undergoing deep brain stimulation in areas connected to the BNST, body weight increases were observed (Baldermann et al., 2019). As such, the BNST is linked to energy homeostasis through feeding and metabolic control.

1.4 GPR55

GPR55 is a rhodopsin-like seven transmembrane G-protein coupled receptor that responds to cannabinoids and other lipid mediators (Oka et al., 2007). Although able to interact with other cannabinoids and lipid mediators, LPI appears to be GPR55's endogenous ligand (Oka et al., 2007). GPR55 is widely distributed throughout the brain and peripheral tissues. It is present in many areas of the CSTC loop implicated in compulsions, such as the nucleus accumbens, caudate nucleus, striatum, and thalamus (Tudurí et al., 2017). Additionally, GPR55 may be implicated in energy homeostasis, supporting its potential role in mechanisms involved with SIP and psychogenic polydipsia (Tudurí et al., 2017).

1.5 GPR55/CB1 Role in Energy Homeostasis

The maintenance of energy homeostasis has been closely tied to behavioural outcomes observed in the SIP paradigm. Metabolic state strongly impacted bi-directional signalling at GABA synapses, with CB1 driving GABA depression in a hunger state and GPR55 driving GABA potentiation in a sated state (Hawken et al., 2019). Bi-directional signalling refers to GABA signalling plasticity, in which GABA synapses can alternate between LTD and LTP. These findings are in line with the large body of work that implicates the endocannabinoid system in feeding behaviour and energy homeostasis. The CB1 receptor, in particular, has been implicated in feeding regulation, energy expenditure, and reward (Cota, 2007; Silvestri & Di Marzo, 2013). Activation of CB1 receptors has been found to cause hyperphagia and appetite stimulation (Cota, 2007). Endocannabinoid signalling has also been implicated in governing lipid and glucose metabolism (Silvestri & Di Marzo, 2013), adding to the evidence of the system's prominent role in energy homeostasis and metabolism. While the role of GPR55 is less clear, there is evidence suggesting that it may play a role in energy homeostasis as well. GPR55 knock

out (KO) mice have been shown to have decreased energy expenditure, although no difference in food intake was found (Bjursell et al., 2016; Meadows et al., 2016). Additionally, there is evidence that GPR55 receptors may be involved in non-CB1/CB2 receptor-mediated effects of endocannabinoid signalling. Responses to both CB1 and CB2 agonists, in both CB1 and CB2 KO mice, have been detected (Baker et al., 2006). As the endocannabinoid system is heavily involved in energy metabolism, this supports the notion that GPR55 also plays a role.

Angelis (2019) and Hawken (2019) showed that deficient control of CB1 mediated signalling by GPR55 in the BNST was correlated with compulsive, high drinking behaviours in the SIP rat paradigm. CB1 inhibits GABA signalling, while GPR55 promotes GABA signalling. OCD and other compulsive disorders are strongly implicated with the dysfunction of inhibitory processes. Further, both endocannabinoid system signalling and the BNST are involved with homeostatic control and energy metabolism.

1.6 Endocannabinoid Signalling, Energy Homeostasis, and Psychiatric Disorders

Deficits in energy homeostasis are implicated in a wide range of pathologies. The endocannabinoid system (ECS) has frequently been a target of pharmacological interventions for conditions involving metabolic dysregulation and adiposity, such as in obesity and diabetes. However, dysregulated energy homeostasis is not unique to metabolic disorders alone. Mutations and malfunctions to genes involved in mitochondrial energy function have been implicated in several severe psychiatric disorders, including schizophrenia, major depressive disorder, and bipolar disorder (Lindberg et al., 2015). Specific to compulsions, OCD is associated with abnormal glucose metabolism (Swedo et al., 1989). Interestingly, eating disorders co-occur at high rates with OCD, suggesting shared etiology (Altman & Shankman, 2009). Together, this

posits a clear connection between energy homeostasis and neuropsychological pathologies. While there are many metabolic and energetic regulators in the body, the ECS's multifaceted contribution to energy homeostasis and targetability makes it a promising area of study. Increasing evidence suggests that energy and metabolic homeostasis are important, yet understudied processes involved in the development and maintenance of many pathologies. Additionally, compulsivity is a prominent feature in a wide range of pathologies. As previously discussed, one underlying neural substrate to investigate with relation to compulsive behaviours, is the bi-directional GABA plasticity mediated by signalling between GPR55/CB1 in the BNST. As found by Angelis (2019) and Hawken (2019), deficient control of CB1 mediated signalling by GPR55 was correlated with compulsive, high drinking behaviours. CB1 inhibits GABA signalling, while GPR55 promotes GABA signalling. OCD and other compulsive disorders are strongly implicated with the dysfunction of inhibitory processes. These findings raise the question of whether both GPR55 and CB1 play a role in compulsivity. There are a wide range of factors already shown to attenuate SIP (e.g., feeding or schedule factors, alterations to stress signalling), which may increase the chance of spurious findings (Cole & Koob, 1994; Falk, 1967). Inducing SIP would provide stronger evidence in support of the notion that GPR55 and CB1 are involved in the modulation of SIP. High-drinking behaviour is associated with deficient GPR55 signalling. Additionally, the CB1 receptor is one of the most widely distributed g-protein coupled receptors in the brain, which may increase the chance of off target effects. Therefore, pharmacological intervention of GPR55, not CB1, is a stronger first target and warrants further investigation to better determine its influence on compulsive drinking in the SIP rat model.

1.7 Objectives and Hypothesis

This project uses a novel pharmacological approach, paired with a validated behavioural paradigm, to investigate whether deficient GPR55 signalling leads to the development of compulsive drinking behaviours in the SIP rat model. A secondary post-hoc objective was to characterize the behaviour that developed over the SIP protocol and explore profiles that may contribute to the development of compulsive, high drinking behaviour. In addition to completing a descriptive characterization of the behavioural paradigm, we sought to answer the question of whether weight and food intake were associated with drinking phenotypes in post-hoc analyses. The BNST and GPR55/CB1 receptors have all been implicated in feeding-behaviours and metabolic control. As CB1 receptors prevail in hunger states and high drinking animals were found to be in CB1 mediated LTD^{GABA}, it was predicted that both free-food intake and weight would negatively correlate with water intake during SIP training.

The primary objective of this project is to investigate if there is a causal link between deficient LPI/GPR55 signalling within the brain and the development of compulsive drinking in the SIP rat model. Previous research has shown LPI/GPR55 signalling appears to be downregulated in the subset of rats that meet the criteria as high drinkers. Therefore, we examined the effects of pharmacologically blocking GPR55 in low drinking rats (Figure 1). We hypothesize that the pharmacological blockade of GPR55 will alter the behaviour of low drinking rats to meet the criteria for high drinking rats.

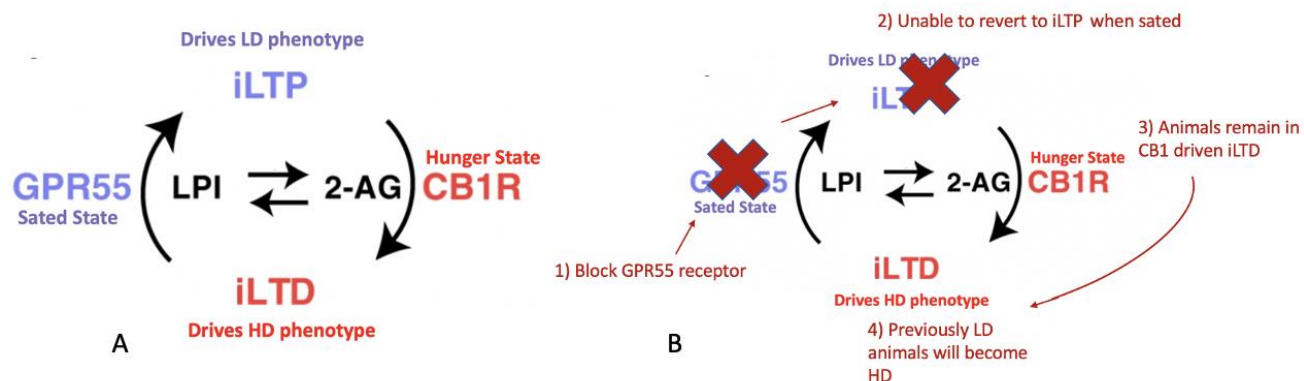


Figure 1. Proposed signalling mechanisms between metabolic state and CB1/GPR55 mediated bi-directional GABA plasticity (adapted from Hawken et al., 2019). **A.** Previously proposed mechanistic interplay between feeding state and bi-directional GABA-IPSC plasticity in the rat ovBNST **B.** Proposed adaptation to investigate causal link between deficient LPI/GPR55 signalling and the development of a HD phenotype. *Note.* 2-AG = 2-arachidonylglycerol, CB1R = cannabinoid type I receptor, GPR55 = G-protein-coupled receptor 55, HD = high drinking, LD = low drinking, LPI = lysophosphatidyl inositol, iLTP = long term potentiation, iLTD = long term depression

Chapter 2

Methods

2.1 Animals

Forty-eight (48) male Long Evans rats (Charles River Laboratories, St. Constant, Quebec) weighing 250-275g upon delivery were initially pair-housed in Plexiglass cages (45 x 23 x 20 cm) lined with bedding. Cages were placed in a climate-controlled room ($21 \pm 1^\circ\text{C}$; humidity 40-70%) in a 12-hour reverse light/dark cycle. Rats were acclimated for a 7-day period with *ad libitum* access to food and water in their home cages. Beyond the acclimation period, rats were individually housed for the duration of the study. All experiments were approved by the Queen's University Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care Guidelines.

2.2 Apparatus

Training and testing were conducted in 12 operant chambers (24 x 30 x 29 cm) (Med Associates, ST-Albans, VT), situated in ventilated sound-attenuating cabinets (Figure 2). A food dispenser was located opposite to a metal, ball-bearing, drinking spout within each chamber. The operant conditioning chambers were controlled, and data were acquired by a computer running MED-PC-V (Med Associates Inc., St. Albans, VT).



Figure 2. Operant conditioning box within a sound-attenuating cabinet.

2.3 Drugs

A stock solution of the GPR55 antagonist CID 16020046 (CID) (Tocris Bioscience, Oakville, ON) was made by dissolving CID in 100% Dimethyl sulfoxide (DMSO). The animals were separated into two groups, a lower 10 μ M dose ($n = 12$) and a higher 10 mM dose ($n = 36$). The first group was tested using the lowest dose (10 μ M) expected to produce a behavioural effect based on previous evidence (Montecucco et al., 2016). To prepare dose 1, the stock solution was diluted with saline to a concentration of 10 μ M and 0.01% DMSO. When no behavioural effect was observed, the dose was increased for the second group (dose 2). To prepare dose 2 drug injections, the stock solution was diluted with saline to a concentration of 10 mM and 10% DMSO. Dose 1 vehicle injections were 0.01% DMSO in saline. Dose 2 vehicle injections were 10% DMSO in saline. All injections were given intraperitoneally (IP).

2.4 Experimental Procedure

2.4.1 Schedule Induced Polydipsia Procedure

The rats had *ad libitum* water access in their home cage for the entire experimental protocol, but were restricted to one or two-hour free-feeding periods for the duration of the protocol. Free-feeding intake was measured by weighing the pellets before and after the free-feeding period. Home cage water intake was determined by weighing cage water bottles every 24 hours. The SIP procedure consisted of a seven-day pre-SIP food restriction (diet day 1 – 7) followed by a minimum 21 days of SIP training (Figure 3). During the seven-day pre-SIP food restriction, free-feeding time was kept at two hours to ensure rats remained within 85-90% of their original weight. Once SIP training had commenced, free-feeding time was reduced to one-hour to counterbalance the 120 food pellets dispensed during the training session (Angelis et al., 2019; Hawken et al., 2019).

