The effect of chronic sucrose consumption during adolescence on dopamine D2 receptor function in a rodent model

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Abbreviations

AMYG - amygdala

ANOVA – analysis of variance

appK_D – apparent affinity of receptor

B_{avail} – receptor binding availability

cAMP – cyclic adenosine monophosphate

CPu – caudate-putamen

CT – computerised tomography

D1 – D_1-like receptors

D2 – D_2-like receptors

DAT – dopamine transporter

DMSO – di-methyl sulfoxide

FDT – final day of treatment

GPe – globus pallidus external

GPi – globus pallidus internal

HIP – hippocampus

MRI – magnetic resonance imaging

NAc – nucleus accumbens

PET – positron emission tomography

PFC – prefrontal cortex

PND – post natal day

PT – six weeks post treatment

ROI – region of interest

SN – substantia nigra

SNc – substantia nigra pars compacta

SNr – substantia nigra pars reticula

STN – subthalamic nucleus

THAL - thalamus

VOI – volume of interest

VTA – ventral tegmental area

WHO – World Health Organisation

Abstract

Sugar consumption has been suggested to alter the dopaminergic motivation-reward systems of the brain. Adolescence is a major period of development in the dopaminergic motivation-reward systems, making this system particularly vulnerable during this period, yet the consequences of chronic excess sugar consumption on dopamine receptor function during adolescence have not been thoroughly examined. This study investigated the effects of unrestricted access to sucrose solution throughout adolescence (PND35-60) on dopamine D2 receptors in the striatum, using a male rodent model. Positron emission tomography imaging using [^{11}C]raclopride was conducted on one cohort of animals (N=12) on the final day of treatment and following a 6 week washout. Data were analysed using partial saturation analysis to give measures of functional D2 receptor density and apparent D2 receptor affinity. Mixed ANOVA found a range of significant main effects and interactions between treatment group and time in functional D2 receptor density in three rostro-caudal levels of striatum. There were no significant treatment group or time effects on apparent D2 receptor affinity. Autoradiography imaging using [^{3}H]raclopride was conducted on a second cohort of animals (N=12), following a 10 day washout. There was no significant difference in total D2 receptor density between treatment groups in any region of interest in the striatal area. These results suggest that chronic excess sugar consumption during adolescence impacts functional, but not total, D2 receptor density in the striatum and these effects may last into adulthood.

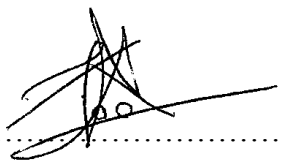
Statement of Authentication and Ethical Accordance

I hereby declare that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text. The material, either in full or in part, has not been submitted for a degree or diploma at this or any other academic institution.

All animal research carried out for the PET component (Experiment 1) of this thesis was approved by the University of Sydney Animal Ethics Committee (AEC Number: 2015/781) in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes, 8th Edition (National Health and Medical Research Council, 2013). I completed all animal care tasks in Experiment 1, including feeding, and took part in PET/CT acquisition.

All animal research carried out for the autoradiography component (Experiment 2) of this thesis was conducted previously, under approval by the Macquarie University Animal Ethics Committee (AEC Number: 2011/029-5) in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes, 8th Edition (National Health and Medical Research Council, 2013). I completed the autoradiography component of this experiment, under the supervision of Dr Paul Callaghan.

Jessica Boh

Signature  Date: 24/12/2015

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1. Literature Review: The Motivation-Reward System on Sugar

1.1. Introduction

The impact of sugar consumption on our health is a contentious issue, with popular media regularly discussing the risk of adverse health outcomes related to refined sugar intake. Most commonly, the issues relate to physical health risks, such as obesity (Apovian, 2004; Berkey, Rockett, Field, Gillman, & Colditz, 2004; Bocarsly, Powell, Avena, & Hoebel, 2010; Ebbeling et al., 2006; Elliott, Keim, Stern, Teff, & Havel, 2002; Hu & Malik, 2010; Johnson et al., 2007; Malik, Schulze, & Hu, 2006; Pollard et al., 2015; Stanhope, 2015), diabetes (Apovian, 2004; Hu & Malik, 2010; Johnson et al., 2007; Ruff, 2015; Singh et al., 2015; Stanhope, 2015) and heart disease (Johnson et al., 2007; Ruff, 2015; Singh et al., 2015; Stanhope, 2015). However, the physical effects of refined sugar consumption are not straightforward and it has been proposed that some physical health outcomes, such as diabetes, may involve initial diet-induced neurological changes (Welberg, 2014).

The idea that excess sugar consumption may impact the physiology of the brain to cause psychological and behavioural issues has led to increased interest in the effects of sugar intake on mental health and resilience. For example, research has begun to identify neurological links to memory deficits (Beilharz, Maniam, & Morris, 2014; Cao, Lu, Lewis, & Li, 2007; Chepulis, Starkey, Waas, & Molan, 2009; Francis & Stevenson, 2011; Hsu et al., 2014; Kendig, 2014; Kendig, Boakes, Rooney, & Corbit, 2013; Ross, Bartness, Mielke, & Parent, 2009) and changes in feeding, decision making and reward behaviours (Alsiö et al., 2010; Grimm, Fyall, & Osincup, 2005; Grimm et al., 2011; Kendig, 2014; Sheludiakova, Rooney, & Boakes, 2012) following sugar consumption.

More recently, research has begun to investigate the way that chronic excess sugar consumption influences the dopaminergic motivation-reward system in the brain, causing neurological outcomes similar to those seen in drug addiction (Ahmed, Guillem, & Vandaele,

2013; Avena, Rada, & Hoebel, 2008; Kendig, 2014). These studies strongly imply the involvement of striatal dopamine D2 receptors, which are linked to a number of behavioural symptoms and psychological disorders (e.g. psychosis, Seeman et al., 2005; depression, Shah, Ogilvie, Goodwin, & Ebmeier, 1997; and social phobia, Schneier et al., 2000).

Within this relatively new research area, adolescents have emerged as a particularly vulnerable population for changes to the motivation-reward system following chronic sugar intake (Kendig et al., 2013; Vendruscolo, Gueye, Darnaudéry, Ahmed, & Cador, 2010). The reason for this is twofold: first, adolescence is an important period of brain development, with adolescents widely recognised to have altered dopaminergic activity compared to both childhood and adulthood (Bjork et al., 2004; Blakemore & Choudhury, 2006; Casey, Jones, & Hare, 2008; Laviola, Macrì, Morley-Fletcher, & Adriani, 2003; Spear, 2000; van Leijenhorst et al., 2010; Wahlstrom, Collins, White, & Luciana, 2010; Wahlstrom, White, & Luciana, 2010); and second, adolescents are reported to be the biggest consumers of refined sugar (Newens & Walton, 2015; Popkin & Nielsen, 2003).

The current literature review provides an overview of the present understanding of the motivation-reward system, with particular emphasis on the striatum, and the development and function of this system during adolescence. The existing literature investigating the impacts of chronic excess sugar consumption on the motivation-reward system will then be discussed, with a focus on psychological and behavioural outcomes.

1.2. The motivation-reward system

The motivation-reward system is evolutionarily important in ensuring humans' survival, in particular by providing motivation to seek high energy food sources such as sugar. When the taste of sweetness is experienced there is a pleasurable sensation that is associated with reward, through activation of the motivation-reward system (Ahmed et al., 2013;

Yamamoto, 2003). The importance of reward motivation for survival and reproduction means that specialised brain mechanisms have developed to interpret and encode reward. Of these, the dopaminergic projections between the ventral tegmental area (VTA) and nucleus accumbens (NAc), and the substantia nigra (SN) and caudate-putamen (CPu) are most prominently studied (Figure 1). The pathway from the VTA to the NAc has been linked to sensations of reward and reward reinforcement (Martel & Fantino, 1996; Pierce & Kumaresan, 2006; Spanagel & Weiss, 1999). The pathway between the SN and CPu is primarily recognised for its role in the regulation of sensorimotor functions (Dayan & Balleine, 2002; Schultz, 2002; Previc, 2009; Smith & Kieval, 2000; Wise, 2009), but has also been recognised as important in the learning of motor movements that result in reward attainment. For example, dopamine cells in the SN respond to signals preceding rewarding events in addition to the rewarding events themselves (Berke & Hyman, 2000; Dayan & Balleine, 2002; Schultz, 2002; Smith & Kieval, 2000; Wise, 2009). Thus, this pathway has been closely studied in relation to learning and habit formation (Dayan & Balleine, 2002; Gerdeman, Partridge, Lupica, & Lovinger, 2003).

These two major motivation-reward pathways cross over at the striatum, which encompasses both the CPu and NAc (Figure 1). The motivation-reward pathways between VTA – NAc and SN – CPu are often described within the mesocorticolimbic and basal ganglia systems. Although commonly investigated independently, the mesocorticolimbic and basal ganglia systems have been recognised to work together in motivation-reward responses (Wise, 2009). For this reason, the following depiction of the motivation-reward system is described in terms of brain regions and specific projections, rather than overarching motivation-reward pathways.

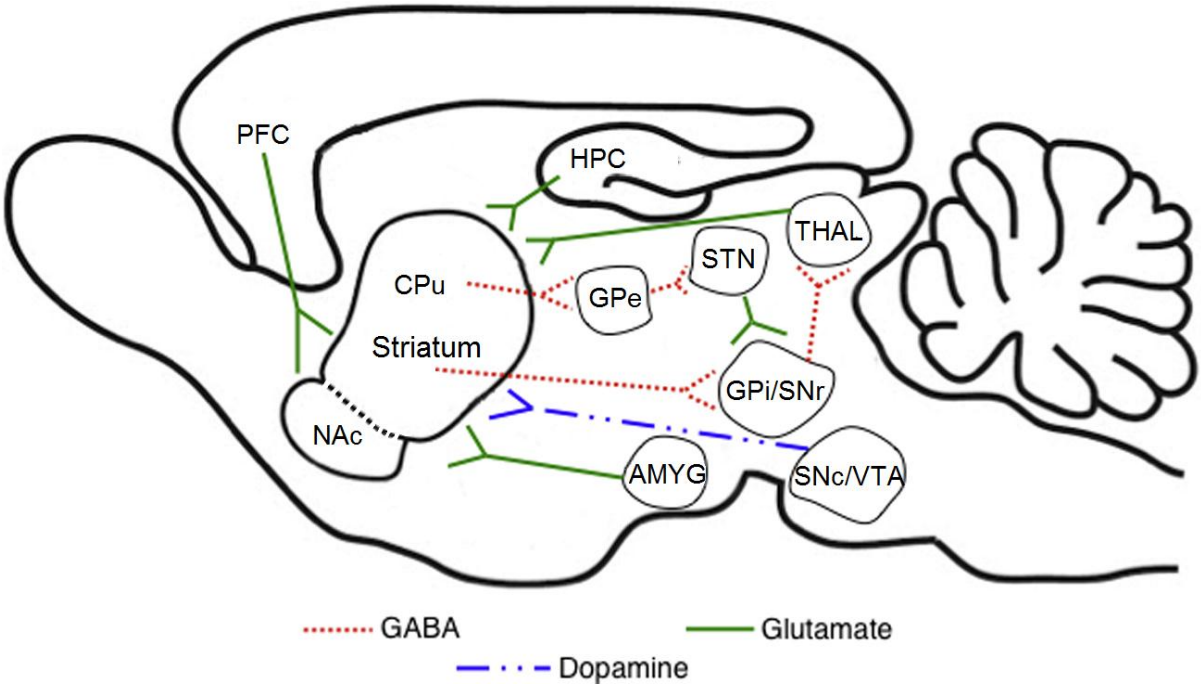


Figure 1. Schematic summary of the motivation-reward system, modified from Yager, Garcia, Wunsch, & Ferguson (2015). The striatum receives glutamatergic afferents from the prefrontal cortex (PFC), hippocampus (HPC), thalamus (THAL) and amygdala (AMYG), and dopaminergic afferents from the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). The striatum projects GABAergic afferents to the globus pallidus external (GPe), globus pallidus internal (GPi) and substantia nigra pars reticula (SNr), which are also involved in a feedback loop with the subthalamic nucleus (STN) and thalamus (THAL).

1.2.1. Dopamine and Dopamine Receptors

Dopamine is a neurotransmitter that is involved in a number of different behaviours, including motivation and reward, locomotor activity, mood, and stress coping (Badgaiyan, 2010; Beaulieu & Gainetdinov, 2011; Beninger, 1983; Cabib & Puglisi-Allegra, 2012; Dayan & Balleine, 2002; Di Chiara & Bassareo, 2007; Missale, Nash, Robinson, Jaber, & Caron, 1998; Previc, 2009; Schultz, 2002; Spanagel & Weiss, 1999; Wise, 2009). There are two broad families of dopamine receptors: D_1-like (D1), which includes D_1 and D_5 receptors, and D_2-like (D2), which includes D_2, D_3 and D_4 receptors (for more detailed descriptions, see Beaulieu & Gainetdinov, 2011; Girault & Greengard, 2004; Missale et al., 1998). The

families of dopamine receptors differ in a number of ways, reflecting different functions. For example, the families differ in location, with D1 receptors found only post-synaptically on dopamine receptive cells, while D2 receptors are found both pre-synaptically on dopaminergic neurons and post-synaptically on dopaminergic and non-dopaminergic neurons (Beaulieu & Gainetdinov, 2011; Wahlstrom, White, et al., 2010). Dopamine D1 and D2 receptors are understood to interact to control behaviours in which they are involved (Beaulieu & Gainetdinov, 2011; Girault & Greengard, 2004; Wise, 2002). The motivation-reward system illustrates this clearly, with excitatory D1 receptors and inhibitory D2 receptors working in balance to moderate reward sensitivity, motivation and response behaviours (Girault & Greengard, 2004; Ikemoto, Glazier, Murphy, & McBride, 1997; Schultz, 2002; Wahlstrom, Collins, et al., 2010; Wise, 2002).

The efficacy of dopamine receptors follows an inverted-U function, such that dopamine transmission induces normal behavioural responses within a small window of optimal function but impairs cognitive and behavioural performance at higher or lower concentrations (Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007; Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010). Dopamine in the synapse binds to dopamine receptors to produce on of a number of flow on effects, such as stimulating or inhibiting the production of cyclic adenosine monophosphate (cAMP), and modulating the sodium and potassium channels of the neuron (Beaulieu & Gainetdinov, 2011; Girault & Greengard, 2004; Missale et al., 1998). Dopamine has been described as a neuromodulator, which alters the response of target neurons to other neurotransmitters (Beaulieu & Gainetdinov, 2011; Schultz, 2002; Wahlstrom, Collins, et al., 2010), as dopamine interacts with other neurotransmitters at secondary messenger level within the neuron to influence behaviours and responses (Beaulieu & Gainetdinov, 2011; Girault & Greengard, 2004; Missale et al., 1998). For example, dopamine regulates the activity of glutamate receptors that

mediate cortico-striatal neurotransmission (Girault & Greengard, 2004). Likewise, dopamine activity is often moderated by the activation of other neurotransmitters. Studies in brain stimulation reward have found that dopamine neurons are secondarily activated after other substrates, such as serotonin and glutamate (Ikemoto et al., 1997; Shah et al., 1997). As such, interpretation of behavioural and neurological changes relating to reward is difficult, as changes may relate directly to dopaminergic changes or to alterations along other pathways or systems. Greater understanding of the brain regions and projections in which these differences arise would assist in tracing the origin and understanding the mechanisms of neurological changes relating to motivation and reward.

1.2.2. Effects of Dopamine within the Motivation-Reward System

Based on animal conditioning experiments, dopamine has been described to signal the difference between reward expectation and outcome in the motivation-reward system (Dayan & Balleine, 2002; Girault & Greengard, 2004; Schultz, 1998, 2002). That is, dopamine receptors signal both the presence of reward and a lack of expected reward. However, studies also describe a more complex system of mechanisms through which dopamine is involved in motivation and reward. For example, it has been observed that dopamine release in the striatum strengthens synapses that were active immediately before a reward was obtained (Girault & Greengard, 2004), suggesting a process through which dopamine aids the learning and reinforcing of reward-motivated behaviours.

It has been proposed that each of the behaviours impacted by dopamine, such as the control of locomotor activity and general goal directed behaviours (Beaulieu & Gainetdinov, 2011; Beninger, 1983; Dayan & Balleine, 2002; Previc, 2009), are related to various aspects of motivation and reward in a complex system. A simplified model of the steps involved in the initiation and attainment of motivation-reward behaviours (Figure 2) provides a structure

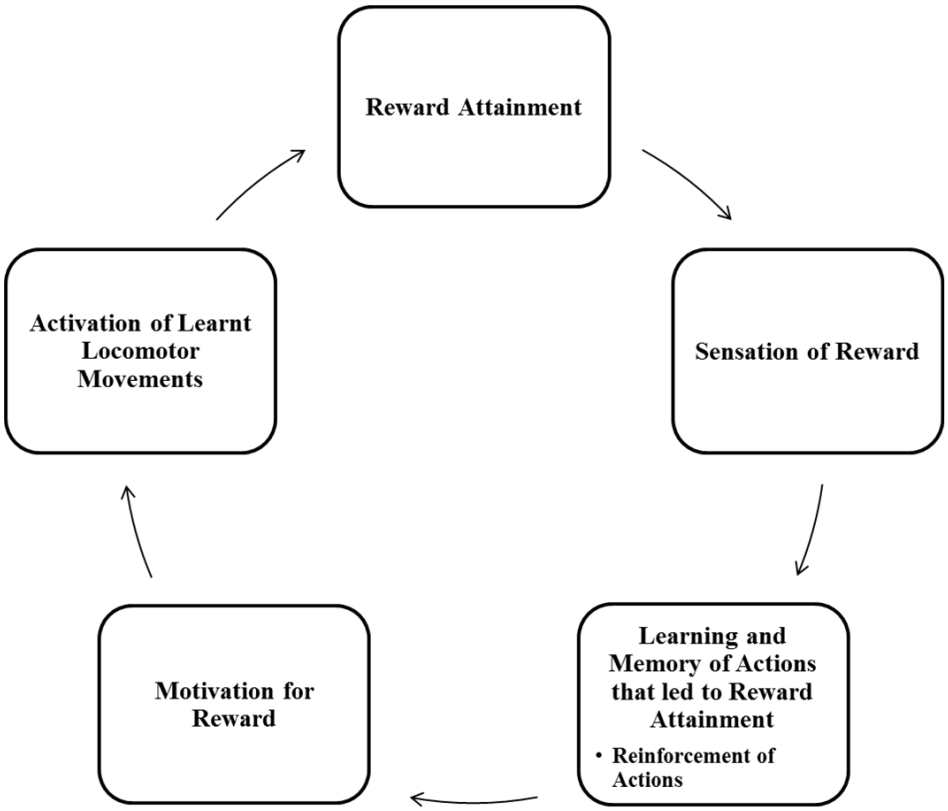


Figure 2. Simplification of processes in a motivation-reward response based on key literature (Dayan & Balleine, 2002; Schultz, 1998, 2002; Wise, 2002, 2009).

through which the role of these dopamine-related behaviours can be illustrated. However, the difficulty in experimentally separating these highly entwined processes makes clear empirical support for this theory challenging.

Sensation of Reward

When a reward is presented, dopamine release induces positive emotion or sensation of reward (Schultz, 2002). The substantia nigra pars compacta (SNc) and VTA have been suggested to initiate this process, as dopaminergic neurons in these regions are selectively activated by rewarding events and cues predicting reward (Berke & Hyman, 2000; Dayan & Balleine, 2002; Schultz, 1998; Smith & Kieval, 2000). Dopaminergic projections from the

SNc and VTA then activate striatal dopamine receptors, which moderate the positive reward response (Badgaiyan, 2010; Wise, 2002).

Previous studies indicate that D1 receptors play a primary role in the value coding of reward. For example, administration of D1 receptor antagonist, SCH 23390, was found to sufficiently devalue sucrose reward, while the D2 receptor antagonist, raclopride, had no effect (Smith & Kieval, 2000). The area of striatum activated by the reward may also influence reward valence. It has been suggested that the ventral striatum (NAc and olfactory tubercle) processes positive sensations in response to reward and mediates food related reward (Alsiö et al., 2010; van Leijenhorst et al., 2010), while the dorsal striatum (CPu) processes negative emotions (Badgaiyan, 2010). In support of this, dopamine concentration in the NAc increases during and after presentation of sucrose rewards (Cabib & Puglisi-Allegra, 2012; Hajnal, Smith, & Norgren, 2004), while negative emotional stimuli elicit the release of dopamine in the CPu (Badgaiyan, 2010; Connolly, Gollan, Cobia, & Wang, 2015). Thus, there is evidence to support that the activation of dopamine receptors in the striatum plays a central role in the initial valuation of rewards.

Learning and Memory of Actions or Reinforcement of Actions

The positive sensations of initially attaining reward propagate the dopamine signal, which is then involved in learning. Dopamine influences the learning and memory of actions by regulating the responses of target neurons and altering their synaptic plasticity (Gerdeman et al., 2003; Girault & Greengard, 2004; Jones & Bonci, 2005; Kreitzer & Malenka, 2008). Conditioned reward studies suggest that this process involves reciprocal interaction between D1 and D2 receptors, with D2 receptor agonists observed to enhance learning of behaviours while D1 receptor agonists impair acquisition of response behaviours (Beninger, 1983; Sharpe, Clemens, Morris, & Westbrook, 2015). Abnormality or dysfunction of either type of

dopamine receptor leads to slower and less efficient learning (Dayan & Balleine, 2002; Schultz, 2002).

The repetition of learning activation patterns results in reinforcement of the response behaviours. Similar to initial learning, both D1 and D2 receptors are required for reinforcement, with the individual administration of D1 or D2 receptor antagonists both observed to reduce cocaine reward reinforcement (Pierce & Kumaresan, 2006). Dopamine activation has different reinforcing effects depending on the specific brain region examined. For example, dopamine neurotransmission in the ventral striatum enhances reinforcement signalling (Dayan & Balleine, 2002; Pierce & Kumaresan, 2006), whereas dopaminergic activation in the dorsal striatum shows no reinforcement effect (Pierce & Kumaresan, 2006).

Interestingly, while dopamine neurons signal the extent that reward outcome deviates from prediction during conditioned learning, dopamine activation in response to reward presentation gradually diminishes as the reward becomes increasingly predicted (Dayan & Balleine, 2002; Schultz, 1998, 2002). The diminishing dopamine response to reward may reflect that the response behaviour has been learned, and that these learned behaviours are consequently activated by other neurotransmitters (Dayan & Balleine, 2002; Schultz, 1998, 2002). This suggests that the primary role of dopamine in learning and reinforcement may be to modulate the response of other neurotransmitters and promote the strengthening of synaptic connections involved (Girault & Greengard, 2004).

Motivation for Reward

The learned positive sensations and dopaminergic activation from reward attainment provide motivation for further reward. Dopamine D2 receptors play an inhibitory control in motivation, moderating drive for reward and reward-related behaviours (Beaulieu & Gainetdinov, 2011; Ghahremani et al., 2012; Girault & Greengard, 2004). In humans, D2

receptors have been suggested to regulate social behaviour (Schneier et al., 2000), particularly with regard to inhibitory control, salience attribution and emotional reactivity (Volkow et al., 2008). For example, D2 receptor abnormality in the striatum has been related to impulsivity and irrational choice (Cocker, Dinelle, Kornelson, Sossi, & Winstanley, 2012; Colantuoni et al., 2001; Ghahremani et al., 2012), providing further support that D2 receptors act in an inhibitory manner on motivation-reward behaviours.

The activation of D2 receptors in different areas of the striatum has also been linked to distinct motivation behaviours. For example, nicotine craving has been associated with lower availability of D2 receptors in the dorsal striatum but higher D2 receptor levels in the ventral striatum (Fehr et al., 2008), reflecting the different roles of striatal regions that relate to the inhibitory and motivational functions of the motivation-reward system. Furthermore, activation of projections between the striatum and prefrontal cortex has been associated with impulse control, as measured by performance on a go/no-go task (Ghahremani et al., 2012; Liston et al., 2006). Meanwhile, dopaminergic activity within the mesolimbic pathway, particularly the NAc, has been positively related to drug seeking behaviour, locomotor response, and novelty seeking (Grimm et al., 2011; Ikemoto, 2010; Pierce & Kumaresan, 2006; Spear, 2000). The mesolimbic pathway is also activated prior to making risky choices (Laviola et al., 2003), with the NAc suggested to integrate input from the VTA and ventral pallidum regarding motivational state (Spear, 2000). Correspondingly, impaired dopamine neurotransmission in the NAc leads to motivational deficits in approach behaviour and reward-directed learning (Schultz, 2002). Together, these findings suggest that the combination of reduced impulse control by D2 receptors in the CPu and increased motivation by D2 receptors in the NAc provides motivational drive for reward-seeking behaviours (Di Chiara & Bassareo, 2007).

Activation of Learnt Locomotor Movements

The presence of sufficient motivational cues will prompt activation of behaviours that have previously been learned to result in reward attainment. Locomotor activity is commonly described to result from activation of the dopaminergic nigrostriatal pathway (Beaulieu & Gainetdinov, 2011; Previc, 2009; Schultz, 2002; Wise, 2009). Much of the evidence for this comes from studies in Parkinson's Disease, a chronic disorder in which there is reduced dopamine release and degeneration of the nigrostriatal pathway (Beaulieu & Gainetdinov, 2011; Beninger, 1983; Wise, 2009), characterised by symptoms such as impaired planning, initiation and control of movements (Schultz, 2002). Comparable deficits have been experimentally induced by lesioning the nigrostriatal dopamine system and blockade of dopamine neurotransmission using dopamine receptor antagonists, supporting the role of the nigrostriatal pathway in locomotor activity (Schultz, 2002). Similarly, reduced motor function has been associated with decreased D2 receptor binding in the striatum in patients with major depression (Shah et al., 1997). However, the mesolimbic pathway also plays a role in locomotor activity, with the VTA and SN having overlapping projection fields (Beninger, 1983; Wise, 2009). Lesions in the VTA result in altered levels of locomotor activity, either increasing or decreasing locomotion depending on the area of damage (Beninger, 1983). Moreover, increases in locomotor activity are observed following activation of dopamine receptors in the VTA from localised administration of psychostimulant drugs, such as amphetamine or cocaine (Vezina, 1996; Wallace, Gudelsky, & Vorhees, 1999). Thus, there is strong evidence that locomotor activity is related to dopaminergic function along both the mesolimbic and nigrostriatal pathways.

Few behavioural studies have considered the location of the receptors in relation to the synapse, however there is evidence to suggest that the activation of presynaptic D2 receptors has an inhibitory effect on locomotor behaviour (Beaulieu & Gainetdinov, 2011). That is,

activation of presynaptic D2 receptors provides feedback which may lead to a decrease in dopamine release, thereby reducing locomotor activity. Meanwhile, the complementary activation of both D1 and D2 postsynaptic receptors is required for the normal manifestation of locomotor behaviour (Beaulieu & Gainetdinov, 2011). This suggests that a specific balance between D1 and D2 receptor activation is required in the motivation-reward system to induce normal reward motivated movements, with presynaptic D2 receptors moderating dopaminergic activity.

1.2.3. Development of the Motivation-Reward System in Adolescence

Adolescence is the period of developmental transition from dependence to independence occurring between childhood to adulthood, characterised by neurological and corresponding behavioural changes (Casey et al., 2008; Dahl, 2004; Laviola et al., 2003; Spear, 2000; Wahlstrom, Collins, et al., 2010). The adolescent period in humans is generally accepted to be from 12 to 18 years of age, but can also include ages from 9 up to 25 years (Laviola et al., 2003; Spear, 2000). Adolescence is better characterised by behavioural features that are commonly observed during this period, such as increased novelty seeking and risk-taking behaviour, social interaction with peers, and emotional reactivity (Blakemore & Choudhury, 2006; Casey et al., 2008; Dahl, 2004; Spear, 2000). These behaviours reflect a heightened drive for reward, which arises from the changing dopaminergic motivation-reward system (Blakemore & Choudhury, 2006; Casey et al., 2008; Spear, 2000; van Leijenhorst et al., 2010; Wahlstrom, Collins, et al., 2010).

Current theory suggests that amplified reward sensitivity during adolescence is the result of increased concentrations of dopamine acting upon brain regions at different stages of development, specifically a disparity between the cortex and striatum (Kendig, 2014; van Leijenhorst et al., 2010; Spear, 2000; Wahlstrom, Collins, et al., 2010). In the primary

processing regions of motivation and reward, dopamine receptor and dopamine transporter (DAT) density has stabilised to adult levels by the end of childhood, such that higher levels of dopamine promote behaviours that result in immediate reward (Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010). For example, impulsive and emotionally reactive behaviour during early adolescence is related to increased dopamine release and correspondingly dopamine receptor activity in the NAc (Casey et al., 2008), as well as increased dopamine activation of the ventral striatum in response to received rewards (van Leijenhorst et al., 2010). In contrast, dopaminergic projections in the prefrontal cortex undergo rapid change during adolescence (Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010). Within the cortical regions, there is lower dopamine regulation by pre-synaptic D2 receptors and DAT activity, and enhanced dopamine signalling due to synaptic pruning (Casey et al., 2008; Spear, 2000; Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010). These increased levels of dopamine overload the developing prefrontal system and heighten responsiveness of motivation-reward areas, such that the reward-motivated systems are less regulated by inhibitory signals of the prefrontal cortex (Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010). The impulsive and risk-taking behaviour that is typical of adolescents is suggested to result from an imbalance between the developed mesolimbic system and developing mesocortical prefrontal control system, causing decisions to be based on heightened sensitivity to immediate reward and emotions rather than logical reasoning (Casey et al., 2008; van Leijenhorst et al., 2010; Wahlstrom, Collins, et al., 2010; Wahlstrom, White, et al., 2010).