Animals were run in four separate cohorts, consisting of 12 animals each. Following the seven days of pre-SIP food restriction, rats began SIP training and were placed in operant conditioning chambers for two hours per day. The first three groups ($n = 36$) underwent SIP training for 22 consecutive days, while the last group ($n = 12$) underwent SIP training for 33 days. During each two-hour session, a 45 mg dustless precision food pellet (Cederlane Labs, Burlington, ON) was delivered in a food dispenser tray on a fixed-time 60-second schedule, resulting in a total of 120 pellets delivered per session. Rats had unlimited access to the waterspout during each SIP session, and intake was determined by weighing the bottles before and after the session. To be in line with previous studies (Hawken et al., 2019; Angelis et al., 2019) and for characterization of the behaviour developed throughout the procedure, rats were classified as High Drinking (HD) if they drank 15 mLs or above for three consecutive days.

Otherwise, rats were classified as Low Drinking (LD). Within the protocol, we expected ~50% of rats to develop high drinking behaviour by day 21 of SIP training.

2.4.2 Effects of GPR55 Antagonism on SIP

Two injection protocols, a single administration and repeat administration, were utilized in this study (Figure 3). For single administration, animals ($n = 36$) received two vehicle injections and one drug injection of the GPR55 antagonist CID (Tocris Bioscience, Oakville, ON). Within the first protocol, 12 animals received a 10 μ M dose and 24 received a 10 mM dose. Vehicle injections were given on day 9 and 19, while drug injections were given on day 21 of SIP training. The 10 μ M dose group received vehicle injections of 0.01% DMSO in saline (0.6 mL, IP) and a 10 μ M CID injection diluted in 0.6 mL of 0.01% DMSO in saline (IP). The 10 mM dose group received vehicle injections of 10% DMSO in saline (0.15 mL, IP) and a 10 mM CID injection diluted in 0.15 mL of 10% DMSO in saline (IP) (Figure 3). The last cohort of animals ($n = 12$) underwent a repeat administration protocol with the 10 mM dose. Similar to single administration, vehicle injections (0.15 mL, 10% DMSO in saline, IP) were given on day 9 and 19. Four consecutive days of CID injections (10 mM CID diluted in 0.15 mL 10% DMSO, IP) were given day 21 through 24 of SIP training. Animals then underwent SIP training for four days with no injections. Finally, the animals underwent four consecutive control injections (0.15 mL, 10% DMSO in saline, IP) on day 29 through 33 of SIP training. Following injections for both protocols, animals were put in their home cages for fifteen minutes and then given access to the operant conditioning chambers where they completed two hours of SIP training as per usual. Animals underwent SIP training for an additional day following CID injection (single administration) or the last repeat control injection (repeat administration).

2.4.3 Post-training Procedures

On day 23 (single administration) and 33 (repeat administration), rats were humanely euthanized using carbon dioxide gas (flow rate = 4.90 liters/minute). Following cessation of breathing, heartbeat, and no response to toe pinch, a secondary measure of euthanasia by decapitation was completed.

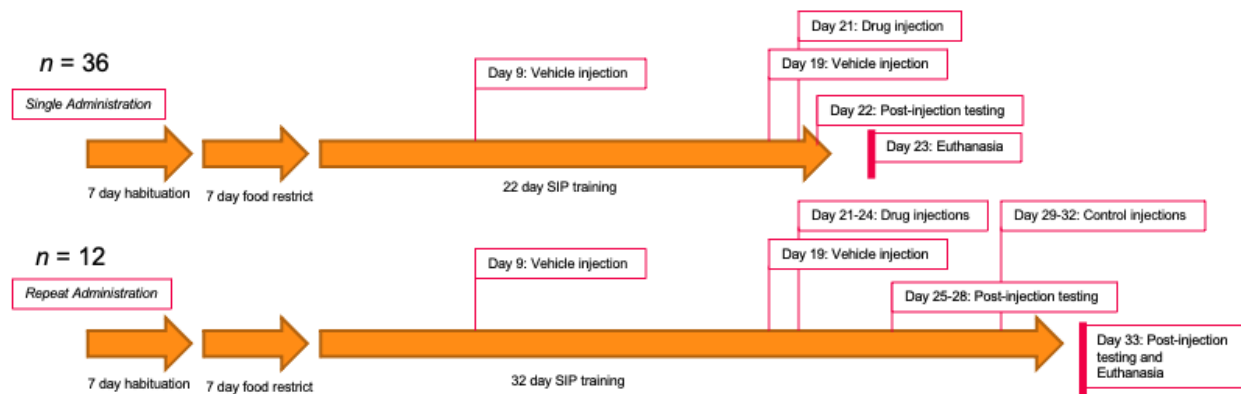


Figure 3. Experimental protocol for SIP paradigm with adaptation for drug injections.

Separate timelines are outlined for the single versus repeat administration protocol.

Note. SIP = schedule-induced polydipsia

2.5 Measures

Throughout the experiment animal weight (g) and food intake (g) were measured each day. In the final cohort, home cage water intake (mL per 24 hrs) was also measured daily. Water intake was measured for every SIP training session by taking the difference in bottle weight before and after each session. An overview of measures used in analyses is outlined in Table 1.

The primary measure of baseline water intake (Baseline A) is the average water intake during SIP training on the three days prior to the second vehicle injection (SIP 16-18). Due to animals naturally increasing their water intake as SIP training progresses, an alternative baseline (Baseline B) was also calculated for comparison (Baseline B, Table 1). For animals undergoing repeat administration, a third baseline (Baseline C) was calculated between repeat drug and vehicle injections. In line with previous work, animals were classified as HD if they consumed 15 mL or greater during SIP training in the three consecutive days prior to the drug injection (HD Criteria 1). As there is no consensus on criteria for HD classification in the literature, two alternative ways of classifying HD were compared (Table 1, HD Criteria 2 and 3). For the primary outcome, CID-induced water intake is the difference between water intake following CID injection (day 21) and water intake following the vehicle injection (day 19).

Several secondary measures were used in post-hoc analyses. Free food intake, average weight, end weight, percent change in body weight, and average home cage water intake were assessed (Table 1).

Table 1. Summary of Measurement Criteria

Measure	Criteria
Baseline water intake (Baseline A, primary measure used)	Average water intake (mL) during two-hour SIP training on days 16-18 (three days prior to the second vehicle injection)
Baseline B	Average water intake (mL) during two-hour SIP training on days 18-20 (three days prior to the CID injection)
Baseline C	Average water intake (mL) during two-hour SIP training on days 25-28 (during recovery period between repeat drug and vehicle injections)

HD Criteria 1 (primary measure used)	Consumed ≥ 15 mLs during two-hour SIP training on 3 consecutive days leading up to CID injection
HD Criteria 2	Consumed an average of ≥ 15 mLs during two-hour SIP training between the 3 consecutive days leading up to CID injection
HD Criteria 3	Consumed above the group median (mLs) on day 20 (the day prior to CID injection)
CID-induced water intake	Difference score (mLs) between SIP training water intake following CID injection and vehicle injection
Free food intake	Average free-food intake per body weight (g/g) over the first seven days of SIP training (prior to when food intake had to be controlled to keep animals within 85-90% weight)
Average weight	Average body weight (g) over days 16-18, the same days used to calculate Baseline A
End weight	Body weight (g) on day 20 of protocol
Percent change in body weight	Percent change in body weight (%) from body weight on day 20 (g) to body weight at commencement of food restriction (g)
Average home cage water intake	Average home cage water intake in 24 hrs between days 16-18, the same days used to calculate Baseline A

2.6 Statistical Analysis

This protocol had a sample size of $n = 48$. However, only 47 animals (10 μ M dose, $n = 12$ and 10 mM dose, $n = 35$) were included in data analysis due to malfunctioning equipment that led to measurement error for one of the animals.

2.6.1 Characterization of Behavioural Procedure

The secondary objective of this project was to explore and characterize the behavioural components of the SIP procedure. A post-hoc exploratory approach was taken following data collection. Descriptive and inferential statistics were run to characterize behavioural and physiological factors that may impact outcomes in the SIP paradigm. Normality was assessed using the Shapiro-Wilks test of normality. Non-parametric statistical tests were used if data was not normal, changes are noted below. Differences between Baseline A and Baseline B were first evaluated using a paired samples Wilcoxon Signed Ranks test. Next, frequencies of LD vs. HD animals based on classification criteria were explored. Pearson correlations were then run to assess the relationships between free food intake, average weight, and average home cage water intake to the primary baseline measure (Baseline A). Finally, predicting factors for HD behaviour, such as average weight, percent change in weight, and average food intake, were examined using logistical regression.

2.6.2 Preliminary Pharmacological Analyses

A preliminary related-samples Friedman's Two-Way ANOVA (non-parametric) was run to assess if there was an effect of the vehicle injection on water intake by comparing water intake at three-time points, pre-injection, vehicle injection, post-injection. The within subject's variable

was water intake at the three respective time points. Pre-injection measures were the animal's baseline drinking score, calculated by averaging water intake of the three days prior to the second vehicle injection. Vehicle injection measures were the animal's water intake on the second vehicle injection. Post-injection scores were the animal's water intake on the day following the vehicle injection.

A preliminary one-way ANCOVA was run to assess if there was a significant difference between LD and HD groups on CID-induced water intake following administration of the pilot dose (10 μ M). As LD and HD groupings are somewhat arbitrary, baseline water intake (Baseline A) was included as a co-variate to assess the relationship between prior drinking levels and CID-induced water intake.

Differences in CID-induced water intake between the 10 μ M dose and 10 mM dose were then assessed using a Mann-Whitney U test (non-parametric).

2.6.3 Analysis of Effect of GPR55 Antagonist CID

Pearson's correlations were run to assess the association between the variables of percent change in body weight, average weight, average home cage water intake, and average food intake with CID-induced water intake.

Differences in measurement criteria for CID-induced water intake were assessed. Difference scores for water intake were calculated between water intake during SIP training on day 21 following CID injection and Baseline A, Baseline B, vehicle injection (day 19), and day 20. A qualitative comparison was completed for the four different measures of CID-induced water intake.

The Shapiro Wilk's test of normality determined that CID-induced water intake was not normally distributed for the 10 mM dose ($n = 36$). As such, a one-way Quade's ANCOVA (non-parametric) was then conducted to determine if there was a significant difference between LD and HD groups on CID-induced water intake. Similar to above, baseline water intake (Baseline A) was included as a co-variate. Using the Quade method of co-variate assessment in non-parametric data, residuals from a linear regression of the ranked covariate to the ranked outcome variable (CID-induced water intake) are assessed (Cangür et al., 2018). This allowed for the comparison of means of the dependent variable (CID-induced water intake) across the levels of the independent variable (LD vs HD classification) while accounting for the effects of the covariate (baseline drinking). HD Criteria 1 was used for classification of LD and HD.

A paired samples T-test was first conducted to assess if there was a significant increase in drinking from baseline water intake (Baseline A) to the end of repeat administration (day 24). This was followed up with an ANCOVA investigating if repeat CID-induced water intake was significantly different between LD and HD groups, with the co-variate of baseline drinking.

Another paired samples T-test was then conducted to compare repeat CID injections to repeat vehicle injections. Repeat CID-induced water intake was measured as the difference score from the fourth CID injection and Baseline A. Repeat vehicle-induced water intake was measured as the difference score between the fourth repeat vehicle injection and newly established Baseline C.

Chapter 3

Results

3.1 Characterization of Behavioural Protocol

3.1.1 Validity and Replicability of the Schedule-Induced Polydipsia (SIP) Model

Animals ($n = 47$) gradually increased water intake during the two-hour daily SIP training as the protocol progressed (Figure 4). As expected, SIP training elicited high drinking behaviour in a subset of animals ($n = 30$, 64%). Most high drinking animals reached the high-drinking criteria in 10 days \pm 3.