The changing balance of dopaminergic activity and neurological development promotes the behaviours that typify adolescence, and is suggested to increase the psychological vulnerability, or susceptibility to negative mental health outcomes, of this age group (Blakemore & Choudhury, 2006; Wahlstrom, Collins, et al., 2010). For example, while

relative insensitivity to the effects of drugs of abuse has been observed due to reward deficiency during this period, adolescent progression to greater substance use and subsequent dependence is also suggested to be more rapid for the same reason (Spear, 2000). Likewise, the symptoms of many psychological disorders, such as schizophrenia, depression and anxiety, often manifest during adolescence (Casey et al., 2008; Lewis, 1997; Spear, 2000; Wahlstrom, Collins, et al., 2010). This suggests that these symptoms and their corresponding mental health disorders may result from abnormal development of neural connections and cross-regional balance of neurotransmitters during the adolescent period. For instance, disrupted development of prefrontal cortex circuitry during adolescence may be related to early symptoms of schizophrenia, such as deterioration of academic performance and sociability, as well as more overt symptoms in later stages, such as psychosis and reduced inhibition (Lewis, 1997). The development of connections between cortical and subcortical regions that are required for cognitive control during adolescence therefore makes this a crucial and vulnerable developmental period for mental health.

1.3. Sugar in Our Diet

Sugar is a natural and vital part of the human diet. Originally sourced from fruit and vegetables, sugar plays the important role of providing the cells of the human body with energy. Thus, the motivation-reward system is activated by sweet tastes that indicate the presence of sugar in food to promote their consumption (Goldfein & Slavin, 2015). However, with the development of sugar refinement, the availability of sugar has increased, allowing sugar consumption to reach levels higher than previously possible (Popkin & Nielsen, 2003). The metabolism of sugar for energy use in the body has long been studied and investigated in relation to physical health disorders, such as obesity and diabetes. However, it is increasingly

recognised that chronic excess sugar consumption can also cause neurological changes and thereby negatively impact psychological health.

1.3.1. Types of Sugar

Sugar comes in a number of forms: monosaccharides (simple sugars such as glucose and fructose), disaccharides (compound sugars such as sucrose and lactose) and polysaccharides (complex sugars such as starch and cellulose). These occur both naturally and artificially in a range of foods, for example, fructose is found naturally in fruits, while sucrose is the refined sugar of normal table sugar.

Refined sugar is defined by the World Health Organisation (WHO) as "all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer, plus sugars naturally present in honey, syrups and fruit juices" (World Health Organisation & Consultation, 2003). That is, refined sugar encompasses all simple and compound sugars that have been processed from the natural form (with honey deemed to be processed by bees) or added to food or drink (World Health Organisation & Consultation, 2003). The refined sugars most commonly added to our foods, and implicated in relation to adverse health outcomes, are sucrose and fructose. Sucrose is broken down by the body into one fructose and one glucose molecule, with fructose responsible for most of the sweet flavour of food and glucose providing the energy source (Akram & Hamid, 2013; Cao et al., 2007). These two molecules are metabolised by, and influence, the body and brain in different ways. In brief, glucose is primarily metabolised directly by cells in the brain and body to provide energy in the form of ATP, while fructose is metabolised in the liver to make glycogen, lactate, fatty acids and triglycerides (for more details of these processes, see Elliott et al., 2002; Malik & Hu, 2015; Mergenthaler, Lindauer, Dienel, & Meisel, 2013; Tappy & Lê, 2015).

It has been suggested that fructose is the primary contributing factor to detrimental health outcomes, particularly in regards to physical health ailments (Akram & Hamid, 2013;

Elliott et al., 2002; Ha et al., 2015; Johnson et al., 2007; Malik & Hu, 2015). For example, fructose consumption has been related to insulin resistance, weight gain and hypertension in both human and animal studies (Dornas, de Lima, Pedrosa, & Silva, 2015; Elliott et al., 2002). However, studies often fail to specify or control for the food source of sugar consumed (e.g. Alsiö et al., 2010; Beilharz et al., 2014; Collins et al., 2015; Molteni, Barnard, Ying, Roberts, & Gomez-Pinilla, 2002), and there is not sufficient evidence that similar outcomes would not be found if studies specifically investigated other sugars such as glucose (Tappy & Lê, 2015). Nevertheless, while the exact dietary factor contributing to these outcomes must be clarified, there is a strong body of evidence which indicates that chronic excess sugar consumption is detrimental to health.

1.3.2. Chronic Excess Sugar Consumption

The amount of sugar in the average diet, particularly Western diets, has increased dramatically with the development of sugar refining techniques. Epidemiological studies report an increase in sugar consumption that is largely due to an increase in both the frequency and quantity per serving of sweetened beverage consumption (Bleich, Wang, Wang, & Gortmaker, 2009; Popkin & Nielsen, 2003).

While a more recent study suggests a positive change in our habits, with a decrease in added sugar consumption from 1999-2000 to 2007-2008 (Welsh, Sharma, Grellinger, & Vos, 2011), the average amounts of added sugar consumed around the world are still in excess of dietary guidelines (Newens & Walton, 2015). It is recommended by the WHO that the average person receive less than 10% of their daily energy intake (a maximum of approximately 200-250 calories) from sugar (Morris, 2015). However, studies suggest that sugars comprise from 13.5-24.6% of energy intake in adults (Newens & Walton, 2015). In 2012, Australians were conservatively reported to consume approximately 430 calories of

sugar on average per day (Green Pool Commodity Specialists, 2012). It has been suggested that sugar sweetened beverages are the largest source of added sugars and largest single food source of dietary calories, alone comprising 7.1% of the total energy intake of the average American (Apovian, 2004; Berkey et al., 2004). Moreover, studies suggest that adolescent consumption of sugar is higher than average reported levels (Newens & Walton, 2015; Popkin & Nielsen, 2003). For example, in Australia, adolescents are reported to consume an average of 121.05g of sugar per day (22% of energy intake), compared to an adult average of 104.8g (19.1% of energy intake; Newens & Walton, 2015). Similarly, sugar sweetened beverages alone account for 10-15% of total energy intake in American children and adolescents (Wang, Bleich, & Gortmaker, 2008).

These levels of sugar intake are far in excess of the requirement for function of the body and brain, in addition to being consumed in a chronic manner. That is, sugar is commonly ingested frequently and over long periods of time. While it is known that energy dense foods high in sugar and fat are highly palatable and promote overconsumption of energy (Alsiö et al., 2010), the impact of this dramatic increase in daily sugar consumption is not yet fully understood. Nevertheless, it is recognised that humans are biologically ill-prepared to process high concentrations of sugar (Ahmed et al., 2013), particularly in liquid forms such as sugar-sweetened beverages (Apovian, 2004). For example, studies have found that fructose sweetened beverage consumption is related to increased production of uric acid, as there is no feedback mechanism regulating the enzymes involved in fructose metabolism (Tappy & Lê, 2015; Viazzi, Genovesi, Ambruzzi, & Giussani, 2015). However, there is also evidence to suggest that males and females may be differently affected by chronic excess sugar consumption, with changes to locomotor sensitisation following access to sucrose solution developing more rapidly in female rodents (Collins et al., 2015). While gender is statistically controlled for in human studies, the majority of animal studies investigating the

effects of sugar consumption are conducted on male rodents. It is important for the role of gender to be further investigated to better understand the effects of chronic excess sugar consumption.

Links between sugar intake and risk of diabetes and obesity are most commonly investigated, yet other detrimental physical outcomes for the overuse of sugar, such as cancer and asthma, have also been suggested (Apovian, 2004; Berentzen et al., 2015; Berkey et al., 2004; Bocarsly et al., 2010; Elliott et al., 2002; Johnson et al., 2007; Malik & Hu, 2015; Ruxton, Gardner, & McNulty, 2009). There is also increasing interest in the effects of sugar intake on the brain and mental health, with studies relating excess sugar consumption and poor diet to memory deficits and psychological disorder (Kendig, 2014; O'Neil et al., 2014; Shi, Taylor, Wittert, Goldney, & Gill, 2010). As such, it is important to further investigate the neurological effects of chronic excess sugar consumption.

1.3.3. Sugar Consumption Alters Behaviour and Cognition

Sugar intake can impact the brain via two different pathways in parallel: directly by metabolism of glucose that crosses the blood brain barrier, and indirectly through activation of the dopaminergic motivation-reward system. The metabolism of glucose for energy has long been studied and investigated in relation to physical disorders. However, studies suggest that abnormal glucose metabolism in the brain, from chronic excess sugar consumption, may be the cause of memory deficits (Beilharz et al., 2014; Kendig et al., 2013; Ross et al., 2009). Chronic high sugar intake may also cause neurological changes through over-activation of the dopaminergic motivation-reward system, similar to that seen in drug abuse (Avena et al., 2008). While previous studies in motivation and reward have tended to focus on the effect of chronic excess sugar consumption on addiction-like behaviours, recognition of dopaminergic

changes have led to increasing interest in the relationship between sugar and psychological disorders related to dysfunction of the dopamine systems.

Learning and Memory Deficits

The relationship between dietary sugar intake and memory deficits is prominently discussed in the media, due to its strong implications for Alzheimer's Disease and other dementias. Chronic excess sugar consumption is related to deficits in hippocampal-dependent spatial and episodic memory in both adult and adolescent populations (Beilharz et al., 2014; Chepulis et al., 2009; Cisternas et al., 2015; Davidson et al., 2012; Francis & Stevenson, 2011; Hsu et al., 2014; Jurdak, Lichtenstein, & Kanarek, 2008; Kendig et al., 2013; Ross et al., 2009), and that these effects may be long lasting (Kendig et al., 2013). High sugar intake has also been reported to exacerbate memory deficits in a rodent model of Alzheimer's Disease (Cao et al., 2007). However, while object recognition was negatively impacted by chronic sugar intake (Jurdak & Kanarek, 2009), later studies have not found evidence to support this result (Beilharz et al., 2014; Chepulis et al., 2009).

Memory deficits related to sugar consumption have been consistently observed alongside increased hippocampal inflammatory markers, oxidative stress markers, insulin resistance and increased triglyceride levels (Beilharz et al., 2014; Cao et al., 2007; Chepulis et al., 2009; Cisternas et al., 2015; Hsu et al., 2014; Kendig, 2014; Ross et al., 2009). This has led to the hypothesis that chronic excess sugar consumption reduces the synaptic plasticity required for memory by causing insulin resistance in the hippocampus (Cao et al., 2007; Hsu et al., 2014; Molteni et al., 2002; Ross et al., 2009), and is consistent with non-sugar related findings of disrupted energy metabolism in the brain in those with Alzheimer's Disease (Kapogiannis & Mattson, 2011). Dysfunction in glucose metabolism therefore offers a

potential mechanism through which high sugar intake relates to memory deficits and Alzheimer's Disease.

The reduced synaptic plasticity and memory deficits observed in sugar-fed animals may be related to dopaminergic changes. Chronic excess sugar consumption is proposed to lead to an excess of extracellular dopamine in the motivation-reward system, to suggest that dopamine plays an important role in regulating the synaptic plasticity required for learning and memory (Beninger, 1983; Mameli & Lüscher, 2011). For instance, it has been observed that high doses of dopamine receptor agonists and antagonists can impair spatial memory (Wahlstrom, White, et al., 2010). Similarly, sugar consumption disrupted the formation of food-cue associations with these effects reversed by administration of a D2 receptor agonist, suggesting the learning deficit is due to altered dopaminergic function (Sharpe et al., 2015). However, the complex nature of memory and learning, and relatively unexamined effects of chronic excess sugar consumption mean that further investigation is required to clarify the underlying mechanisms in the development of sugar-related memory and learning impairments.

Addiction

The impact that chronic excess sugar consumption has on the dopaminergic motivation-reward system has been less well studied. As noted, the sweet taste of sugar activates the motivation-reward pathway, due to the importance of sugar as an energy source for the brain. However, sugar is now chronically consumed in higher quantities and concentrations than would be naturally available (Bleich et al., 2009; Popkin & Nielsen, 2003; Welsh et al., 2011).

The effects of chronic sugar intake closely reflect drug-induced alterations in behaviour, mood and cognitive function (Ahmed et al., 2013; Avena et al., 2008; Avena,

Rada, Moise, & Hoebel, 2006; Spangler et al., 2004), leading to the theory that sugar may be addictive. Indeed, animal studies demonstrate that chronic excess sugar consumption fulfils the behavioural criterion of addiction (i.e. bingeing, withdrawal, craving and cross-sensitisation), particularly when administration is intermittent (Avena et al., 2008; Avena et al., 2006; Colantuoni et al., 2002; Kendig, 2014). For example, restricted access to sugar resulted in cross-sensitisation to drugs of abuse, including methamphetamine (Avena & Hoebel, 2003; Franklin, 2015; Franklin et al., 2013; Sharpe et al., 2015) and cocaine (Avena et al., 2008; Gosnell, 2000; Levine, Kotz, & Gosnell, 2003). The relative strength of sugar addiction is still in question, with some studies suggesting that the reward response to sugar is more robust than that of cocaine (Ahmed et al., 2013), while others maintain that any dependence is smaller in magnitude (Avena et al., 2008). Nevertheless, there is strong evidence to suggest that chronic excess sugar consumption can be addictive, particularly when consumed on an intermittent regimen.

It has been proposed that excess sugar consumption can impact the brain via over-activation of the motivation-reward system, causing altered striatal D2 receptor density or affinity in a similar way to addictive substances (Ahmed et al., 2013; Kendig, 2014; van Wieringen, Booij, Shalgunov, Elsinga, & Michel, 2013). Indeed, it has been shown that chronic excess sugar consumption is related to decreased levels of D2 receptor and D2 receptor mRNA in the striatum (Alsiö et al., 2010; Bello, Lucas, & Hajnal, 2002; Spangler et al., 2004). Lower levels of D2 receptor in the striatum have been linked to cravings (Fehr et al., 2008), poor decision making (Cocker et al., 2012), reduced behavioural inhibition (Ghahremani et al., 2012), and compulsive eating (Alsiö et al., 2010; Johnson & Kenny, 2010). As low striatal D2 receptor density is linked to addiction behaviours (Schneier et al., 2000), this offers a neurobiological basis for the suggestion that sugar intake may be addictive. However, it has been proposed that the relative proportion of high affinity state D2

receptors may be more important in addiction pathology than the expression of D2 receptors (Seeman et al., 2005; van Wieringen et al., 2013). For example, increased high affinity D2 receptor density has been linked to both dopamine hypersensitivity and addiction, with rodents showing increased density of high affinity state D2 receptors in cocaine, nicotine and caffeine models of addiction (van Wieringen et al., 2013). Alternatively, the behavioural effects of chronic excess sugar consumption may be related to changed dopamine activity in the mesolimbic reward pathway, particularly increased extracellular dopamine in the NAc (Avena & Hoebel, 2003; Avena et al., 2008; Avena et al., 2006). These mechanisms are not incompatible, as all indicate a difference in the functional activity of dopamine within the motivation-reward system.

The nature of addiction necessitates the primary use of adult subjects when conducting experiments, as changes in more stable adult brains are more easily interpreted. That is, the changing nature of developing brains in children and adolescents can mean that the same drug has vastly different impacts depending on the timing of administration, and therefore different long term outcomes. However, the vast changes occurring in the dopaminergic motivation-reward system and altered reward response during adolescence suggest that this cohort may be particularly vulnerable to addiction-like outcomes. In support of this, animal studies have found that younger subjects are more sensitive to the effects of sugar consumption on the motivation-reward system (Kendig et al., 2013; Vendruscolo et al., 2010). Similarly, increased sugar consumption has been positively correlated to reward sensitivity in adolescents (De Cock et al., 2015). Thus, it is important to more closely investigate the impact of chronic excess sugar consumption on the motivation-reward system in adolescent groups, particularly the dopaminergic pathways involved in addiction responses.

Impulsive Behaviour

The consumption of sugar sweetened beverages has been correlated with hyperactivity, aggression and conduct problems in children and adolescents (Lien, Lien, Heyerdahl, Thoresen, & Bjertness, 2006; Oddy et al., 2009; Solnick & Hemenway, 2012; Suglia, Solnick, & Hemenway, 2013). Similarly, maternal sugar consumption during pregnancy has been linked to increased likelihood of behavioural problems in the child's later life (Choi et al., 2015; Steenweg-de Graaff et al., 2014). As previously discussed, chronic excess sugar consumption has been linked to reduced striatal D2 receptor levels in adult populations (Bello et al., 2002; Spangler et al., 2004). Low D2 receptor density in the striatum is suggested to reduce inhibition and increase impulsivity (Cocker et al., 2012; Ghahremani et al., 2012), which may provide some explanation for findings that link higher sugar intake with poor behavioural outcomes in children and adolescents.

It should be recognised that many studies investigating the long-term health effects of sugar sweetened beverages do not control for the effects of caffeine, which is found in many popular soft drinks and has also been suggested to increase aggression and hyperactivity (Lien et al., 2006; Solnick & Hemenway, 2012). In one study, fruit juice, which has a similar sugar content to soft drink, was associated with lower scores of aggression in children (Suglia et al., 2013), suggesting that sugar was not the causal factor of aggressive behaviour. Similarly, in the short-term, sugar consumption can promote a helping attitude and increased self-control (Ahmed et al., 2013), thereby increasing social rather than anti-social behaviours. As such, the relationship between sugar intake and hyperactive or aggressive behaviours is questionable, with many researchers concluding that one does not exist (Benton, 2008; Kendig, 2014; Kruesi & Rapoport, 1986; Wolraich, Wilson, & White, 1995).

Psychological Disorder

Dopamine and the activation of D2 receptors play a large role in stress-coping and emotion regulation, particularly within the NAc (Badgaiyan, 2010; Cabib & Puglisi-Allegra, 2012; Hajnal & Norgren, 2001; Spear, 2000; Żurawek et al., 2013). The role of dopamine in promoting active coping with stress suggests that dopaminergic abnormality will have important implications for psychological resilience. Indeed, dopamine imbalances have been associated with a number of psychological symptoms. A reduction of D2 receptor function in the striatum has been linked to reduced stress resilience, as well as depression and anxiety disorders (Said et al., 2015; Schneier et al., 2000; Shah et al., 1997; Żurawek et al., 2013). Meanwhile, an increase of D2 receptors in high affinity states has been suggested to promote psychotic symptoms, and has been linked to schizophrenia (Previc, 2009; Seeman et al., 2005). Therefore, chronic excess sugar consumption could negatively impact psychological health, by altering dopamine receptor function in the brain.

Epidemiological studies have reported that mental health issues, predominantly depression, are associated with poor diet quality and high sugar consumption in both adult and adolescent populations (Jacka, Kremer, et al., 2010; Jacka, Pasco, et al., 2010; Jacka, Rothon, Taylor, Berk, & Stansfeld, 2013; O'Neil et al., 2014; Oddy et al., 2009; Pan, Zhang, & Shi, 2011; Quirk et al., 2013; Shi et al., 2010). While many of these studies do not specify which independent dietary elements are detrimental to mental health (e.g. Jacka, Kremer, et al., 2010; Jacka, Pasco, et al., 2010; Jacka et al., 2013; O'Neil et al., 2014; Oddy et al., 2009; Westover & Marangell, 2002), those specifically investigating soft drink consumption have found similar results (e.g. Lien et al., 2006; Pan et al., 2011; Shi et al., 2010). For example, Lien et al. (2006) reported a strong association between soft drink consumption and mental distress in adolescents, when controlling for social, behavioural and food related factors. Likewise, soft drink consumption has been positively correlated with depression, stress

related issues and suicidal ideation (Pan et al., 2011; Shi et al., 2010). However, causality cannot be inferred from correlational data, as individuals under stress or with mental health issues may be more prone to consume unhealthy or sweet foods. Moreover, the interpretation of soft drink consumption is confounded by the presence of caffeine, which is suggested to have negative psychological correlates (Solnick & Hemenway, 2012; Wang, Shen, Wu, & Zhang, 2015).

Greater experimental control means that animal studies are able to more clearly illustrate the impact of chronic excess sugar consumption on mental health. For example, Chepulis et al. (2009) found that rats fed a sugar-supplemented diet over a prolonged period of time show significantly higher levels of anxiety than controls. However, anxiety in rodents may not directly reflect anxiety in humans, and could instead represent stress. Indeed, studies have more often found human sugar consumption to be related to stress (Lien et al., 2006; Shi et al., 2010) than anxiety (Jacka, Pasco, et al., 2010). Nevertheless, the study provides evidence to suggest that chronic excess sugar consumption is related to adverse mental health outcomes, with changes to striatal D2 receptor offering a mechanism through which chronic excess sugar consumption has the potential to negatively impact psychological resilience.

1.3.4. Adolescent Vulnerability to Effects of Chronic Excess Sugar Consumption

It has been suggested that adolescents may be particularly vulnerable to the effects of chronic excess sugar consumption, as there is altered reward sensitivity due to significant changes in the dopaminergic system during this developmental period (Blakemore & Choudhury, 2006; Casey et al., 2008; Clark, Kirisci, & Tarter, 1998; Spear, 2000; van Leijenhorst et al., 2010; Wahlstrom, Collins, et al., 2010). Evidence from animal studies supports this, with adolescent rodents showing a greater increase in sugar consumption and heightened sensitivity to sugar reward than adults (Kendig et al., 2013; Vendruscolo et al.,

2010). Correspondingly, epidemiological studies of human behaviour consistently indicate that adolescents consume a higher percentage of energy from sugar in comparison to adults (Newens & Walton, 2015; Popkin & Nielsen, 2003).

As described above, adolescence is a period of substantial changes to the dopaminergic motivation-reward system, suggesting that the motivation-reward system may be particularly vulnerable to the effects of chronic excess sugar consumption during this period. Moreover, the movement towards stabilisation of brain circuitry and function during adolescence poses the threat that the effects of chronic excess sugar consumption during this time may be retained in later life. For example, rodents given free access to sugar solution during adolescence were observed to have decreased motivation for reward in adulthood (Vendruscolo, Gueye, Darnaudéry, Ahmed, & Cador, 2010). Similarly, several studies demonstrate significant residual effects of prenatal and perinatal exposure to sugar in later life (such as behavioural problems and altered reward responses; Bocarsly et al., 2012; Choi et al., 2015; Ong, Gugusheff, & Muhlhausler, 2012; Ong & Muhlhausler, 2011; Steenweg-de Graaff et al., 2014), and deficits in spatial learning and memory (Kuang et al., 2014). Nevertheless, while it has been suggested that adolescence is a period of heightened vulnerability to chronic excess sugar consumption, the long term outcomes of sugar consumption during adolescence remain relatively unexplored.

1.4. Conclusion and Current Study

There is strong evidence to suggest that chronic excess sugar consumption could affect psychological function, through over-activation of the dopaminergic motivation-reward system. In particular, studies have found reduced levels of D2 receptor mRNA and D2 receptor density in striatal brain regions. This is of greatest significance for adolescents, who

are the main consumers of refined sugar and particularly vulnerable to neuropsychological effects due to rapid brain development. Thus, many epidemiological studies in chronic excess sugar consumption have focused on adolescent populations. The overlap between behavioural and psychological symptoms expressed independently in relation to chronic excess sugar consumption, dopaminergic alterations in the motivation-reward system, and adolescence, also suggest similar neurological bases. However, as an emerging area of interest, the current literature has not yet provided convincing evidence of a relationship between chronic excess sugar consumption, changes to the dopaminergic reward pathway, and mental health problems. This is partly because methodological variations and issues within studies make it difficult to reach clear conclusions regarding causal factors. Further research is still required to clarify the effects of chronic excess sugar consumption during adolescence on mental health, and the mechanisms through which sugar impacts on the brain.

Based on findings in the literature, the aim of the current body of work is to investigate the effects of chronic excess sugar consumption during adolescence on D2 receptor density and affinity in striatal regions of the brain. These effects will be examined in two separate receptor-binding experiments using a rodent model with free access to 10% sucrose solution, making use of positron emission tomography (PET) imaging and autoradiography to provide in vivo and in vitro data, respectively.

The present study will address a number of gaps in the current literature, being one of few to consider the effects of free access to sugar on D2 receptors, and effects of chronic excess sugar consumption in an adolescent animal model. As such, it will provide important contributions towards resolving the nature of the relationship between chronic excess sugar consumption, the dopaminergic motivation-reward system, and mental health issues.

2. Research Project: The effects of chronic excess sugar consumption during adolescence on dopamine D2 receptors in a rodent model

2.1. Introduction

Chronic excess sugar consumption causes neurological changes in the dopaminergic motivation-reward system in the brain. Similarities between chronic excess sugar consumption and drug addiction (Ahmed et al., 2013; Avena et al., 2008) have led studies to investigate the impact of sugar intake on addiction-related behaviours and striatal function (Ahmed et al., 2013; Avena & Hoebel, 2003; Avena, Rada, & Hoebel, 2009; Bello et al., 2002; De Cock et al., 2015; Hajnal et al., 2004; Kendig, 2014; Ong & Muhlhausler, 2011; Vendruscolo et al., 2010). Striatal D2 receptor expression is observed to decrease following consumption of a sugar supplemented diet (Bello et al., 2002; Colantuoni et al., 2001; Spangler et al., 2004). Altered D2 receptor function has been implicated in such addiction-related behaviours as craving (Fehr et al., 2008), impulsivity and poor decision making (Cocker et al., 2012; Colantuoni et al., 2001; Ghahremani et al., 2012), and conditioned learning and reinforcement (Kendig et al., 2013; Sharpe et al., 2015). Substantial development of the dopaminergic motivation-reward system throughout adolescence has been suggested to increase vulnerability to these neurological and behavioural outcomes of chronic excess sugar consumption during this period. Furthermore, it has been suggested that the effects of chronic excess sugar consumption during this period may be long-lasting. However, previous studies have primarly focused on the immediate effects of sugar consumption. The current study will take steps to bridge this gap in the literature by longitudinally examining the effects of chronic excess sugar consumption during adolescence on the function of D2 receptors.

Previous research has demonstrated that chronic excess sugar consumption is related to decreased D2 receptor levels in the striatum (Bello et al., 2002; Colantuoni et al., 2001; Spangler et al., 2004). However, these changes may be impacted by the dietary regimen of

subjects. Many rodent studies place animals on a restricted diet, in which they are given access to highly palatable food for only a certain period of time. Indeed, a majority of studies investigating chronic excess sugar consumption utilise the intermittent access protocols developed by Avena & Hoebel (2003) or Kendig et al. (2013), in which animals are given access to sugar solution for a limited number of hours each day, to promote sugar-bingeing behaviours. Yet, it has been observed that dietary access itself can impact on D2 receptor levels, with both obese and lean animals on restricted food access showing higher levels of striatal D2 receptors than those with unrestricted access (Thanos, Michaelides, Piyis, Wang, & Volkow, 2008). Moreover, it has been suggested that people who consume excess amounts of sugar do so in a consistent manner, maintaining regular sugar intake rather than bingeing on sugar at intervals. Thus, the current study will investigate the impact of chronic excess sugar consumption on striatal D2 receptor density and function in animals with unrestricted access to sucrose solution.

Previous studies investigating D2 receptor variation have tended to focus on acute and short-term residual effects of chronic excess sugar consumption. For example, Bello et al. (2002) found that animals with restricted access to sucrose for only 7 days, showed lower D2 receptor binding in the CPu and NAc on the final day of treatment. Similarly, Spangler et al. (2004) and Colantuoni et al. (2001) found reductions in striatal D2 receptor density at 21 and 30 days of restricted access to sugar, respectively. On the other hand, recent behavioural studies suggest that the negative impacts of chronic excess sugar consumption on spatial memory and reward may remain after removal of sugar from the diet (Grimm et al., 2005; Kendig et al., 2013). However, a corresponding enduring effect of long-term sugar consumption on D2 receptors has yet to be described.

There is a shortage on information about the longitudinal effects of brain function following chronic sucrose administration. One of the few studies to longitudinally examine

the effects of unrestricted access to sugar during the period of adolescence, found a selective decrease in motivation for natural reward in adulthood (Vendruscolo et al., 2010). However, interpretation of this study is difficult, as the methods were not clearly reported and results are yet to be replicated. By examining D2 receptor density and affinity at the time of sugar treatment and 6 weeks following the end of treatment, the current study will provide further information to evaluate the possibility of long lasting effects of chronic excess sugar consumption during adolescence on the dopaminergic motivation-reward system.