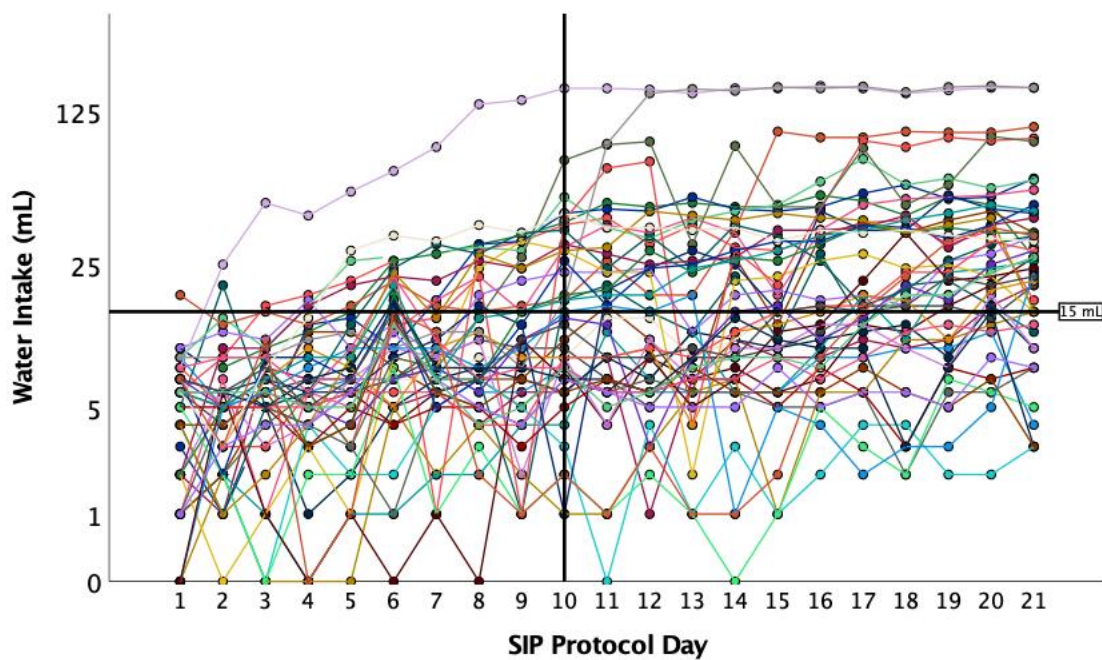


Figure 4. Progression of water intake during the 2 hrs of daily SIP training over the 21-day protocol. Intersecting black lines represent the 10-day mark where most animals reached high drinking criteria.

3.1.2 Comparison of Baseline Drinking Criterion

A paired samples Wilcoxon Signed Ranks test showed there was a statistically significant difference in baseline water intake measures when using Baseline A (average water intake during SIP training days 16-18) compared to Baseline B (average water intake during SIP training days 18-20), $Z = -3.14$, $p = .002$. Baseline B ($Mdn = 24.00$) was significantly greater than Baseline A ($Mdn = 17.33$) (Figure 5).

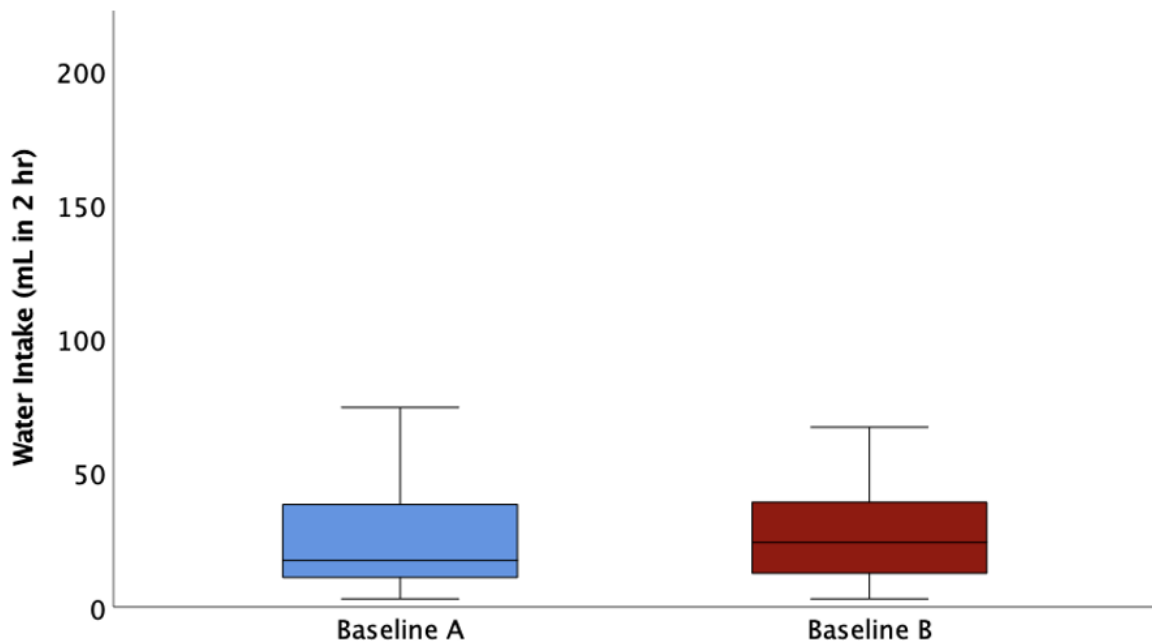


Figure 5. Comparison of median baseline water intake during daily two-hour SIP training for Baseline A and Baseline B.

3.1.3 Classification of Low and High Drinking Behaviour

Descriptive statistics were used to compare LD vs. HD frequencies using the main classification (HD Criteria 1, >15 mLs for three consecutive days prior to CID injection) to two alternative criteria's, HD Criteria 2, and HD Criteria 3 (Figure 6). Using HD Criteria 1, there were 30 (63.8%) high drinkers and 17 (36.2%) low drinkers. Using HD Criteria 2 (an average of

>15 mLs over the three days prior to CID injection), there were 34 (72.3%) high drinkers and 13 (27.7%) low drinkers. HD Criteria 3 was based on the group median on day 20. The median was 24 mLs, 9 mLs higher than what the threshold for HD behaviour was for the other two criteria. As expected, the split was 24 (51.1%) high drinkers and 23 (48.9%) low drinkers.

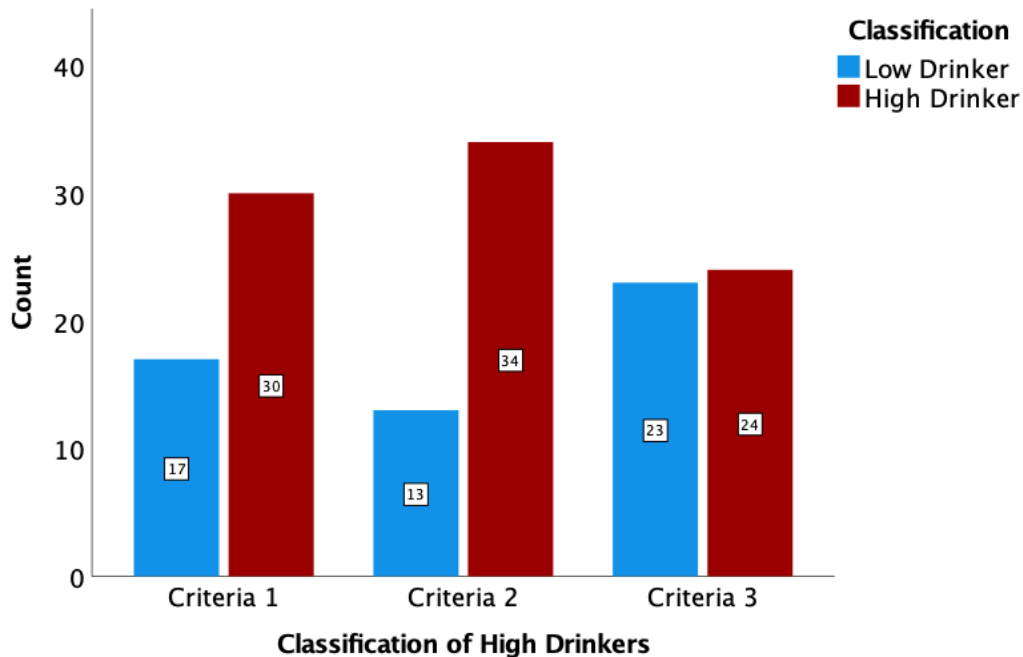


Figure 6. Count of low drinkers to high drinkers for three different classification criteria. Criteria 1 is based on drinking ≥ 15 mLs for three consecutive days prior to CID injection. Criteria 2 is based on drinking an average of ≥ 15 mLs between the three days prior to CID injection. Criteria 3 is based on consuming above the group median ($Mdn = 24$) for water intake the day before CID injection.

3.1.4 Relationship between Food Intake, Weight, and Drinking Behaviour

A Pearson's coefficient correlation was run to assess the relationship between average food intake and baseline water intake (Figure 7, A). There was no significant correlation between the average free-food intake at the beginning of SIP training and baseline water intake (Baseline A), $r(45) = .209$, $p = .159$; correlating with Baseline B yielded similar results.

Average weight and baseline water intake (Baseline A) were also correlated (Figure 7, B). There was no significant correlation between average weight and baseline water intake, $r(45) = .093$, $p = .533$; similar results were found when using Baseline B.