The use of a novel PET analysis technique, partial saturation analysis, allows for a repeated measures longitudinal study design to assess both the immediate and longer-term residual effects of chronic excess sugar consumption (Wimberley, Fischer, Reilhac, Pichler, & Gregoire, 2014). Partial saturation analysis is also unique in that it enables a measure of functional D2 receptor density and affinity (Wimberley, Fischer, et al., 2014). That is, where previous techniques, such as autoradiography, provide a relative measure of the total D2 receptor population within the synaptic terminal and on the membrane surface at a single time point, partial saturation analysis is able to provide a measure of functional D2 receptor that is available for binding in the living organism across multiple time points (

Figure 3). Utilising both PET and autoradiography imaging techniques, the current study attempts to provide greater insight into the mechanisms of the D2 receptor variation observed in models of chronic excess sugar consumption by differentiating between total and functional D2 receptor density. The assessment of apparent D2 receptor affinity may also provide useful information regarding the nature of D2 receptor alterations in chronic excess sugar consumption, and potential downstream effects to impact on brain function and behaviour. Previous studies suggest that altered D2 receptor affinity is related to various psychological symptoms, for

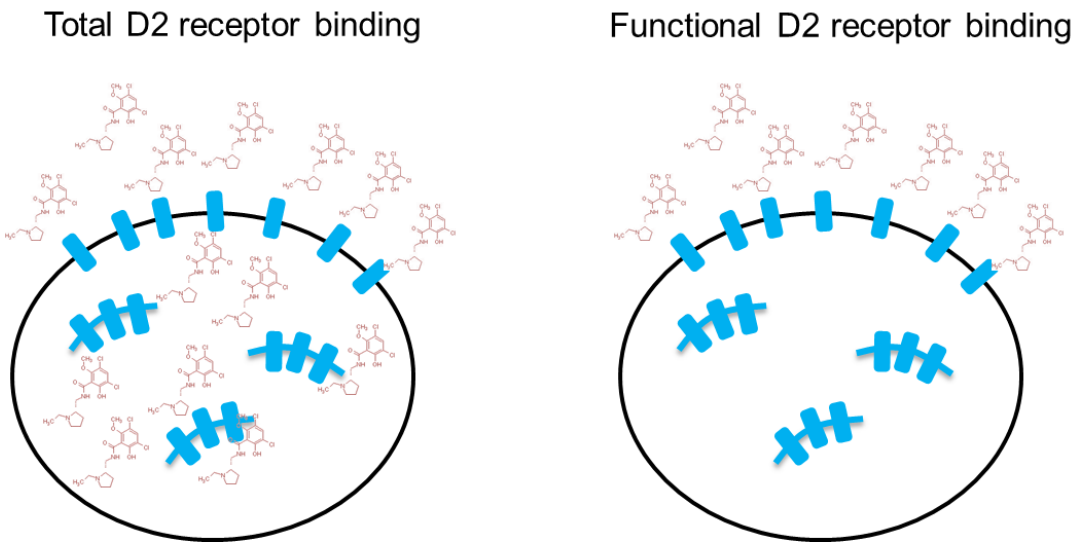


Figure 3. Schematic illustration of total versus functional D2 receptor (blue) binding, with raclopride (red).

example, schizophrenia has been linked to increased density of high affinity state D2 receptors (Seeman et al., 2005; van Wieringen et al., 2013). As chronic excess sugar consumption has been linked to changes in D2 receptor density, it is essential to further elucidate the functional change of this receptor to understand the potential consequences of chronic excess sugar intake for psychological health.

2.1.1. Aims and Hypotheses

This novel study will utilise PET and autoradiography techniques to image the D2 receptor binding in adult rodents given free access to sugar during adolescence. Relative measures of total and functional D2 receptor density, as well as apparent D2 receptor affinity, will be assessed to provide a more detailed characterisation of the impacts of chronic excess sugar consumption on the motivation-reward system. Moreover, both immediate and residual effects of chronic sugar intake will be measured within subjects, to examine the stability of any observed differences produced by sugar consumption when compared to water controls.

Based on previous literature, the following hypotheses have been formed.

Hypothesis 1: Chronic excess sugar consumption during adolescence will result in reduced functional D2 receptor density in the striatum in early adulthood, compared to controls.

Hypothesis 2: Chronic excess sugar consumption during adolescence will alter the affinity of D2 receptors in the striatum in early adulthood, compared to controls.

Hypothesis 3: Chronic excess sugar consumption during adolescence will result in reduced total D2 receptor density in that striatum in early adulthood, compared to controls.

Hypothesis 4: Differences observed in functional D2 receptor density and D2 receptor affinity on the final day of treatment will be sustained after sugar treatment has ended.

Hypothesis 5: Chronic excess sugar consumption during adolescence will result in reduced total D2 receptor density in the striatum in early adulthood, compared to controls.

2.2. Materials and Methods

2.2.1. Experiment 1: *In vivo* PET

Twelve age-matched adolescent male Sprague-Dawley rats (PND ~28; Animal Resources Centre Canning Vale, WA, Australia) were housed three to a cage in standard laboratory conditions. Rodents were housed in temperature regulated rooms (21 ± 1°C) on a 12h light-dark cycle, with lights on at 07:00 h. The study was approved by the University of Sydney Animal Ethics Committee, and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013).

Upon arrival, rats underwent a three day acclimation period followed by three days of handling, prior to treatment. Treatment began when animals were at the start of middle adolescence (approximately PND 35) and continued to the end of adolescence (approximately PND 60). Throughout the experiment, rats had *ad libitum* access to standard rat chow and toys. Rats were divided into two groups, each containing 2 cages of 3 animals, that were randomly assigned to receive *ad libitum* access to either 10% sucrose solution (100g/L sugar in tap water; sugar; n=6) or normal tap water (control; n=6) for the treatment period. Body weight, chow and fluid consumption were measured every morning, beginning on the first day of treatment to the final day of the experiment. Chow and drinking bottles were refilled each morning after measurement.

On the final day of treatment (Day 28), the D2 receptor binding of all rodents was imaged using the PET and computerised tomography (CT) protocol described below. PET/CT imaging was conducted during the light period over two days, with sugar animals having free access to sucrose solution until imaging to ensure any observed differences were not due to sucrose removal. After the initial imaging study, animals were removed from the scanner and placed in a warm cage to recover from anaesthesia, before being returned to the home cage.

Following the treatment period, all animals were provided with *ad libitum* access to normal tap water for 6 weeks. Rodents were then re-imaged according to the PET/CT imaging protocol described below. Following the final imaging study, the rats were euthanised while under anaesthesia. Brain, liver and blood samples were removed and snap-frozen in liquid nitrogen for analyses in future studies, not detailed here.

PET/CT Acquisition

Imaging studies were performed using two dedicated small animal PET/CT imaging systems (Inveon, Siemens AG, Germany). A previous study, by Callaghan et al. (2014), has verified that the inter-scanner variability in binding potential using kinetic modelling techniques between these systems is not significantly different to test-retest variability within scanners.

Rodents were placed in an induction chamber with 5% isoflurane in medical oxygen. Once anaesthetised, animals were moved to the scanner bed and secured on a temperature controlled heating pad, with anaesthesia maintained through a nose mask. Animals' lateral tail vein was then cannulated with heparinised saline (1:50 dilution) in preparation for scanning. Respiration rate and temperature were monitored using BioVet (m2m Imaging Corp, USA), and isoflurane levels were adjusted as necessary to maintain anaesthesia throughout the procedure.

Each PET scan commenced 10 seconds prior to injection start to ensure all events were captured, and lasted a total of 60 minutes. Ten seconds after PET start, the animals were injected with [^{11}C]raclopride (injected volume 300μL, injected dose 10-33 MBq, specific activity 134±9.2 MBq/mmol; produced on site at the ANSTO Camperdown facility) over a period of 1 minute using an infusion pump (Harvard Apparatus, USA). After the PET scan, a 15 minute CT scan was systematically completed for use in later attenuation and scatter correction calculations. An example of the PET/CT images obtained is provided in Figure 4.

PET/CT images were not obtained from three animals (one sugar and two control) at the final day of treatment, due to radiotracer injection issues.

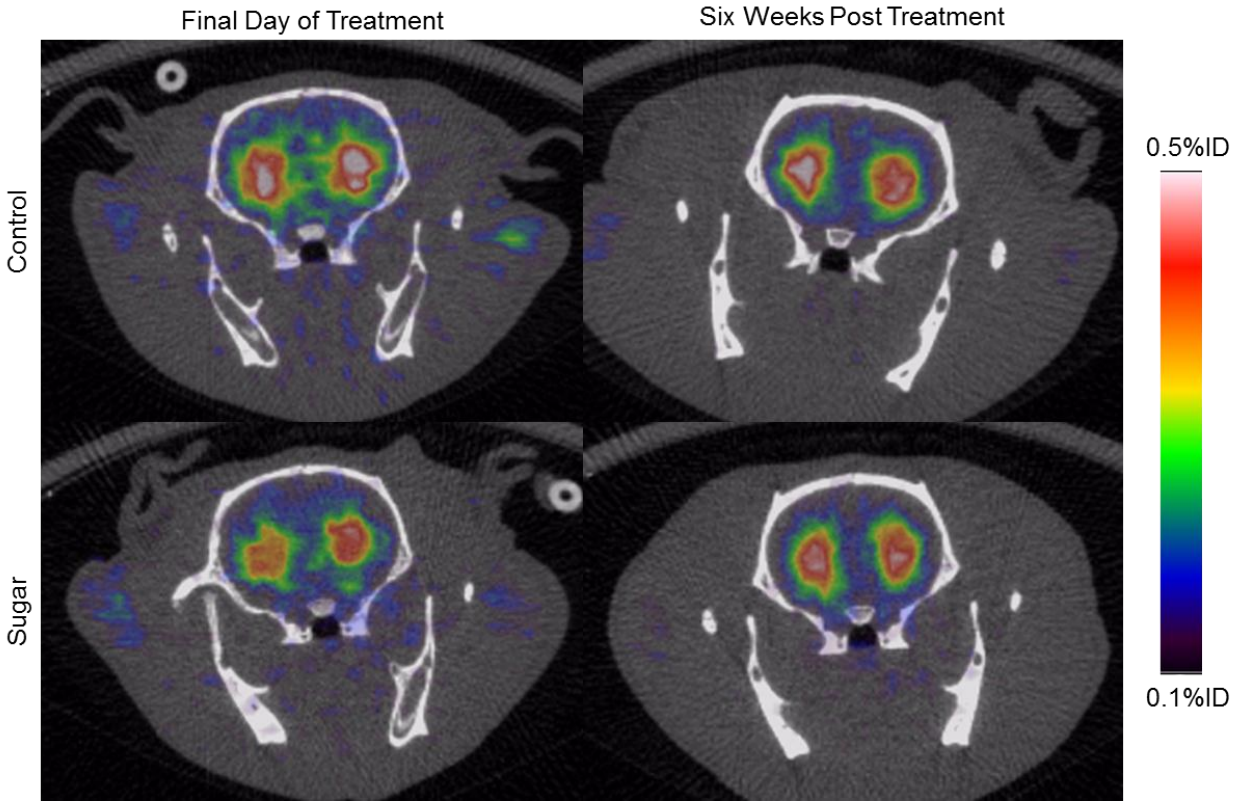


Figure 4. Coronal sections of late frame images of brain showing [^{11}C]raclopride uptake (percentage of injected dose per mm^3) with coregistered PET/CT at the final day of treatment and six weeks post treatment, in sugar and control group animals. The calibrated lookup table for the PET images is shown on the right.

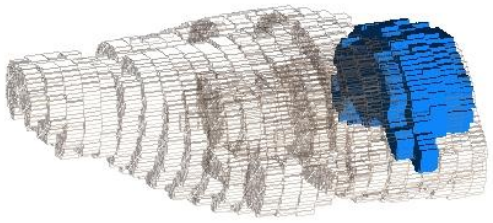
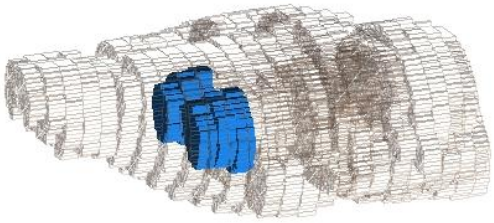
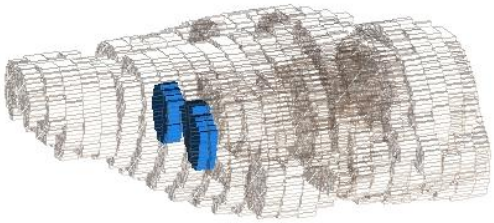
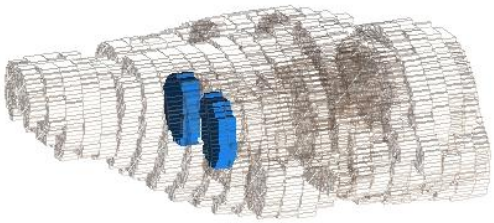
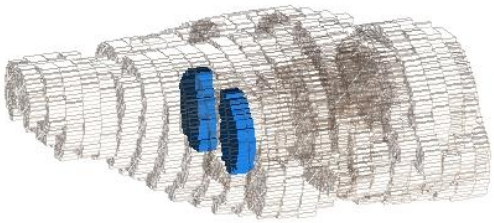
PET Data Analysis

List-mode data were reconstructed with an analytical reconstruction (2D filtered back-projection) including attenuation and scatter correction, achieving a reconstructed spatial resolution of 1.5mm. The dynamic PET data were converted into histograms in 14 frames (6 × 120s, 8 × 360s) before reconstruction. Respective PET and CT volumes were automatically co-registered for each of the scans. All CT volumes were manually co-registered to an arbitrarily determined reference CT for the corresponding scan cohort, using Anatomist/BrainVisa (V4.2.1, 2012, http://brainvisa.info/). The reference CTs were then manually aligned to an in-house magnetic resonance imaging (MRI) based rat brain atlas, allowing equivalent volumes of interest (VOI) to be applied across subjects for each scan

cohort. The VOIs utilised in this study, cerebellum and striatum, are shown in Table 1. The cerebellum was chosen as a reference region for kinetic modelling analyses due to minimal expression of D2 receptors. Due to the established heterogeneity within striatum (Berendse, Graaf, & Groenewegen, 1992; Tassin, Cheramy, Blanc, Thierry, & Glowinski, 1976; Willuhn, Sun, & Steiner, 2003; Yager et al., 2015), both the whole structure and three component rostro-caudal levels were used, with Level 1 being most rostral and Level 3 most caudal (Table 1). Finally, transformation matrixes were created from the rat brain atlas to each PET image in each group, using Anatomist/BrainVisa (V4.2.1, 2012, http://brainvisa.info/). Data from the selected VOIs were extracted and converted to activity concentrations (Bq/mm^3) with calibration factors.

Estimations of the functional D2 receptor binding availability (B_{avail}) and apparent affinity ($appK_{D)}$ were then calculated using partial saturation approach analysis (Wimberley, Angelis, et al., 2014; Wimberley, Fischer, et al., 2014). In brief, partial saturation analysis makes use of the tissue activity curves for the regions of interest and the reference region (an example of the population tissue activity curve data for whole striatum is shown in Figure 5) to determine a time range where the receptor and radiotracer are in binding equilibrium. A two compartment mathematical model can then be solved at this point, to give both B_{avail} and $appK_D$. One animal from the control group did not reach the required level of activity (60-80% occupancy of available receptors) for partial saturation analysis to be performed for any region of interest, at both scan time points.

Table 1. SUMMARY OF VOLUMES OF INTEREST, WITH EACH VOLUME DEPICTED WITHIN THE WHOLE RAT BRAIN ATLAS.

Volume of Interest (VOI)	**Image in Brain Atlas**	**Volume (mm^3)**
Cerebellum		210.333
Striatum, whole		57.1289
Striatum, Level 1		14.4470
Striatum, Level 2		20.2698
Striatum, Level 3		22.4121

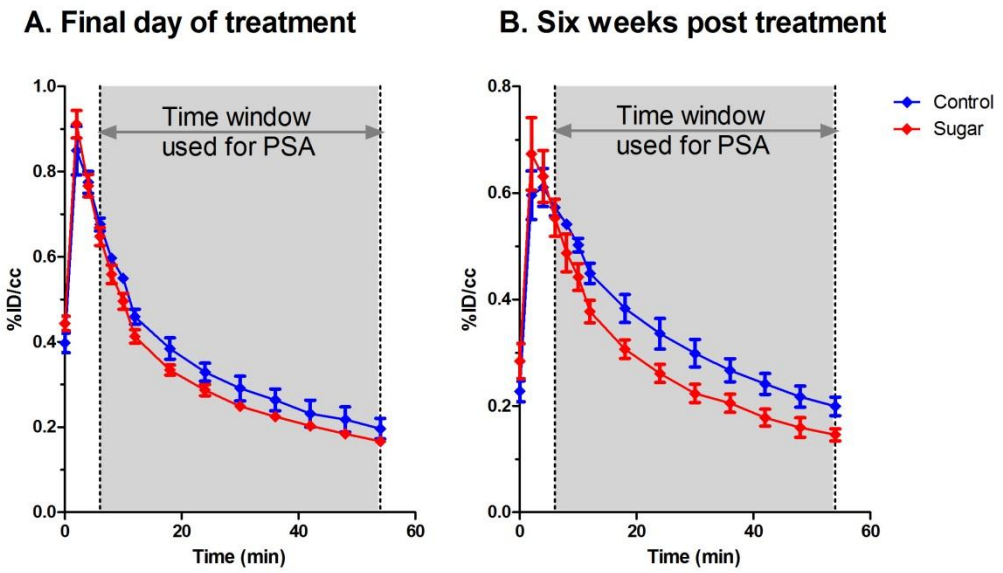


Figure 5. Tissue activity curves for [^{11}C]raclopride within whole striatum showing the mean (±SEM) percentage of injected dose per cm^3 for the control and sugar treatment groups at the final day of treatment (A) and 6 weeks post treatment (B).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (Version 20.0) and Prism (Version 6.0, GraphPad Software Inc). Data are expressed as mean ± SEM in all figures.

Multiple two-way mixed analysis of variances (ANOVAs) were conducted to individually compare weight gain, fluid intake, and food intake between the sugar and control groups. The Greenhouse-Geisser adjustment was applied if the assumption of sphericity was violated. Weight gain (g) was analysed as a measure of individual animal weight at 11 time points, on days spaced approximately one week apart. Fluid (mL) and food intake (g) were recorded per cage on a daily basis. For the purpose of analysis, an average measure of daily intake was calculated for each cage in a given week and divided by the number of animals per

cage, to give an approximate average fluid and food intake per day for 10 time points for each animal.

Multiple two-way mixed ANOVAs were run to compare functional D2 receptor density and apparent D2 receptor affinity across treatment groups and time points, for each specified volume of interest. Missing data meant that using a repeated measures design resulted in reduced sample sizes being available for final analysis (minimum sugar: n=5, minimum control: n=3). Nevertheless, a repeated measures design was maintained over a between groups design, as statistical control of within subjects variance was determined to be of greater importance than the slight increase of power offered by an increased sample size.

2.3.1. Experiment 2: *In vitro* Autoradiography

Twelve age-matched adolescent male Sprague-Dawley rats (Animal Resources Centre Canning Vale, WA, Australia) were housed four to a cage in standard laboratory conditions. Rodents were housed in temperature regulated rooms (21 ± 1°C) on a 12h light-dark cycle, with lights on at 08:00 h. Body weight, chow and fluid consumption were measured every three days, following which chow and drinking bottles were refilled. Animal treatment in this study was approved by Macquarie University Animal Ethics Committee, and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013).

Upon arrival to the animal facility, rats underwent an acclimation period of approximately one week followed by three days of handling, prior to treatment. Treatment began when animals were at the start of middle adolescence (PND 35) and continued to the end of adolescence (PND 60). Throughout the experiment, rats had *ad libitum* access to standard rat chow and toys. Rats were divided into two groups (n=6), that were randomly

assigned to receive either *ad libitum* tap water or a 10% sucrose solution (100g sugar/L tap water) for the duration of the treatment. All rodents underwent locomotor behavioural testing on the first day of treatment and final day of treatment.

Following the treatment period, all animals were provided with *ad libitum* access to water for 9 days, prior to sacrifice for brain analyses. Although not detailed here, locomotor activity in response to an acute saline challenge (intraperitoneal injection of saline; 0.9%, 1mL/kg) was measured on the final day of the experiment and reported in Franklin (2015).

Two hours after the final locomotor testing, animals were euthanized with an I.P. injection of 1mL pentobarbitone sodium (325mg/mL; Virbac, Milperra, Australia) diluted in 1mL saline, followed by guillotine decapitation. The brains were rapidly removed, snap frozen in liquid nitrogen, and stored at -80°C until autoradiography analyses.

Cryosectioning and Autoradiography

Brain tissue was sliced at -20°C into 20µm coronal sections, using a cryostat (Leica CM3050s). Sections were collected and thaw mounted onto poly-lysine coated slides, and stored at -80°C until binding assays were conducted.

In recognition of the heterogeneous structure of the striatum, slides containing three rostro-caudal levels of the CPu were analysed in the autoradiography assay. The figures contained in these sections (Figure 20 [F20], Figure 30 [F30] and Figure 40 [F40], according to the stereotaxic rat atlas of Paxinos & Watson, 2006) were chosen according to previous studies investigating topographical changes in striatal receptor density (Tassin et al., 1976; Willuhn et al., 2003).

The study followed previously validated methods using [^{3}H]raclopride ligand binding for the dopamine D2 receptor. Matched slide mounted sections were removed from the -80°C freezer and allowed to thaw for at least 30 minutes. Slides were then pre-incubated for 20 minutes in Tris-buffer solution (pH 7.4 at room temperature), consisting of 50mM Tris-HCl, 120mM NaCl, 2mM CaCl and 1mM MgCl. Following this, slides were allowed to air dry flat, ready for addition of radio-ligand solution. Total binding and non-specific binding slides were pipetted with 900µL of hot ligand (5nM [^{3}H]raclopride [2890 GBq/mmol; Perkin Elmer, USA] and di-methyl sulfoxide [DMSO] in Tris-buffer solution) or cold ligand (5nM [^{3}H]raclopride [2890 GBq/mmol; Perkin Elmer, USA] and 10µM (+)-butaclamol [Sigma D033, MW 397.98] dissolved in DMSO, in Tris-buffer solution) respectively, and allowed to incubate in a humidified environment for 60 minutes at room temperature. The ratio of vehicle DMSO added to the hot and cold ligands was adjusted to make 0.001% of the total volume, to control for vehicle effects. Immediately after incubation, the slides were washed twice for 5 minutes in ice-cold Tris-buffer and dipped twice in ice-cold distilled water. The slides were then dried under a stream of cold air for 30 minutes, and allowed to dry overnight at room temperature.

Dried sections were exposed to film paper (Carestream-Kodak: Biomax MR) in X-ray film cassettes. One set of slides from each assay contained autoradiographic tritium standards (autoradiographic[^{3}H]micro-scales; Amersham Biosciences, UK). Sections were exposed for 8 weeks before being developed. Films were developed using Kodak GBX developer and fixed with Kodak GBX fixer.

Films were analysed using a computer assisted image analysis system, Multianalyst, connected to a GS-800 Calibrated Densitometer (Bio-Rad, USA). Regions of interest (ROIs) were manually drawn using ImageJ (Fiji) software (Figure 6). Striatal ROIs were drawn on the medial, dorsal and ventral striatal regions according to illustrations by Willuhn et al.

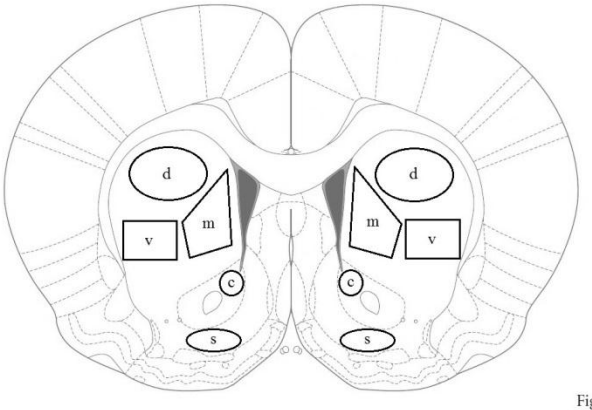


Figure 20
Bregma 1.56 mm

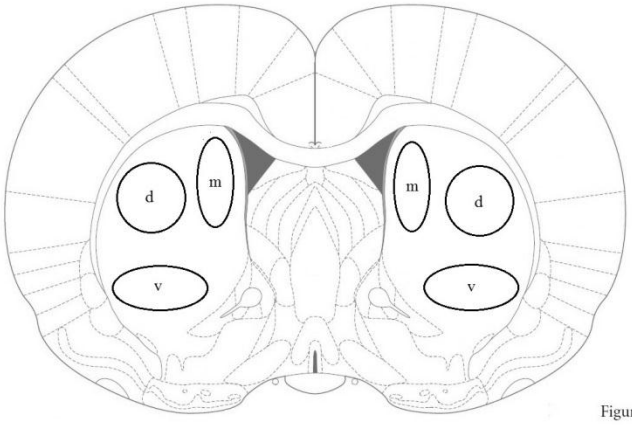


Figure 30
Bregma 0.36 mm

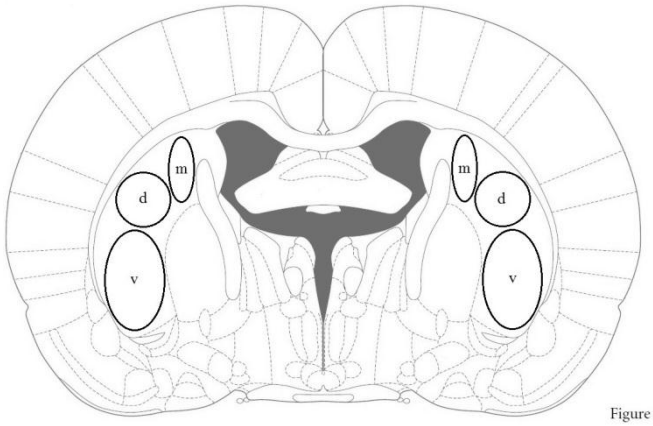


Figure 40
Bregma -0.84 mm

Figure 6. Regions of interest drawn on Figure 20 (F20), Figure 30 (F30) and Figure 40 (F40) of the stereotaxic rat brain atlas of Paxinos and Watson (2006), depicting the medial (m), dorsal (d) and ventral (v) aspects of the striatum, and nucleus accumbens core (c) and shell (s).

(2003). ROIs were also drawn around the NAc core and shell, with the regions identified by comparison to a stereotaxic atlas of the rat brain (Paxinos & Watson, 2006). Quantification of receptor binding in each brain region was performed by measuring the average optical density in adjacent brain sections. Nonspecific binding was subtracted from total binding to give a value for specific binding. Optical density measurements for specific binding were then converted into a tissue equivalent (mCi/mg) according to the calibration curve obtained from the autoradiographic tritium standards. Sections containing cryosectioning artefacts (e.g. missing tissue) were identified by visual inspection, and excluded from analysis for each affected drawn ROI.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (v20) and Prism (v6.0, GraphPad Software Inc).

Multiple two-way mixed analysis of variances (ANOVAs) were conducted to individually compare weight gain, fluid intake, and food intake between the sugar and control groups. The Greenhouse-Geisser adjustment was applied if the assumption of sphericity was violated. Weight gain (g) was analysed as a measure of each individual animals' weight at 6 time points, on days spaced approximately one week apart. Fluid (mL) and food intake (g) were recorded per cage on a daily basis. For the purpose of analysis, an average measure of daily intake was calculated for each cage in a given week and divided by the number of animals per cage, to give an approximate average fluid and food intake per day for 5 time points for each animal.

Unpaired Students t-tests were conducted to compare relative measures of total D2 receptor density between treatment groups, for each ROI. Univariate ANOVA with repeated contrasts was conducted to compare total D2 receptor density between the rostro-caudal levels of striatum (i.e. F20, F30, F40). Likewise, univariate ANOVA with repeated contrasts was conducted to compare total D2 receptor density between the medial, dorsal and ventral regions within each rostro-caudal level.

Statistical analysis was not performed for locomotor activity in the current study. However, results from this cohort of animals have previously been reported, with no significant difference in locomotor activity found between the treatment and control groups on the first day of treatment, final day of treatment, or following a 10 day washout period with a saline challenge (Franklin, 2015).

2.3. Results

2.3.1. Experiment 1: *In vivo* PET

Animal Weight and Diet

On the first day of treatment there were no differences in weight between the sugar and control groups (Figure 7[A]). All animals gained weight at a steady rate over the course of the study [$F(1.321, 13.212)=633.029$, $p<0.0001$]. While the sugar animals appeared to gain weight at a slightly slower rate than controls following the end of sucrose treatment, this did not reach significance.

Mixed two-way ANOVA showed a significant interaction between treatment group and volume of fluid consumed throughout the experiment [$F(1.150, 11.496)=27.331$, $p<0.0001$]. Sugar animals drank significantly more than controls throughout the treatment

period, with this amount reducing to baseline fluid intake levels following the end of treatment (Figure 7[B]). This is consistent with findings from previous studies in which animals were given free access to sucrose solution. Due to an outlier, the control group appeared to significantly increase fluid intake across the period of treatment. This deviation in fluid intake is evident through comparison between the two control group cages in the first week of treatment (Cage3=34.12mL, Cage4=27.75mL), first week post-treatment (Cage3=121.68mL, Cage4=38.83mL), and final week of treatment (Cage3=131.55mL, Cage4=35.84mL), where the outlier is in Cage3. However, due to the group housing this data could not be excluded as fluid intake was determined from cage averages.

All animals increased the amount of food consumed over the period of the study [$F(1.135, 11.349)=58.958$, $p<0.0001$]. A significant interaction was also observed between treatment group and grams of chow consumed [$F(1.135, 11.349)=10.921$, $p=0.006$]. Throughout the treatment period, sugar animals consumed significantly less chow than control animals (Figure 7[C]). This amount increased following the end of treatment. An outlier in the control group skewed results such that the food intake of the control group appears higher than observed in previous studies. This deviation in food intake is evident through comparison between the two control group cages in the first week of treatment (Cage3=25.19g, Cage4=24.45g), first week post-treatment (Cage3=37.25g, Cage4=28.43g), and final week of treatment (Cage3=37.82g, Cage4=30.29g), where the outlier is in Cage3. However, as chow consumption was measured as a cage average, it was not possible to exclude this data from the dataset.