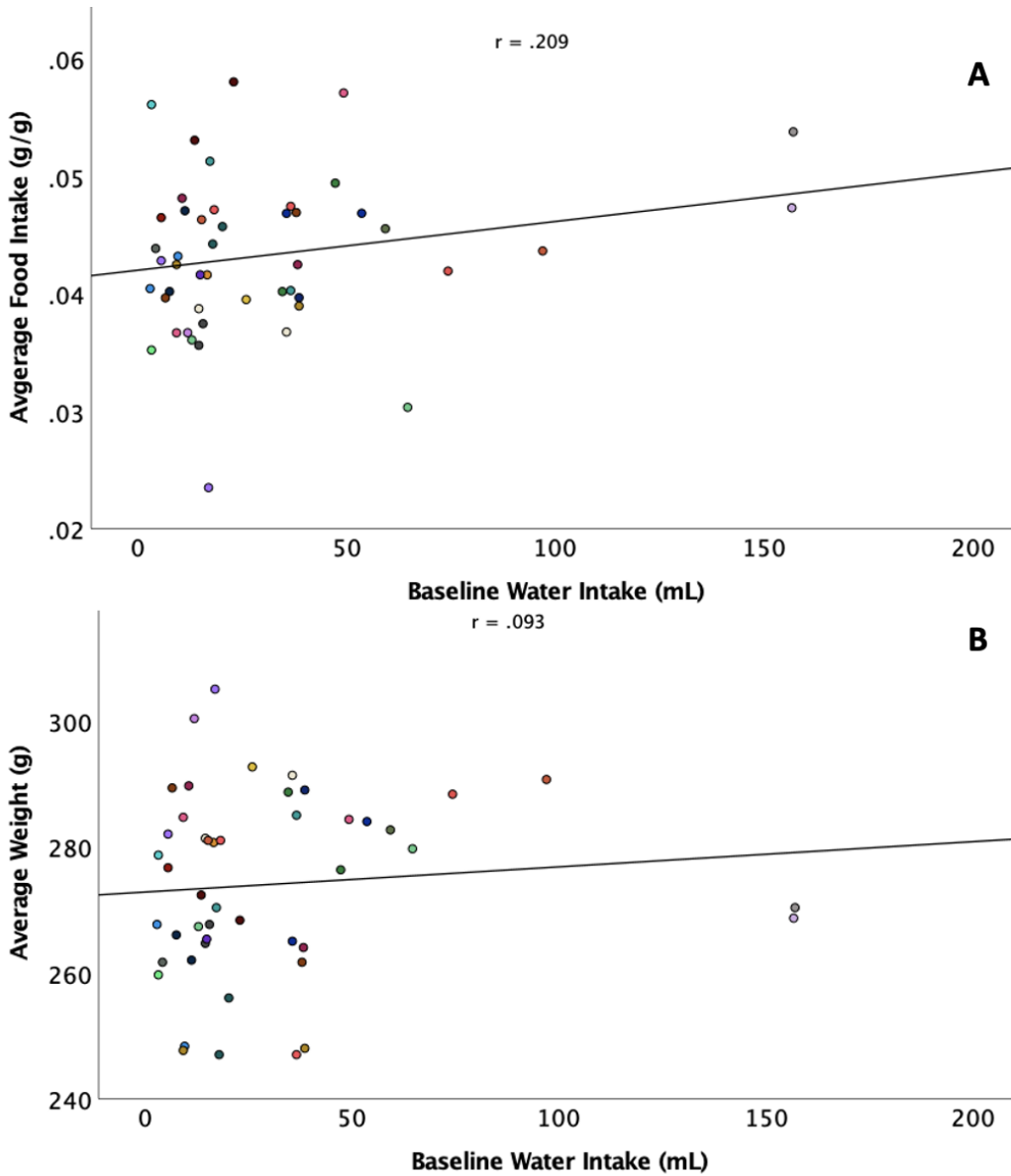


Figure 7. Association between baseline water intake during SIP training (2 hr) and food intake and weight. Each coloured dot represents a rat. **A.** Association to average food intake within the first week (day 1-7). **B.** Association to average body weight day 16-18.

3.1.5 Relationship between Home Cage Water Intake and Baseline Water Intake

A Pearson's coefficient correlation was run to assess the relationship between average home cage water intake and baseline water intake (Baseline A) (Figure 8). There was no

significant correlation between average home cage water intake and baseline water intake, $r(22) = -.176, p = .411$. An Independent Samples T-Test also showed no difference in average home cage water intake between animals classified as low and high drinkers, $t(23) = -1.264, p = .220$.

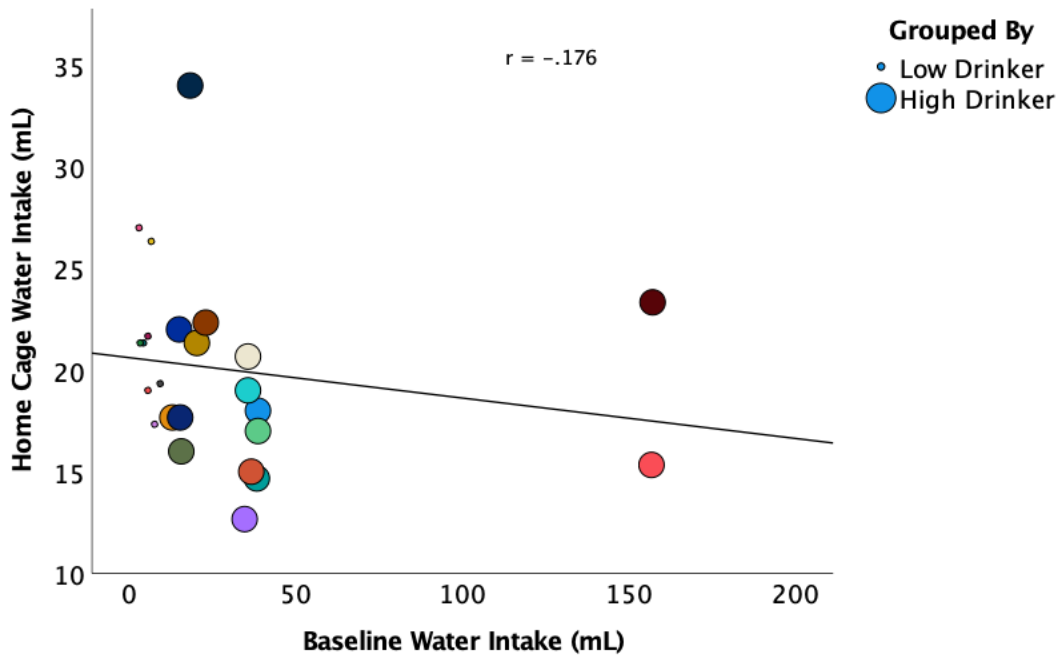


Figure 8. Association between average home cage water intake in 24 hrs and baseline water intake during two-hour SIP training on days 16-18 of the SIP protocol.

3.1.6 Predicting Factors of High Drinking Behaviour

A logistic regression was performed to ascertain the effects of end weight, percent change in weight, and average food intake on the likelihood of becoming a high drinker (Figure 9). The logistic regression model was not significant ($\chi^2(4) = 1.378, p = .711$). End weight, percent change in weight, and average food intake was estimated to explain 4.0% (Nagelkerke R^2) of the variance in drinking classification.

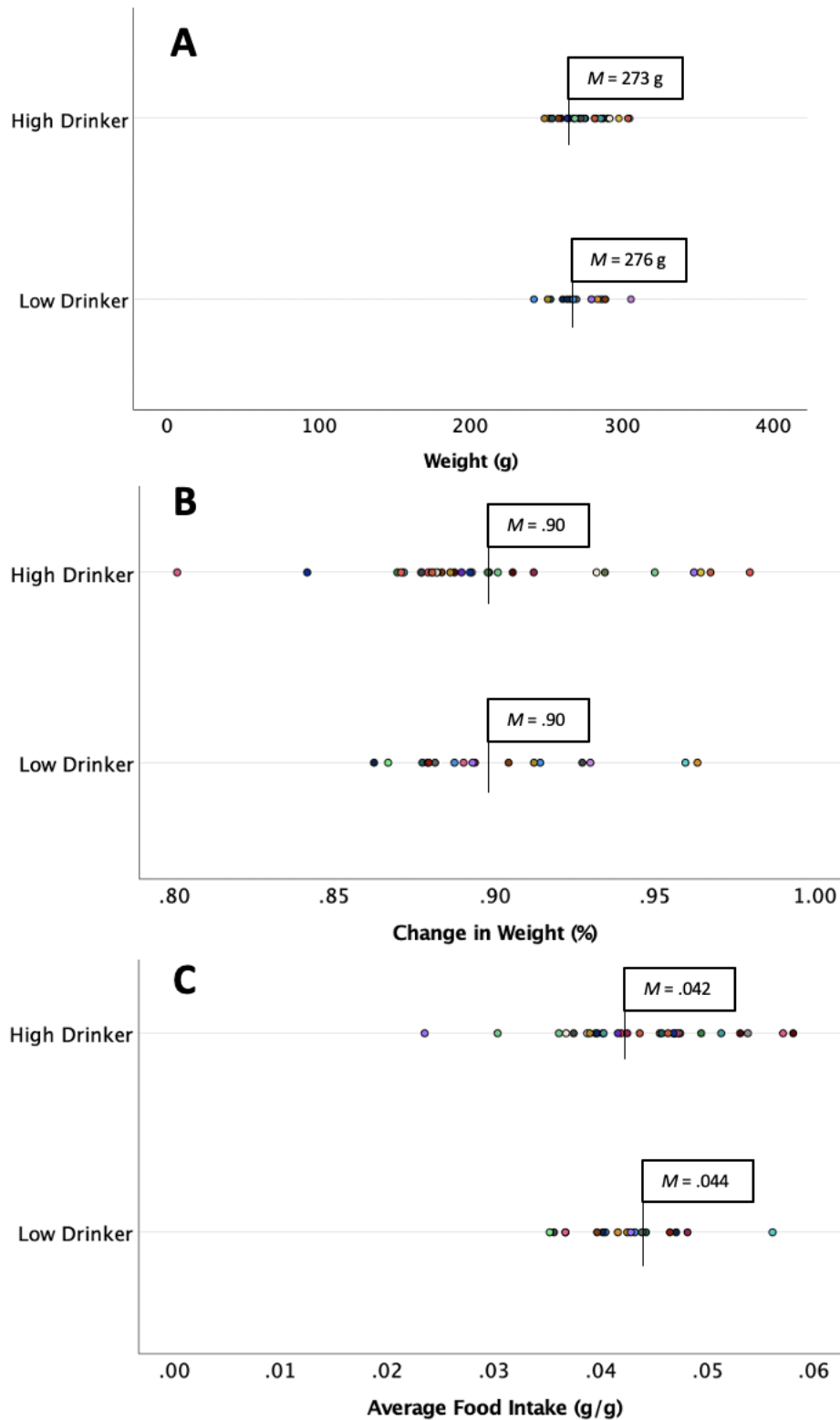


Figure 9. Potential predictors of high drinking behaviour **A.** End weight (g) on day 20 of SIP training for low drinkers and high drinkers. **B.** Average food intake (g/g) in the first week of SIP

training for low and high drinkers. **C.** Percent change from of original body weight at day 20 of SIP training for low and high drinkers.

3.2 Preliminary Pharmacological Analyses

3.2.1 Analysis of Vehicle Injection Effect

A preliminary related-samples Friedman's Two-Way ANOVA (non-parametric) was run to assess if there was an effect of the vehicle injection by comparing water intake at three-time points, pre-injection baseline, vehicle injection, post-injection. There was a significant effect found, $\chi^2_{F(2)} = 10.45$, $p = .005$. Pairwise comparisons revealed pre-injection baselines of water intake were significantly lower than post-injection water intake ($p = .004$). However, pre-injection baseline to vehicle and vehicle to post-injection comparisons were not significant ($p = .330$).

3.2.2 Analysis of pilot dose of GPR55 Antagonist CID

A one-way ANCOVA was conducted for the pilot dose (10 μ M) to determine if there was a significant difference for CID-induced water intake between LD and HD animals while controlling for baseline drinking levels. The co-variate, baseline drinking, was not significantly related to CID-induced water intake, $F(1, 9) = .579$, $p = .466$, $\eta^2 = .060$. There was also no significant relationship between LD vs HD grouping and CID-induced water intake, $F(1, 9) = .824$, $p = .388$, $\eta^2 = .084$.

Differences in CID-induced water intake between the 10 μ M dose and 10 mM dose were then assessed (Figure 10). Animals who received the 10 mM dose had significantly greater CID-

induced water intake ($Mdn = 2.00$, $IQR = -1 - 8$) than animals who received the $10 \mu\text{M}$ dose ($Mdn = -2.00$, $IQR = -10.75 - 1.00$).