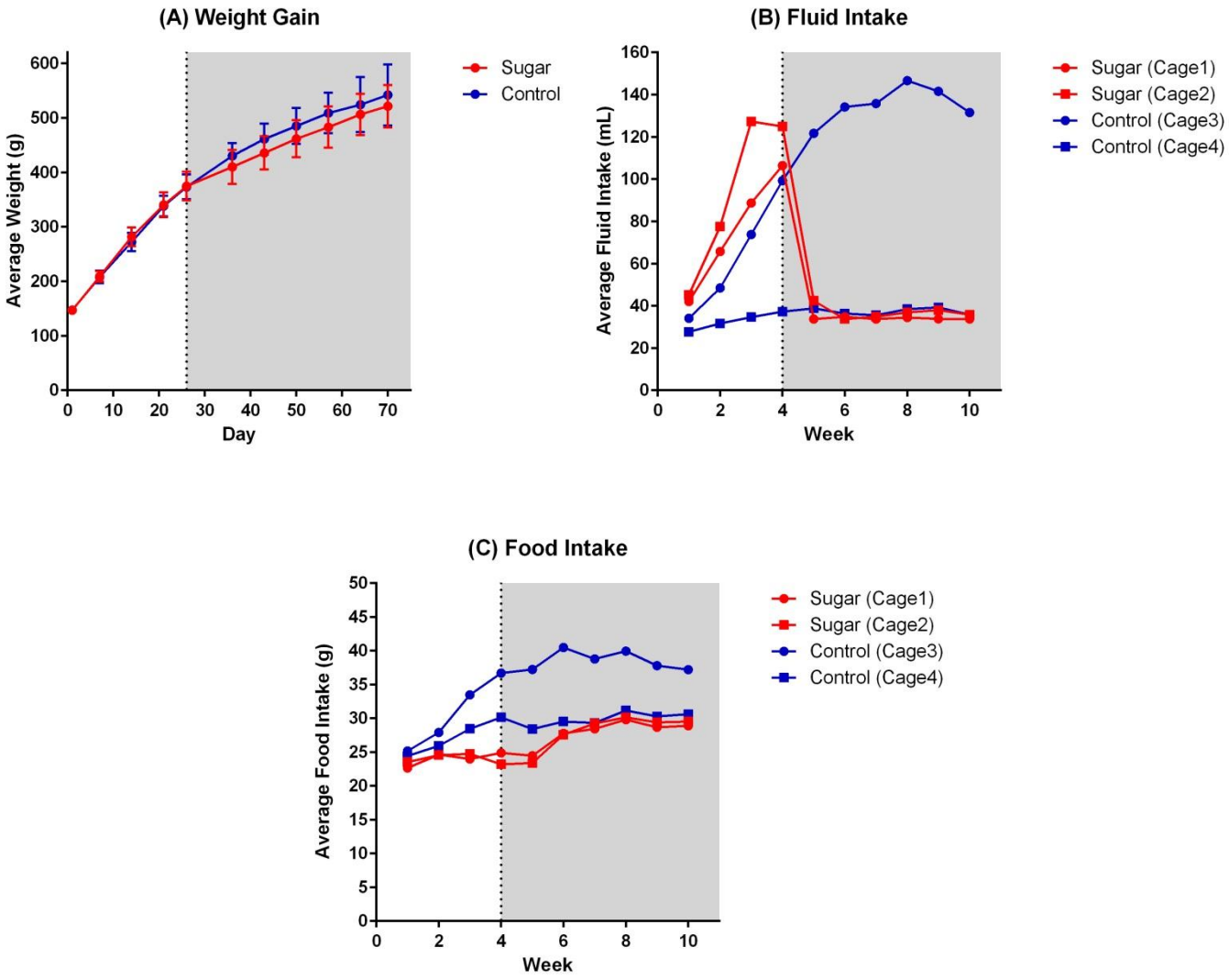


Figure 7. Animal weight, fluid intake and food intake for the period of Experiment 1. Weight (panel A) is shown as mean (±SEM) of daily animal weights (g) from treatment Day 1 to 6 weeks post treatment (Day 70). Fluid intake (panel B) is shown as average weekly fluid intake (mL) by cage, from first week of treatment to 6 weeks post treatment (Week 10). Food intake (panel C) is shown as average weekly food consumption by cage (g) from first week of treatment to 6 weeks post treatment (Week 10). Vertical dashed line represents final treatment point, with the washout period in grey.

Binding Availability

A two-way repeated measures ANOVA found no significant main effects of time [$F(1,6)=0.198$, $p=0.672$] or treatment [$F(1, 6)=0.028$, $p=0.873$] on D2 receptor B_{avail} in the

whole striatum (Figure 8[A]). Likewise, no interaction effect between time and treatment group was observed [$F(1, 6)=2.545$, $p=0.162$]. However, analyses of rostro-caudal levels within the striatum showed variable effects, reflecting the heterogeneity of the striatum.

Level 1, the most rostral level of striatum, showed a significant main effect of time [$F(1, 6)=9.327$, $p=0.022$) and interaction effect between time and treatment [$F(1, 6)=6.871$, $p=0.040$]. There was no main effect for treatment [$F(1, 6)=1.447$, $p=0.274$]. While D2 receptor B_{avail} in the sugar group remained fairly stable from the final day of treatment to 6 weeks post treatment, receptor availability in the control group nearly doubled from the final day of treatment to 6 weeks post treatment (Figure 8[B]).

Level 2, the middle rostro-caudal level of striatum, showed no significant main effects of time [$F(1, 5)=0.184$, $p=0.686$] or treatment [$F(1, 5)=0.682$, $p=0.447$), however a significant interaction effect was observed [$F(1, 5)=12.713$, $p=0.016$]. On the final day of treatment, the sugar and control groups showed a similar D2 receptor B_{avail} (Figure 8[C]). At six weeks post treatment, the D2 receptor B_{avail} of the two groups had moved in contrasting directions, with a decrease in the sugar group and increase in the control group from the final day of treatment.

Level 3, the most caudal level of striatum, showed significant main effects of both time [$F(1,6)=28.349$, $p=0.002$] and treatment [$F=(1, 6)=15.772$, $p=0.007$], but no interaction effect [$F(1,6)=1.353$, $p=0.289$]. The D2 receptor B_{avail} reduced from the final day of treatment to 6 weeks post treatment in both treatment conditions (Figure 8[D]). However, the sugar group had lower levels of D2 receptor B_{avail} at both time points, compared to the control group.

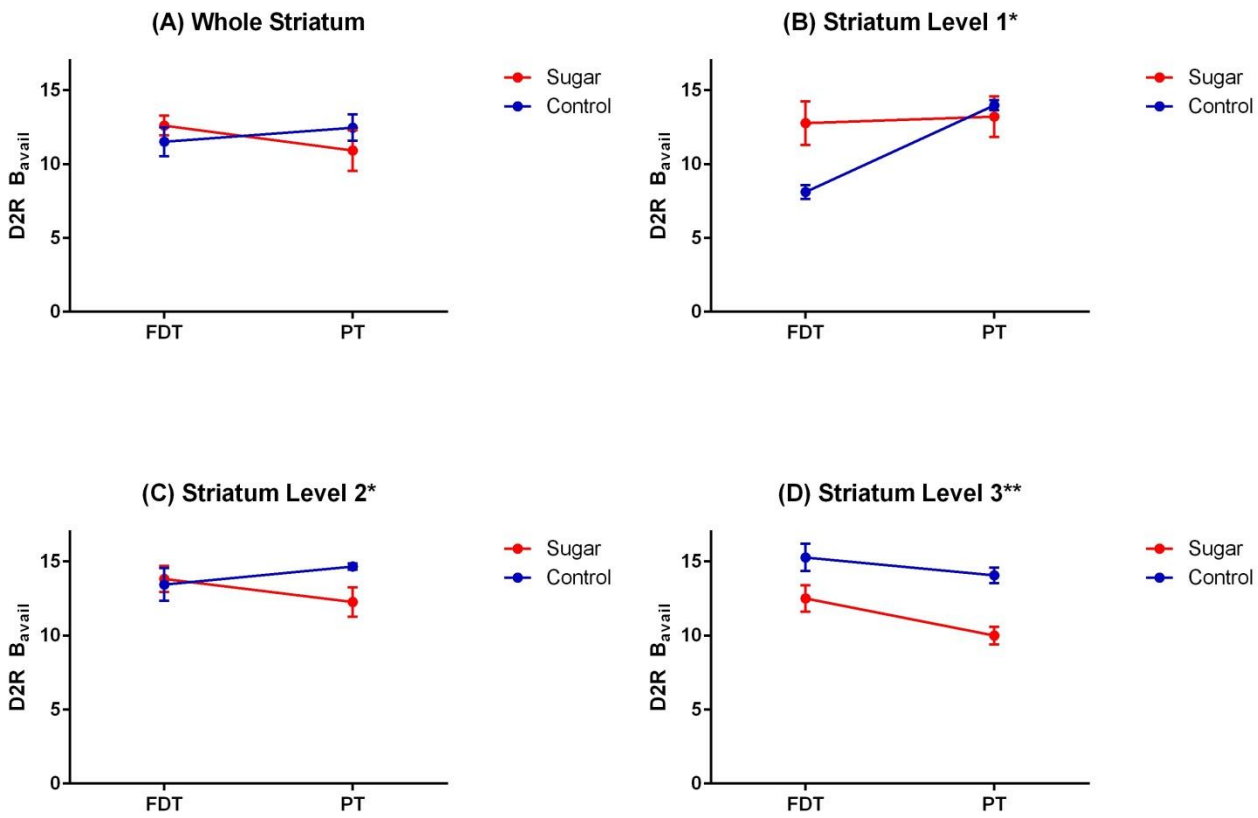


Figure 8. Functional D2 receptor bioavailability (D2R Bavail) at the final day of treatment (FDT) and six weeks post treatment (PT) in the whole striatum (panel A), striatal Level 1 (panel B; *p=0.040 for time×treatment interaction effect), striatal Level 2 (panel C; *p=0.016 for time×treatment interaction effect), and striatal Level 3 (panel D; **p=0.007 for main effect of treatment and p=0.002 for main effect of time).

Apparent Dissociation Constant

A repeated measures ANOVA found a significant main effect of time [$F(1, 6)=8.186$, $p=0.029$], but no main effect of treatment [$F(1, 6)= 5.485$, $p=0.058$] or interaction effect [$F(1,6)=1.015$, $p=0.353$], on the appK_D of D2 receptors in the whole striatum. That is, the appK_D was higher on the final day of treatment than 6 weeks post treatment across both treatment groups (Figure 9(A)).

No significant time [$F(1,6)=1.647$, $p=0.247$] or treatment [$F(1,6)=0.437$, $p=0.533$] main effects, or interaction effect [$F(1,6)=1.137$, $p=0.327$], were observed for the $appK_D$ in level 1 of the striatum (Figure 9(B)).

There was a significant main effect of time [$F(1,5)=8.855$, $p=0.031$] on $appK_D$ in level 2 of the striatum. That is, the $appK_D$ of D2 receptors in the middle rostro-caudal level of striatum, was higher on the final day of treatment than 6 weeks post treatment (Figure 9(C)). No significant main effect for treatment [$F(1,5)=0.090$, $p=0.776$] or interaction effect [$F(1,5)=0.002$, $p=0.963$] were shown.

Similarly, there was a significant main effect of time [$F(1,6)=5.992$, $p=0.050$], but no treatment [$F(1,6)=2.217$, $SD=0.187$] or interaction [$F(1,6)=2.888$, $p=0.618$] effects, on $appK_D$ in level 3 of the striatum. The $appK_D$ of D2 receptors was higher on the final day of treatment than 6 weeks post treatment, in the caudal level of the striatum (Figure 9(D)).

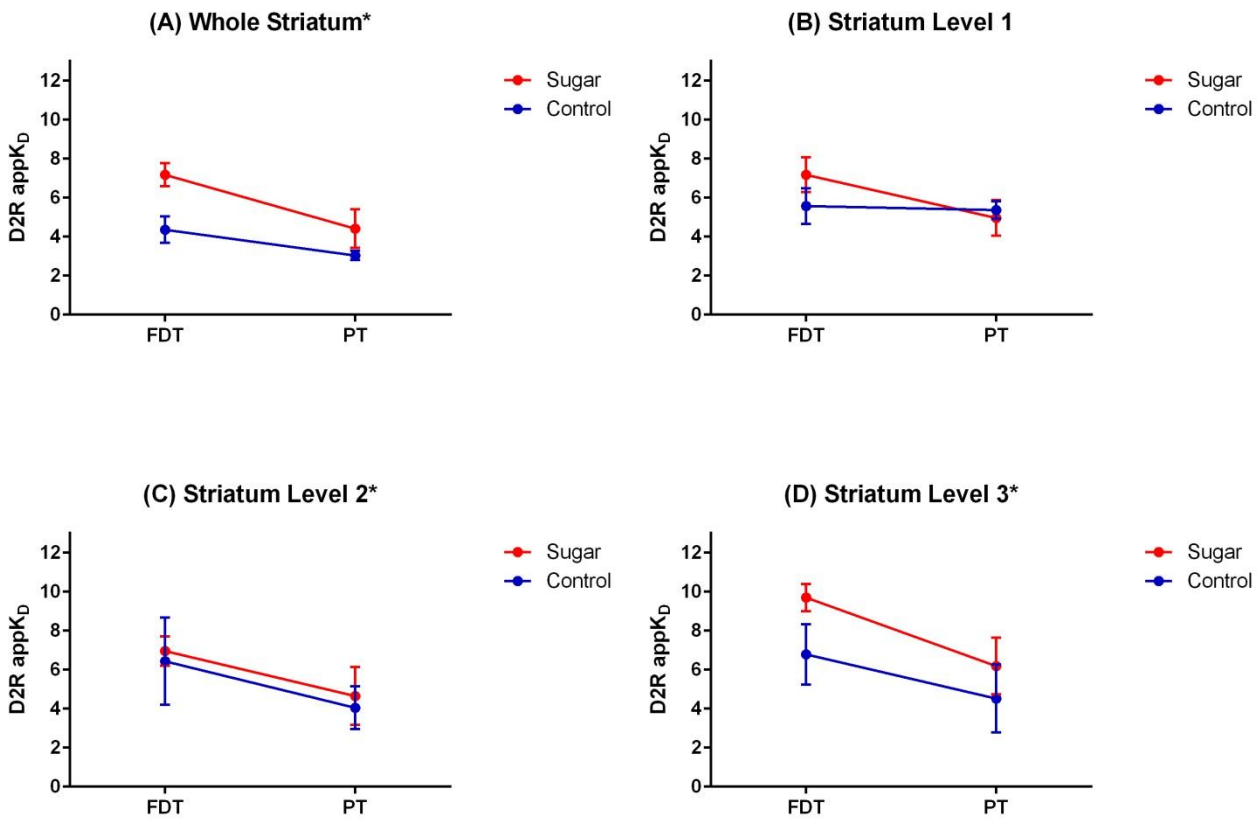


Figure 9. Apparent D2 receptor dissociation constant (D2R appK$_D$) at the final day of treatment (FDT) and six weeks post treatment (PT) in the whole striatum (panel A; *p=0.029 for main effect of time), striatal Level 1 (panel B), striatal Level 2 (panel C; *p=0.031 for main effect of time), and striatal Level 3 (panel D; *p=0.050 for main effect of time). No treatment or treatment×time interaction effects were found.