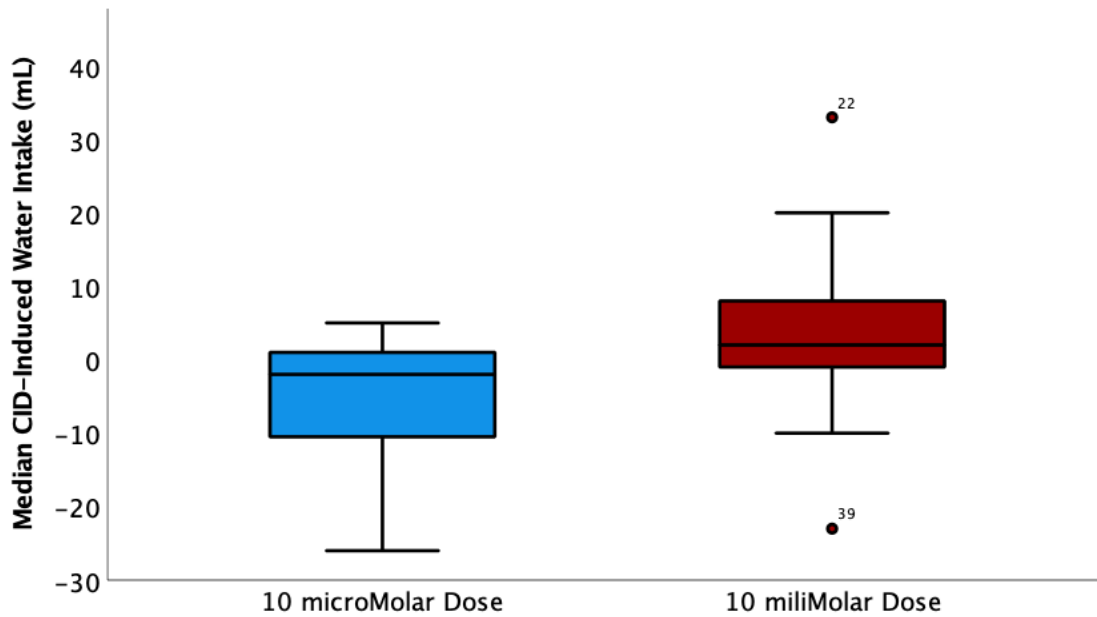


Figure 10. Median CID-induced water intake for $10 \mu\text{M}$ dose and 10mM dose. CID-induced water intake refers to the difference in water intake during 2 hr SIP training between CID injection and vehicle injection.

3.3 Analysis of CID Effect

3.3.1 Correlations between Food Intake, Water Intake, Weight, and CID-Induced Water Intake

Pearson's correlations were run to assess the association between the variables of percent change in body weight, average weight, average home cage water intake, and average food intake with CID-induced water intake. Percent change in body weight was not significantly correlated with CID-induced water intake, $r(33) = .163$, $p = .348$ (Figure 11, A). Average weight was not correlated with CID-induced water intake, $r(33) = .049$, $p = .779$ (Figure 11, B).

Average home cage water intake was not correlated with CID-induced water intake, $r(33) = .295$, $p = .165$ (Figure 12, A). Average food intake was also not correlated with CID-induced water intake, $r(33) = .114$, $p = .513$ (Figure 12, B).

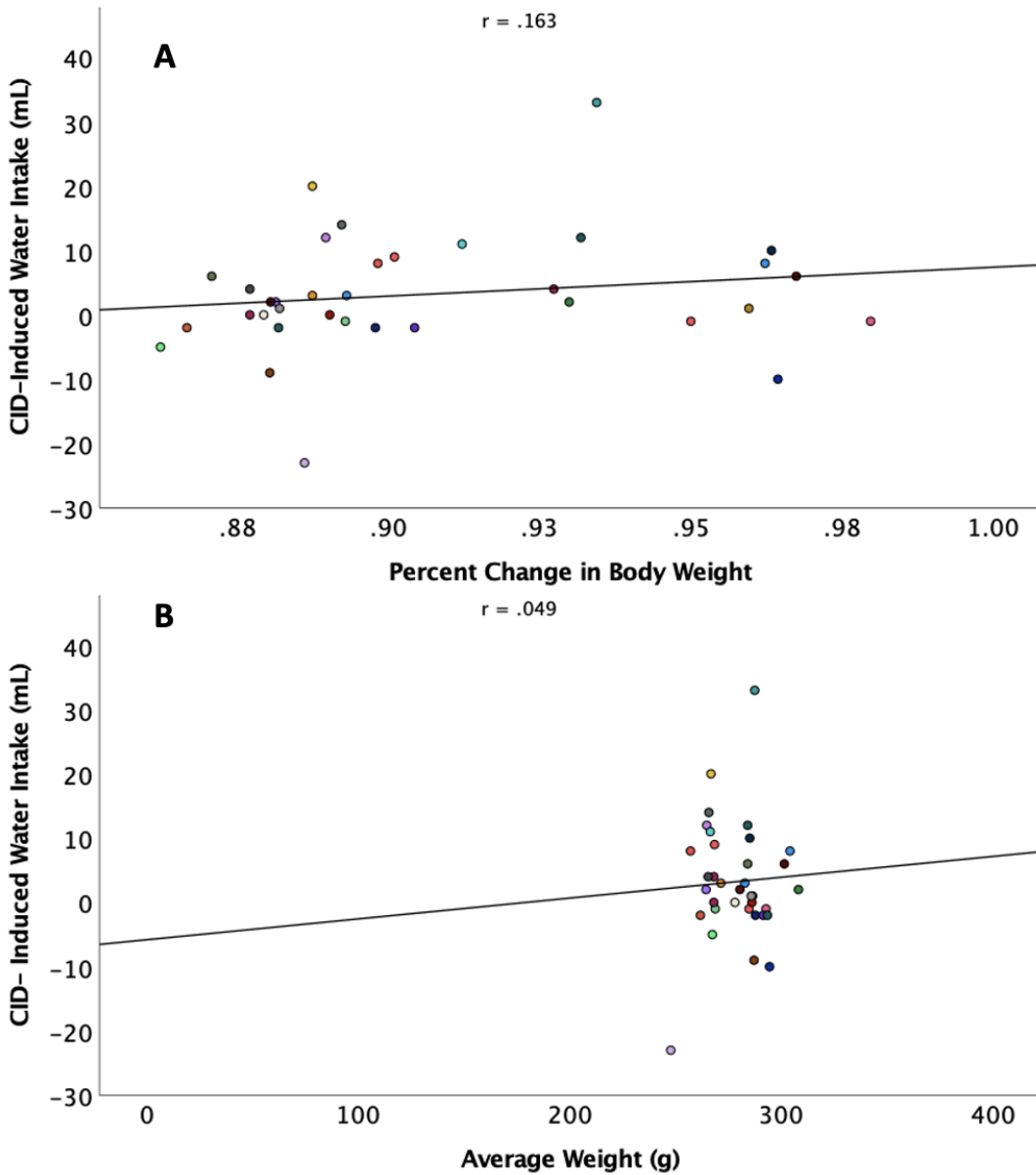


Figure 11. Association between weight and CID-induced water intake. **A.** Relationship between CID-induced water intake and percent change in body weight from start to end of protocol. **B.** Relationship between CID-induced water intake and average body weight (day 16-18)

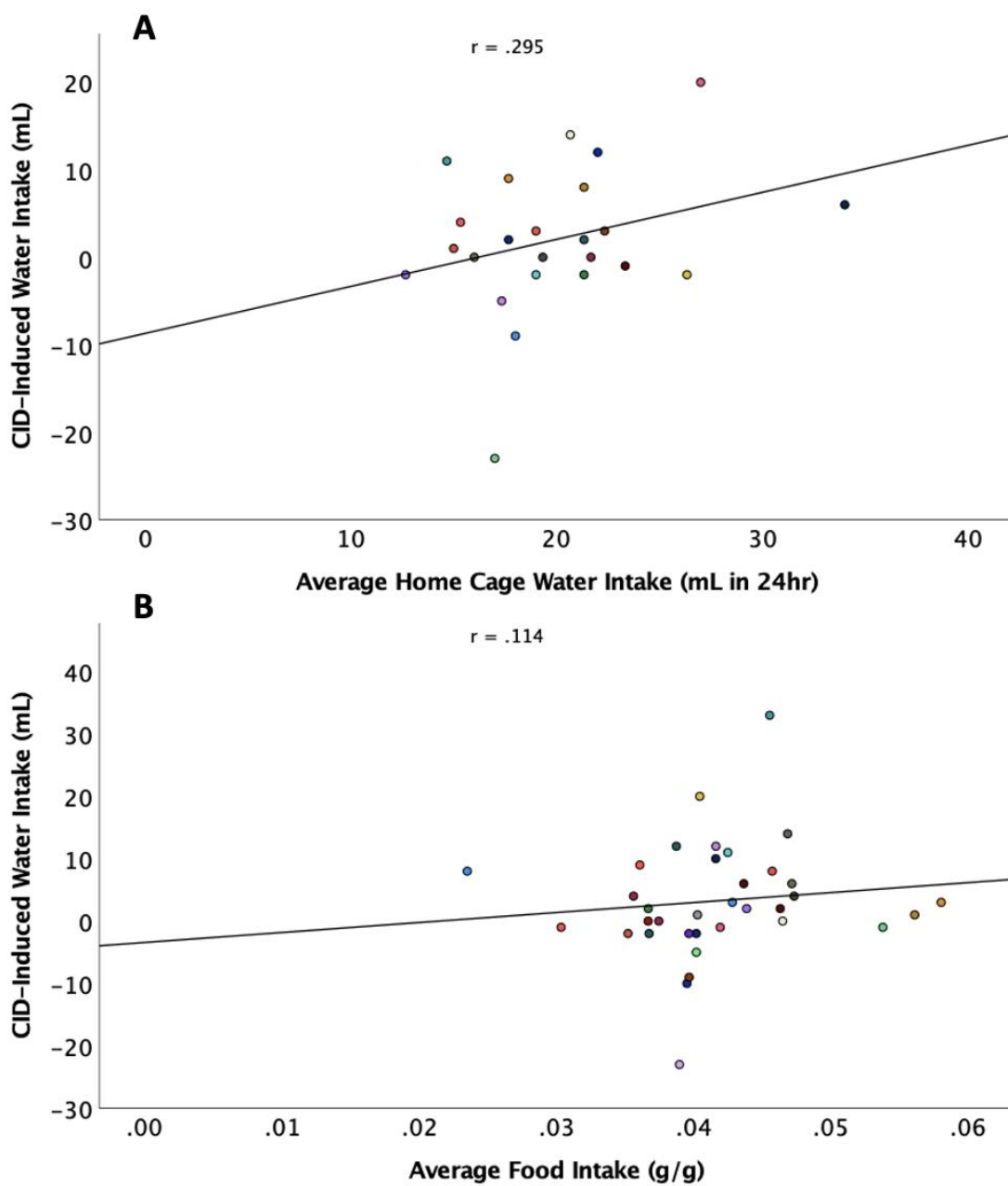


Figure 12. Association between CID-induced water intake and food and water consumption. **A.** Relationship with baseline home cage water intake (Baseline A). **B.** Relationship between average food intake (day 1-7 of SIP training).

3.3.2 Effect of GPR55 Antagonist CID on Water Intake Using Different Measurement Criteria

CID-induced water intake was defined as the difference score between water intake during 2 hr SIP training following CIP injection and Vehicle injection. To assess for the effect of time or measurement criteria, comparisons between water intake following CID injection and several different timepoints and measures was explored. Comparisons between Baseline A, Baseline B, Vehicle Injection, and day 20 were carried out (Figure 13). The greatest overall change, irrespective of LD or HD groupings, was when comparing Baseline A ($Mdn = 17$) to CID ($Mdn = 29$) ($Z = -3.163$, $p = <.01$), followed by comparing Baseline B ($Mdn = 24$) to CID (Figure 13, panel A). The effect began to lessen when comparing CID intake to the vehicle injection ($Mdn = 23$) ($Z = -.2320$, $p = .02$) and lost when comparing CID intake to day 20 intake ($Mdn = 25$) ($Z = -.654$, $p = .513$) (Figure 13, Panel B).

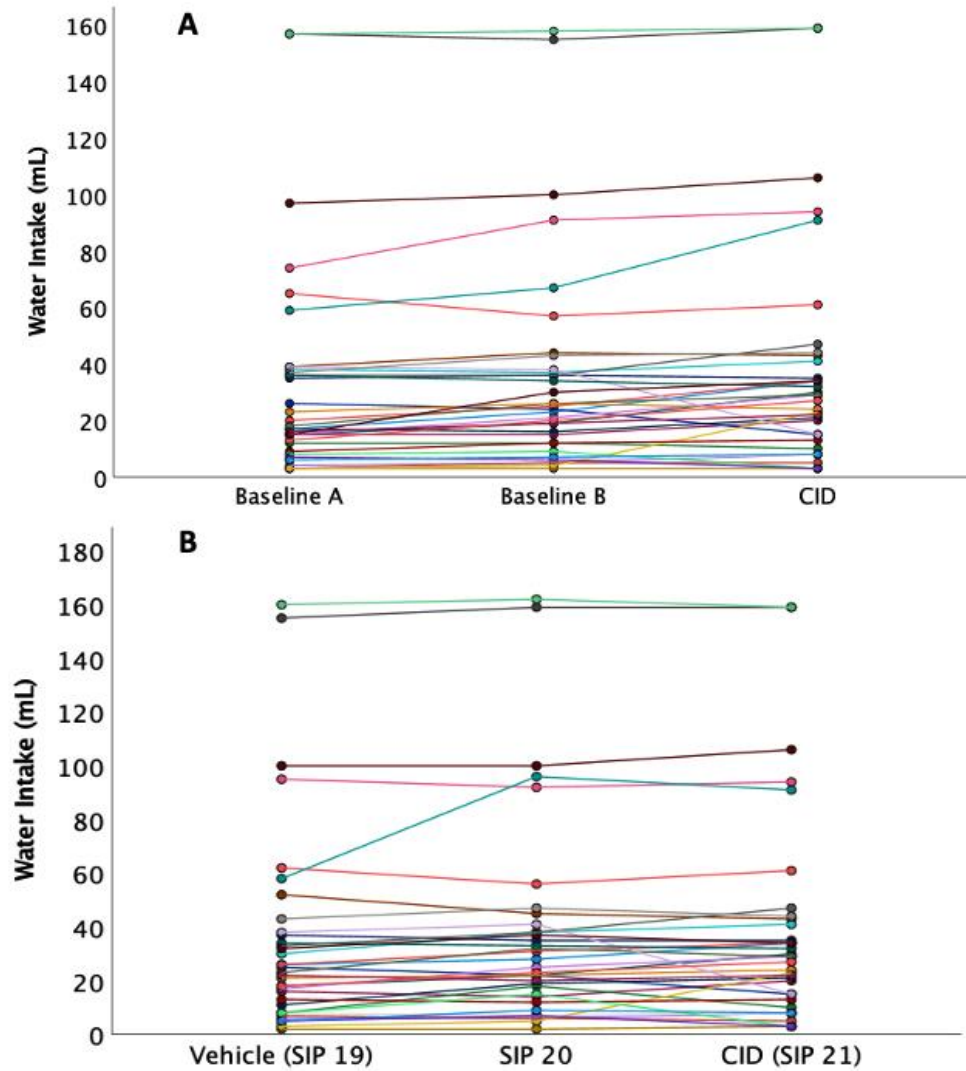


Figure 13. Water intake (mL) during 2 hr SIP training **A.** Water intake on CID injection day compared to Baseline A and Baseline B. Water intake for CID injection compared to vehicle injection and the day prior to CID injection (day 20).

3.3.3 Effect of Single Administration of GPR55 Antagonist CID on Drinking Behaviours

A one-way Quade's ANCOVA (non-parametric) was then conducted for the 10 mM dose ($n = 36$) to determine if there was a significant difference between LD and HD groups on water

intake while including baseline drinking levels as a co-variate (Figure 14). The covariate, baseline drinking, was not a significant predictor of CID-induced water intake, $R^2 = .026$, $F(1, 33) = .022$, $p = .884$. There was no significant effect of LD vs HD grouping on CID-induced water intake after controlling for baseline drinking, $F(1, 33) = .217$, $p = .644$.

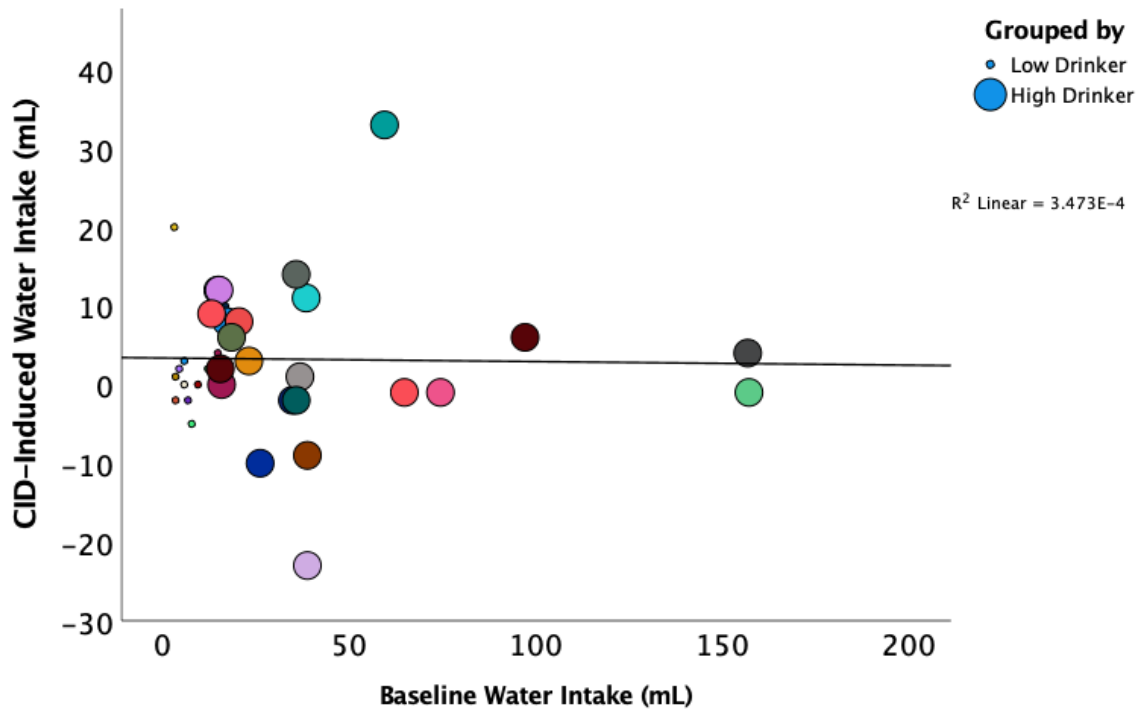


Figure 14. The relationship between baseline water intake (Baseline A) and CID-induced change in water intake (difference score between CID and Vehicle injection) during 2 hr SIP training sessions.

3.3.4 Effect of Repeat Administration of GPR55 Antagonist CID on Drinking Behaviours

An initial paired samples t-test showed a significant increase in water intake between Baseline A ($M = 21.22$, $SD = 13.66$) and water intake at the last day of repeat CID injections ($M = 29.50$, $SD = 16.27$), $t(11) = -2.711$, $p = .020$.

To investigate if this change differed depending on LD or HD grouping or baseline drinking levels, a one-way ANCOVA was run. Although trending towards significance, the covariate of baseline drinking was not significantly related to repeat CID-induced water intake, $F(1, 9) = 5.007, p = .052, \eta^2 = .357$. There was a significant effect of LD vs HD grouping on CID-induced water intake after controlling for baseline drinking, $F(1, 9) = 6.615, p = .030$. High drinkers had a significantly greater increase in water intake ($M = 10.71, SD = 12.27$) compared to low drinkers ($M = 3.42, SD = 3.4$).

However, a Paired Samples T-Test assessing changes to water intake between Baseline C (average of water intake on four injection recovery days) and repeat vehicle injections also showed a significant difference in water intake ($t(11) = -3.941, p = .002$), with significantly higher water intake found after repeat vehicle injections ($M = 35.92, SD = 15.27$) than at Baseline C ($M = 31.08, SD = 16.53$).

Finally, another Paired Samples T-Test assessing water intake between repeat CID-induced water intake to repeat vehicle-induced water intake found no significant difference between the two, $M_{CID} = 8.23, M_{vehicle} = 4.83, t(11) = 1.08, p = .304$ (Figure 15).

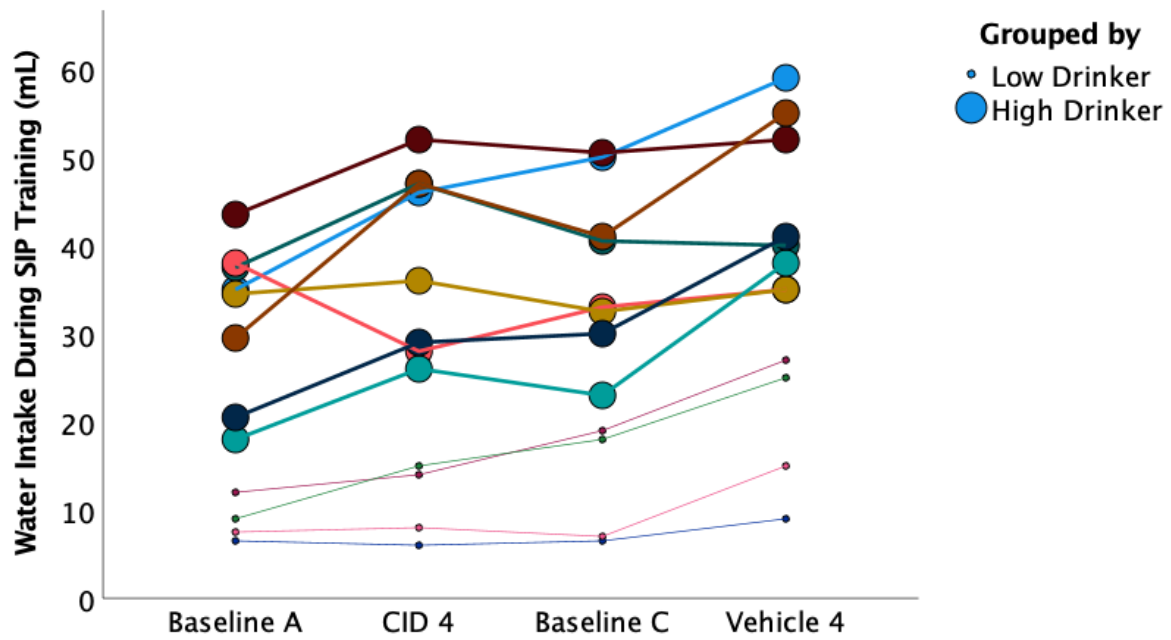


Figure 15. Progression of water intake during 2 hr SIP training for repeat administrations. CID 4 refers to water intake following the last repeat CID injection. Vehicle 4 refers to water intake following the last repeat vehicle injection.

Chapter 4

Discussion

Compulsive-type disorders cause significant impairment in the lives of those that experience them. The exact neurobiological underpinnings remain unclear, greatly hindering the ability to develop efficacious treatment options. Accumulating evidence has shown SIP to be a robust and ecologically valid model to study compulsions (Brett & Levine, 1979; Falk, 1961; Flores et al., 2014; Moreno & Flores, 2012; Platt et al., 2008). Investigations into the subset of animals that developed compulsive drinking uncovered bi-directional GABA plasticity mediated by signalling between LPI/GPR55 and 2-AG/CB1 within the BNST that was heavily influenced by metabolic state (Angelis et al., 2019; Hawken et al., 2019). Neurophysiological correlates revealed that when going from a hunger to a sated state, rats with normal drinking behaviours switched from CB1 mediated GABA depression to GPR55 mediated GABA potentiation (Angelis et al., 2019; Hawken et al., 2019). However, compulsive, high drinking rats remained in 2-AG/CB1 mediated GABA depression leading to a chronic down-regulation of GPR55 signalling and, therefore, inhibition of GABA signalling (Angelis et al., 2019; Hawken et al., 2019). These findings warranted greater investigation into the role of GPR55/CB1 signalling in the development of compulsions.

To further explore the role of GPR55/CB1 signalling, this project used a behavioural pharmacological approach to investigate whether deficient GPR55 signalling leads to the development of compulsive drinking behaviours in the SIP rat model. A secondary objective of this project was to characterize the behavioural procedure and investigate potential profiles associated with drinking behaviour in this model. Assessment of measurement criteria revealed

there were significant differences in baseline drinking outcomes and the proportion of HD rats, depending on which criteria was used. Using a slightly earlier time period for baseline water intake calculations resulted in a significantly lower baseline water intake score. Using 15 mL threshold criteria resulted in a greater number of HD rats compared to using the Median criterion (24 mLs). Neither average food intake, average weight, or home cage water intake was associated with baseline drinking levels in SIP training. HD classification was also not predicted by average weight, percent change in weight, or average food intake. These findings raise an important discussion on how certain measures in the SIP protocol may impact results. Additionally, a lack of association between drinking behaviour and several measures related to energy homeostasis give valuable information on where potential vulnerabilities may not be.

The primary objective of this project was to investigate if there is a causal link between deficient LPI/GPR55 signalling and the development of compulsive drinking. First, an exploratory investigation of measures that may predict or influence the effect of the GPR55 antagonist CID on drinking was completed. The impact of measurement criteria was once again assessed. CID-induced water intake, measured as a difference score, varied depending on which intake measures were subtracted. When intake on day 21 (CID injection) was compared to intake at an earlier time point in the protocol (e.g., Baseline A) the difference score was largest. When intake on day 21 was compared with intake at a later time point (e.g., day 20) the score was smallest. Assessments on measures of body weight, average food intake, or home cage water intake were not associated with CID-induced water intake. These results show that indicators of energy homeostasis did not impact the response to GPR55 antagonism.

Finally, I hypothesized that the pharmacological blockade of GPR55 would alter the behaviour of low drinking rats to meet the criteria for high drinking rats. This investigation

revealed that LD/HD grouping and baseline drinking levels did not predict a change in water intake following administered with GPR55 antagonist CID. Specifically, LD animals did not display high drinking behaviour, as was hypothesized. No drug effect was observed for either single or repeat administrations of CID. Repeat IP injections did induce an increase in water intake for HD animals, but this effect was also seen for repeat vehicle injections. Therefore, this was not deemed a drug effect. Finally, although no drug effect was seen for the primary analyses, differential effects of drug dose were observed. The higher 10 mM dose of CID elicited an increase in water intake compared to the lower 10 μ M dose. One interpretation of these findings is that dosing was not sufficient to induce a noticeable effect. Alternatively, this may give support to the influence of other signalling mechanisms involved with compulsive, high drinking behaviour. The blockade of the GPR55 receptor alone may not be sufficient to induce widespread behavioural change.

Although findings were non-significant, this experiment serves as a useful pilot study to assess the translation of correlational neurophysiological findings to pharmaco-behavioural outcomes. This study supports the reliability of the SIP paradigm to investigate compulsions in rats, while also raising some important considerations on measurement criteria. Additionally, it serves as a good starting point to further investigate the role of GPR55/CB1 signalling in the development of compulsive phenotypes.

4.1 Characterization of Behavioural Protocol

4.1.1 Validity and Replicability of the Schedule-Induced Polydipsia (SIP) Model

A secondary objective of this project was to characterize the behavioural component of the SIP paradigm. These findings support the reliability of the SIP model to induce compulsive

behaviours in rats. Consistent with the established experimental protocol, there was a progressive increase in water intake as the protocol went on (Figure 4) (Falk, 1961, 1966; Platt et al., 2008). Importantly, a subset of animals diverged into high drinkers, while the rest did not, allowing for a unique opportunity to look at triggers or associated risk factors for the maladaptive phenotype (Moreno & Flores, 2012; Rosenzweig-Lipson et al., 2007; Toscano et al., 2008). This closely mirrors human psychiatric conditions, in which some individuals are at a greater risk than others for developing psychiatric conditions. Further, SIP is based on primary polydipsia, which is also seen in humans (Dundas et al., 2007; Hariprasad et al., 1980; Hawken et al., 2009). The replicability and relevancy of SIP to human conditions posits SIP as a strong animal model for compulsivity (Angelis et al., 2019; Bale et al., 2019; Hawken et al., 2019; Moreno & Flores, 2012; Nestler & Hyman, 2010; Platt et al., 2008). Many currently available investigative tools do not allow for the comprehensive study of the underlying neurobiology of compulsive phenotypes. This has left a knowledge gap that needs to be filled before the next generation of treatments can be developed. The split in behavioural profiles seen within the SIP paradigm serves as a starting point to fill this gap and investigate the genetic and neurobiological profiles that may be a core component of compulsive, maladaptive phenotypes.

4.1.2 Considerations for Water Intake Measures and HD Classification

Assessment of several different criteria for measuring baseline water intake and HD classification revealed significant differences depending on which criteria were used (Figure 5). For baseline water intake measures, using an earlier range of days (days 16-18 vs days 18-20) resulted in a significantly lower baseline. This shows the impact of repeated SIP testing, in which water consumption increases significantly as the protocol progresses. Therefore, the obtained

measure could vary depending on which time point the measure was from (e.g., early versus later in the protocol). This could lead to an exaggerated or diminished effect when comparing the baseline consumption to intake following some experimental manipulation. As such, measures using difference scores or comparisons between time points should be carefully assessed for indication of biasing results.

There is little consensus on which criteria to use for HD classification. Previous work that provided the basis for the current project classified rats as HD if they consumed 15 mL or more of water during SIP training for three consecutive days (Angelis et al., 2019; Gregory et al., 2015; Hawken et al., 2019). Other studies classified rats as HD if they were above the group median in water intake (López-Grancha et al., 2008; Moreno et al., 2012; Navarro et al., 2015; Pellón et al., 2011). We investigated if using one criterion over the other would have an impact on the proportion of high drinkers. The group median was 24 mL, as such, when using the 15 mL criteria, a greater proportion of rats met criteria for HD (Figure 6). This indicates that rats within the 15-24 mL range could be classified as either low drinking or high drinking, depending on which criteria was used. This may significantly impact the ability to compare findings across studies, particularly when assessing genetic or neurobiological substrates that may differentiate low drinkers from high drinkers. In the future, it may be useful to classify animals into 3 groups, low drinkers, moderate drinkers, and high drinkers, to better compare distinct differences between animals on either end of the water intake spectrum.

4.1.3 Relationship between Food Intake, Weight, and Drinking Behaviour

To further investigate the influence of energy homeostasis on SIP, we sought to answer the question if weight and food intake were associated with drinking phenotypes. Caloric

restriction is necessary to induce excessive drinking within the SIP paradigm (Falk, 1961; Hooks et al., 1994). Consequently, the conversion of caloric deficit stress into motivated behaviours that re-establish energy homeostasis may be an underlying neural mechanism (Angelis et al., 2019). Contrary to what was expected, no measures of weight or food-intake correlated with baseline water intake measures or predicted which animals would become high drinkers (Figure 7 and 9). Previously, CB1 activation has been shown to cause hyperphagia and appetite stimulation (Cota, 2007). Yet, in the SIP paradigm, it appears that HD animals under chronic CB1 receptor mediation did not have elevated food intake compared to those who were lower drinkers. As CB1 is strongly implicated in reward processing, it could be that the drive for reward was satisfied through polydipsia, and thus the drive to eat was lessened. One consideration is that free-food intake was measured as the average food intake in the first seven days of SIP training. Most animals who increased their drinking into excessiveness did so after day 7, as such they most likely were not in a constant CB1 mediated state. This further supports the idea that compulsive, high drinking is not an inherent difference in energy balance or bi-directional GABA plasticity. Instead, this phenotype is triggered at a certain point, most likely by a combination of genetics and environmental stress. Interestingly, re-feeding animals abolishes high drinking while inducing compulsive water spout checking (Angelis et al., 2019). This shows that polydipsia is, indeed, calorie-deficit driven, however, the transfer to compulsive checking suggests that CB1 mediated LTD^{GABA} may be an underlying neural substrate for compulsivity itself.

A major limitation in exploring this relationship was the need to keep animals at a food restricted weight. By day 9 of SIP training, food given during free-feeding had to be limited, sometimes drastically, to maintain a food-restricted weight. This limited our ability to get valid

measures of free-feeding behaviour throughout the entire protocol to properly assess the relationship between food intake and water intake. As feeding was restricted to 1-2 hours, with similar amounts of food given, conditions may have also been too homogenous to detect differentiation between low and high drinking animals.

While food intake represents behaviour earlier in the protocol, body weight was calculated as the average of day 16-18, once differences between LD and HD behaviour were well established. However, neither body weight nor percent change in body weight showed any discernable differences between low and high drinkers. Literature on the impact between body weight and polydipsia is varied. Falk (1969) reported rats at 80% vs 90% of their original body weight had no differences in SIP. However, greater weight loss was associated with greater polydipsia when the range was expanded to 60% vs 90% original body weight (Freed & Hymowitz, 1972). Within the current protocol, rats did not exceed 85% of their original body weight. A more drastic weight reduction may have been needed for a relationship to appear. Lower body weight inducing greater water intake during SIP training is in line with the hypothesis that high drinking states are “hunger” state driven by CB1-mediated LTD^{GABA}. However, these findings suggest that there is a threshold for body weight loss needed to induce an observable polydipsic effect.

4.1.4 Relationship between Home Cage Water Intake and Baseline Water Intake

Previous studies show that all animals slightly decreased home cage water intake throughout SIP (Angelis et al., 2019; Hawken et al., 2019). However, LD animals’ decreases are proportional to their increases in SIP intake. Conversely, HD animals drink in excess, increasing their total daily intake. We sought to investigate if there was a relationship between average

home cage intake and baseline water intake during SIP training on days 18-20. No relationship was found, in line with previous findings that all animals gradually decreased water intake (Figure 8). There was also no difference in average home cage water intake between animals classified as low and high drinkers. This supports the notion that that HD animals increase their daily water intake in excess, rather than decreasing home cage intake.

4.2 Analysis of CID effect

4.2.1 Correlations between Food Intake, Weight, and CID-Induced Water Intake

We sought to investigate if free-food intake and weight were associated with the response to the pharmacological intervention of GPR55 antagonist CID. As previously discussed, within HD states CB1 is the primary signalling receptor at GABA synapses. Rats that exhibit LD behaviours can toggle between CB1 and GPR55, dependent on metabolic state. Therefore, it was thought that animals who consumed more food and were at a higher weight would more likely be in a sated, or GPR55 driven state, and may have a greater response to GPR55 receptor antagonism. However, no relationship between feeding behaviour or weight and CID-induced change to water intake was found (Figure 11). While GPR55 is distributed in areas that suggest a role in energy expenditure and metabolism, such as the hypothalamus, GI tract, and adipose tissue (Imbernon et al., 2014; Simcocks et al., 2014), it's exact role is unclear (Marichal-Cancino et al., 2017). Results have been varied, and there are issues surrounding the lack of functional selectivity of the receptor. GPR55 mutations are linked to increased vulnerability to anorexia nervosa (Ishiguro et al., 2011). However, GPR55 KO mice did not show any difference in food intake compared to their littermates (Bjursell et al., 2016). The lack of association

between metabolic state and response to GPR55 antagonist CID found in this study further questions the receptors' exact role, if any, on feeding behaviour within the CNS.

There is more evidence supporting GPR55's role in peripheral metabolism. Hyperactivity of the GPR55/LPI system in humans has been associated with obesity (Marichal-Cancino et al., 2017; Tudurí et al., 2017). Additionally, GPR55 agonists increase plasma insulin levels while decreasing plasma glucose levels peripherally (Meadows et al., 2016; Romero-Zerbo et al., 2011). This may give evidence that GPR55 mediated peripheral processes are separate from GPR55 mediated CNS processes. Since drinking behaviour is mediated by the CNS, it may not correlate with a peripherally based measure such as weight.

While previous evidence supports the idea that GPR55 has some role in energy homeostasis and metabolism, it may be that isolated receptor activity does not have a direct relationship with feeding behaviour and weight. Alternatively, feeding and weight restrictions may have impacted the ability to fully assess this relationship.

4.2.2 Assessment of GPR55 Antagonist CID on Drinking Behaviours

The primary objective of this project was to investigate a causal link between deficient GPR55 signalling and high drinking behaviours. Animals with LD behaviour switch to LPI/GPR55 mediated signalling at GABA synapses when sated. As such, it was hypothesized that blocking GPR55 would switch rats into a CB1 mediated state, eliciting HD behaviour in previously LD rats. This study did not support our hypothesis, as neither LD/HD classification nor baseline drinking levels were associated with the effect of GPR55 antagonist CID (Figure 14). Although our hypothesis was not supported by the empirical observations, there are several implications of these findings that are discussed below.

4.2.3 Single versus Repeat Administration

When using a single drug administration protocol ($n = 36$), one dose of the GPR55 antagonist did not elicit any effect. As SIP builds over a number of days, it was reasoned that a one-time administration may not be sufficient to switch LD rats into chronic CB1-mediated LTD^{GABA}. No other repeat drug administration SIP studies have used CID, however, other investigations utilized repeat pharmacological interventions such as SSRI's, dopamine, or amphetamines (Hawken & Beninger, 2014; Íbias et al., 2016; Prus et al., 2015; Woods et al., 1993). Further, GPR55 is thought to have homeostatic functions and, as such, it may take multiple administrations to alter system signalling. Among the few studies that have utilized CID, two investigations that noted a drug effect, although peripheral, used repeat administration (Montecucco et al., 2016; Stančić et al., 2015). As such, the last cohort of rats ($n = 12$) underwent a four day repeat drug administration protocol.

Initially, a significant effect was found when solely considering the repeat CID administration. HD rats showed a significantly greater CID-induced water intake compared to LD rats, an opposite relationship to what was hypothesized. However, after comparing intake between drug- and vehicle treated animals, it was found that there was no difference between the effect of the drug versus vehicle injections (Figure 15). This suggests that the effect was not drug induced and, instead, was elicited by another manipulation. Two plausible explanations that may explain this are the effect of time and the effect of stress.

As the SIP protocol progresses so does water intake. We previously showed that, when comparing baseline measures between days 16-18 and 18-20, a significant increase in water

intake was seen at the later time period. Within the 20–30-day time range, high drinkers in particular experience a steady increase in drinking, which may explain the significant increase.

In addition to time, repeat administrations most likely acted as a stressor. Although the exact relationship is unclear, stress does appear to impact SIP. Food restriction serves as a chronic stressor that underlies the behavioural outcome of excessive drinking in an attempt to regain homeostatic balance. Excessive drinking is attenuated by an adrenalectomy and restored by corticosterone replacement (Levine & Levine, 1989), strongly linking the influence of stress to this phenomena. The BNST is also connected to the hypothalamic–pituitary–adrenal (HPA) axis, with evidence that the ovBNST contributes to maladaptive anxiety states (Kim et al., 2013; Normandeau et al., 2018). There is debate on the exact relationship between stress and SIP. Some propose that SIP may be a coping mechanism to stressful or aversive states (Brett & Levine, 1979, 1981; Moreno & Flores, 2012). Activity of the HPA axis is suppressed in an operant chamber (Brett & Levine, 1979). Additionally, corticosterone levels increase in polydipsic animals when no water bottle is in the operant chamber (Tazi et al., 1986). However, other findings do not always align with this explanation. Direct corticosterone administration and anxiogenic stimulation (e.g. foot shock) attenuate, not increase, SIP (Mittleman et al., 1988; Tang et al., 1984). Central administration of CRF also does not affect SIP (Cole & Koob, 1994). As such, the exact role of stress and the HPA axis in SIP remains undetermined (Banasikowski & Hawken, 2019; Moreno & Flores, 2012). However, the significant increase in water intake following repeat administration seen in this study supports that stress may increase SIP, potentially through the aversive state coping hypothesis. Neuroendocrine measures would need to be examined to better elucidate this observation.

Several additional considerations may underlie the null results, which will be discussed below.

4.2.4 Dosing

One plausible explanation for the null effect is that the dose of CID may not have been sufficient to elicit a response. As the administration of CID during SIP training is a novel adaptation of the paradigm, dosing for this study was based on a mouse study using the same drug (Montecucco et al., 2016). Differences in neurobiological and metabolic processes between mice and rats may have caused issues with proper dose conversion, even when using an mg/kg basis to account for the size difference. Additionally, the mouse study was considering peripheral targets while we were aiming to target the brain. The difference in pharmacokinetics may have contributed to challenges regarding dose conversion. Also, the very phenomenon we were looking to target, bi-directional GABA plasticity between GPR55 and CB1, has only been identified within the BNST. Thus, if CID did not reach the BNST, it is unknown if similar signalling profiles are found elsewhere and would elicit the same response. In general, there are few *in vivo* studies using CID, limiting the utility of previous literature to guide dosing.

Further, existing *in vivo* studies were most often focused on investigating peripheral targets (Montecucco et al., 2016; Stančić et al., 2015). Initial dosing had a concentration of 10 μ M and was based on the 0.5 mg/kg dose used in Montecucco (2016). However, this study was not assessing behavioural measures. Besides Montecucco (2016), there has only been two other studies to date injecting CID IP in rodents. The effect of CID on thermal hyperalgesia in mice was assessed using the hot plate test (Munawar et al., 2017). Although a higher dose of 10 mg/kg IP was used, no significant effect on behaviour was found. Another study assessing if CID

blocked CBD induced reduction of cocaine self-administration using a similar dose of 5 mg/kg IP also did not find an effect. The higher 10 mM dose of the current study was approximately 5 mg/kg IP, and therefore within the dosing range used in previous studies that observed no effect. To our knowledge, there have been no other studies administering CID IP with targets in the brain in a rat model. In the one study that had a brain target and showed a drug effect, CID was directly injected through microinjection into the ACC (Galaj & Xi, 2019). This suggests there may be issues with peripherally administered CID reaching targets within the brain. There have yet to be studies assessing how well CID can cross the blood brain barrier.

In summary, it is possible that with the doses used, a sufficient amount of CID did not reach the brain, and thus no response was seen. One important finding of this study is that there was a significant difference in CID-induced water intake between the 10 μ M and 10 mM doses (Figure 10). While there was not a significant effect of CID within the 10 mM dose group, the greater median CID-induced water intake, compared to the 10 μ M dose, suggests a dose-dependent response. Therefore, dosing may have been too low to produce an observable effect.

4.2.5 Neuropharmacological Considerations

Finally, there are undoubtedly other neurobiological substrates that impact compulsivity beyond bi-directional GABA plasticity in the BNST. Targeting one component may not be sufficient to elicit a widespread behavioural change. Many other receptor signalling systems have been implicated in not only compulsions but also in the SIP model itself. Additionally, blocking the GPR55 receptor could have unintended downstream signalling or modulatory effects.

Specific to the bi-directional GABA plasticity seen in the BNST, LPI and 2-AG, the ligands for GPR55 and CB1, share overlapping synthesis pathways (Hawken et al., 2019). LPI can be synthesized by phospholipase A (PLA) 1 or 2 and 2-AG can be generated by PLA₁ (Murataeva et al., 2014). Stress states inhibit PLA₂ suggesting that the bi-directional plasticity seen within the ovBNST may partly be mediated by PLA_{1/2} activity (Hawken et al., 2019; Shukla et al., 2015). This could lessen the effect of blocking GPR55 as there is another component mediating the bi-directionality. Additionally, since synthesis between the endogenous ligands are intertwined, it could cause modulation to both ligand-receptor pairs.

More broadly, signalling between serotonergic, dopaminergic, or the HPA-axis could have also dampened the effect of GPR55 antagonism. For example, the 5-HT₂ receptors in the serotonergic system are highly implicated in compulsivity (Fineberg et al., 2010). Administration of SSRIs significantly reduces drinking in SIP (Moreno & Flores, 2012). Further, considerable cross-talk has been shown between endocannabinoids and the serotonin system to regulate stress responses (Haj-Dahmane & Shen, 2011). Dopamine antagonists also decrease SIP water intake (Mittleman et al., 1994). And finally, an inverse relationship between corticosterone levels and drinking levels was found, supporting the involvement of the HPA-axis and stress response on SIP behaviour (Dantzer et al., 1988). The involvement of a wide variety of systems confirms that signalling underlying compulsions is complex and multifaceted. As many other systems appear to take part in driving high versus low drinking behaviours in SIP, it may be that the GPR55 receptor does not impact compulsive drinking behaviours strongly on its own.

4.3 Limitations and Caveats

4.3.1 Lack of Available Literature

With any novel approach, a lack of relevant literature can be a challenge. As discussed briefly above, the lack of available literature may have hampered our ability to obtain proper dosing levels. Very few studies have used CID, and in those that have, the majority had peripheral, not brain, targets. Additionally, these studies were in mice, not rat, models. As such, dosing had to be estimated from literature that had pronounced differences to the study at hand. The relevance of these issues is supported by findings that there was a significant difference in CID-induced change in water intake between the two doses used.

4.3.2 Sample Size

Another potential limitation of this project was the small sample size. Preliminary power analyses revealed a sample size of 48 animals was required to obtain 80% power at a 0.05 significance level. Although data from 48 animals was collected, only 36 received the higher dose of CID. Additionally, the previous studies investigating GPR55/CB1 receptor signalling's role in SIP both had samples sizes of over 100. As such, the limited sample size may have constrained our ability to detect an effect through the statistical analysis.

4.4 Future Directions

Several avenues would be useful to investigate in future studies. First, future projects that address the issues surround dosing and route of administration would help validate or dispute the null results observed in this project. This includes investigating higher doses of CID and giving multiple administrations of the drug. Additionally, performing intracranial injections of CID into the BNST, where the bi-directional plasticity signalling between GPR55 and CB1 was originally found, would allow for a more targeted approach.

More broadly, further investigation of signalling between peripheral endocannabinoid and associated receptors and the endocannabinoid system within the brain is needed. Furthermore, it would be useful to evaluate if bi-directional GABA signalling between GPR55 and CB1 is present and functional the same way in other areas of the brain as it is in the BNST.

Conclusion

This experiment served to assess the translation of correlational neurophysiological findings to pharmaco-behavioural outcomes. We sought to assess if there is a causal link between deficient LPI/GPR55 signalling within the brain and the development of compulsive drinking in the SIP rat model. An additional objective was to characterize the behaviour that developed throughout the protocol, including assessing potential predictors for developing compulsive behaviours. Our findings support the validity of the SIP paradigm to studying animal models of compulsion. Variables associated with energy homeostasis, such as free-food intake and weight, do not appear to be associated with CID-induced change to water intake. We hypothesized that the blockade of GPR55 would turn LD rats into HD rats. However, our hypothesis was not supported by our results. There are several reasons an effect may not have been achieved. Issues with dosing and route of administration may have impeded CID from reaching its target in the brain. Additionally, a wide range of receptors and signalling systems have been implicated in the development of compulsive phenotypes. As such, the targeting of one receptor may not have been sufficient to achieve a behavioural change. This project served as a useful starting point to investigate the role of GPR55/CB1 signalling on the development of compulsive phenotypes. Subsequent research should concentrate on identifying other areas that

may be influenced by GPR55/CB1 signalling as well as other signalling systems that may be implicated in energy homeostasis and mediate GPR55/CB1 signalling.

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