2.3.2. Experiment 2: *In vitro* Autoradiography

Animal Weights and Diet

A mixed two way ANOVA found that all animals gained weight at a steady rate over the course of the study [$F(1.262, 12.621)=498.358$, $p<0.0001$]. On the first day of treatment, there were no differences in weight between the sugar and control groups (Figure 10[A]). Although the sugar group gained weight more slowly than the control group, this did not reach significance.

There was a significant interaction between treatment group and volume of fluid consumed throughout the experiment [$F(1.891, 18.908)=42.242$, $p<0.0001$]. Throughout the treatment period, there were significant differences in fluid intake between the sugar and control groups (Figure 10[B]). This difference was greatest on the final day of treatment, where the sugar group drank significantly more than the control group. One week post treatment, there was no difference between the sugar and control groups.

There was a significant interaction between treatment group and amount of food consumed throughout the experiment [$F(1.185, 11.854)=4.805$, $p=0.044$]. Throughout the treatment period, the sugar group consumed less chow than the water group, and this difference was greatest at the final day of treatment (Figure 10[C]). One week post treatment, there was no difference between the sugar and control groups.

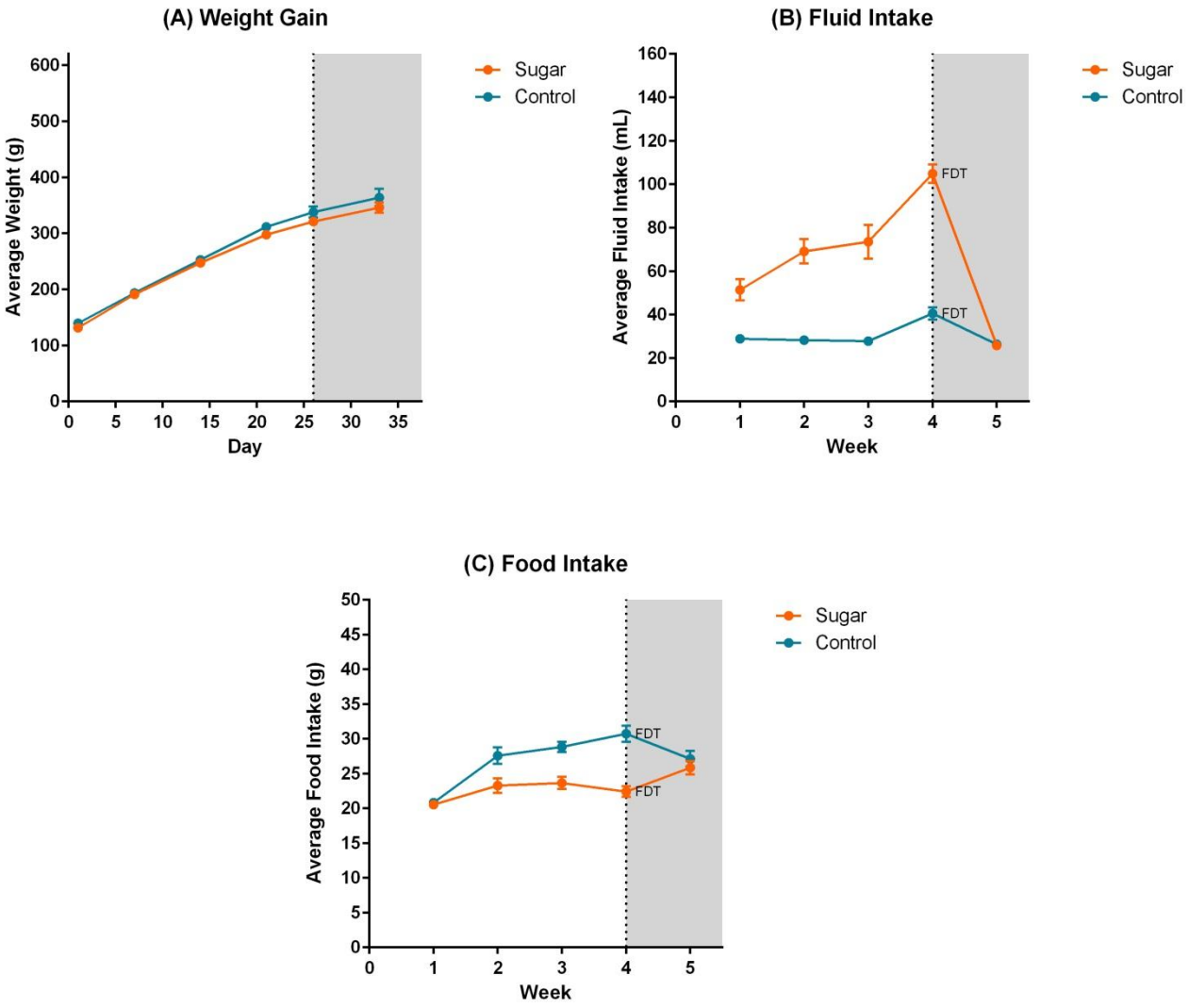


Figure 10. Animal weight, fluid intake and food intake for the period of Experiment 2. Weight (panel A) is shown as mean (±SEM) of daily animal weights (g) from Treatment Day 1 to 10 days post treatment (Day 33). Fluid intake (panel B) is shown as average weekly fluid intake (mL) from first week of treatment to 1 week post treatment (Week 5), where Week 4 is the average for the final day of treatment (FDT). Food intake (panel C) is shown as average weekly food consumption (g) from first week of treatment to 1 week post treatment (Week 5), where Week 4 is the average for the final day of treatment (FDT). Vertical dashed line represents final treatment point, with the washout period in grey.

Autoradiography

Multiple unpaired t-tests found no significant differences in total D2 receptor density between the sugar and control groups in any of the drawn regions of interest (Figure 11).

Univariate ANOVA found differences between the three rostro-caudal levels of the striatum, when compared at the medial, dorsal and ventral regions separately (Figure 11). Specifically, striatal samples for F20 had significantly higher total D2 receptor density than those for F30 in the medial and dorsal regions, but not the ventral region. Meanwhile, total D2 receptor density in F30 was significantly higher than F40 in the medial, dorsal, and ventral regions. Likewise, univariate ANOVA found differences between the medial, dorsal and ventral regions within the three rostro-caudal levels of the striatum (Figure 11). That is, the medial region had significantly lower total D2 receptor density than the dorsal region in striatal samples for F20, F30 and F40. Similarly, the dorsal region was significantly lower in total D2 receptor density than the ventral region in striatal samples for F30 and F40. However, there was no difference in total D2 receptor density between the dorsal and ventral regions in F20.

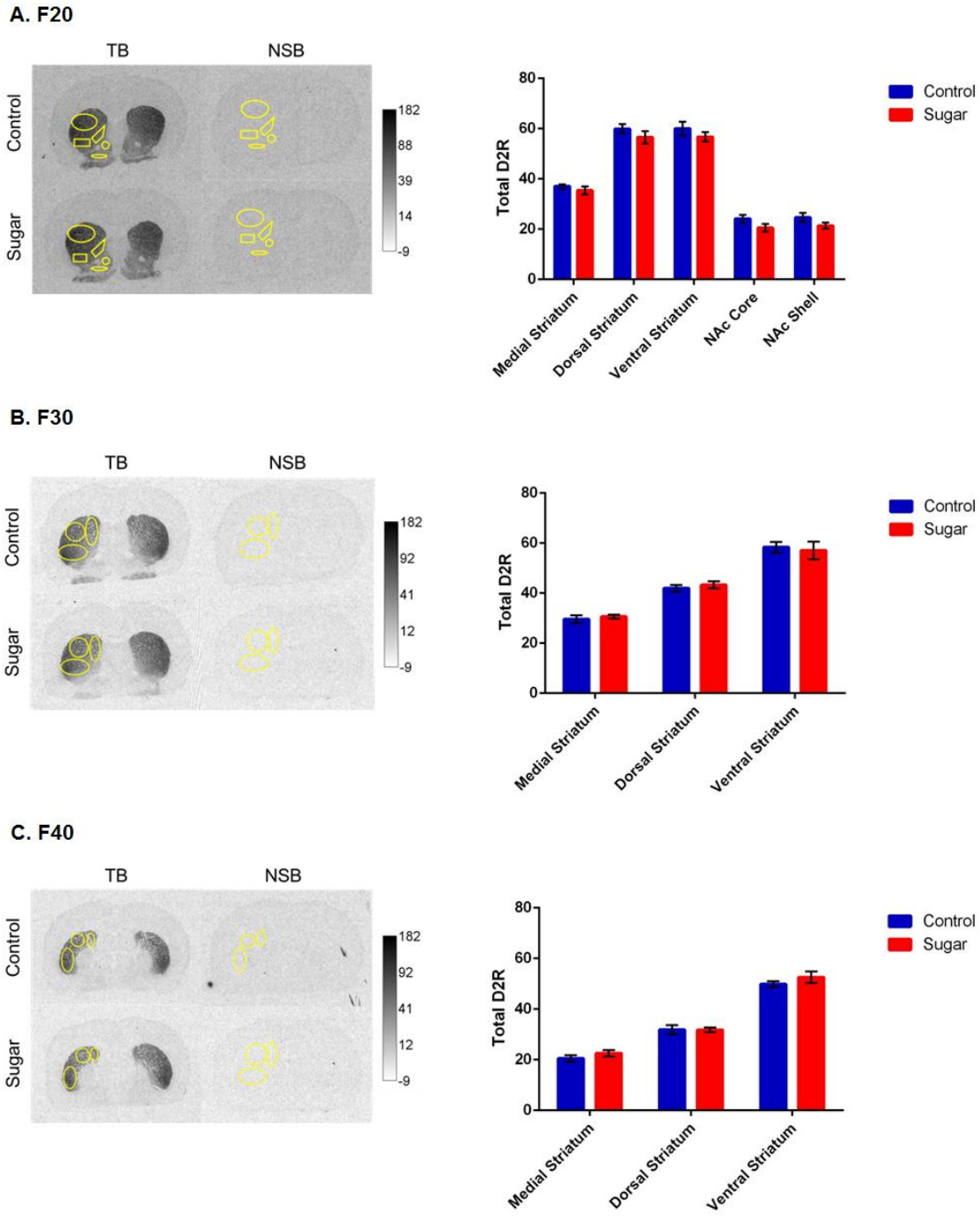


Figure 11. Autoradiography (left) and bar graph (right) representations of total D2 receptor density for regions of interest in F20 (panel A), F30 (panel B) and F40 (panel C). Autoradiography images show of total binding (TB) and non-specific binding (NSB) slides with regions of interest drawn on the left hemisphere. The calibrated lookup table for the autoradiography images is shown to the right. Bar graphs show the means (±SEM) of total D2 receptor density (Total D2R) for the sugar and control groups in the regions of interest.

2.4. Discussion

The current study investigated the impact of chronic excess sugar consumption during adolescence on dopamine D2 receptors in the striatum in early adulthood, utilising a combination of *in vivo* and *in vitro* receptor imaging techniques to assess D2 receptor density across two experimental cohorts. It was found that free access to 10% sucrose solution during adolescence was related to changes in functional, but not total, D2 receptor density in the striatum in early adulthood. Differences in functional D2 receptor density between the sugar and control groups varied in direction across rostro-caudal levels of the striatum, reflecting the heterogeneity of the striatum. Moreover, differences in functional D2 receptor density were observed in animals given free access to sugar on the final day of treatment and six weeks after treatment, indicating both immediate and residual effects. There was no significant difference in D2 receptor affinity between the sugar and control groups, however a reduction in D2 receptor affinity was observed between the final day of treatment and six weeks post treatment in both groups.

The results from the present study do not support all of the hypotheses formed based on previous findings, however these data serve to highlight the novelty of the current study. Past research has tended to assess the immediate effects of restricted access models of sugar consumption on *in vitro* neurological markers within the motivation-reward system. In contrast, the current study investigated both immediate and residual effects in a free access adolescent model of chronic excess sugar consumption. The use of autoradiographic and PET imaging techniques to investigate a free access sugar model has allowed the current study to provide unique insight into the effects of chronic excess sugar consumption on D2 receptor density within the striatum, as an index of changes to the motivation-reward system. Moreover, through the application of partial saturation analysis, the current study was able to examine the effects of chronic excess sugar consumption on functional D2 receptor density

and apparent D2 receptor affinity, two neurological outcomes that have not previously been assessed.

2.4.1. Animal weight, fluid intake and food intake

The current study made use of two cohorts of animals given identical sugar (or control) treatment for the period of adolescence, in order to compare D2 receptor outcomes using PET and autoradiography imaging techniques. In both studies, all animals were observed to gain weight normally with no difference between the sugar and control groups across the treatment period. While animals in the PET study did gain weight at a slightly faster rate than the autoradiography animals, this difference was not large enough to suggest variation between the cohorts. Similarly, both sugar groups significantly increased fluid intake until sucrose solution was removed, whereupon fluid intake dramatically dropped to be comparable with control levels of intake. The two sugar groups were also observed to consume less food than the water groups for the treatment period, and increase food consumption during the washout period. The differences in fluid and food intake between sugar treated and control animals follow a pattern that has previously been suggested to indicate calorie compensation by sugar treated animals (Avena et al., 2009; Kendig et al., 2013; Sheludiakova et al., 2012). Differences in fluid and food intake were observed between the two control groups, however the discrepancy was deemed to result from data of a single animal in the PET group. Although the data from this animal could not be removed, due to the nature of measurements taken in group housing, there is no evidence to suggest that the other animals in the control cage differed in fluid or food intake. Thus, based on the close similarities in weight gain, fluid and food intake between the two animal cohorts given identical treatment, it was concluded that finding from the two sugar and two control groups should be comparable across the cohorts.

2.4.2. D2 Receptor Density

Chronic excess sugar consumption during adolescence was related to altered functional, but not total, D2 receptor density in rostro-caudal levels of the striatum, in early adulthood. The results of the current study offer support for a number of ideas, including the heterogeneity of the striatum and a long-term effect of chronic excess sugar consumption on the motivation-reward system. Comparison of functional and total D2 receptor density findings elucidate limitations of previous research investigating the effects of chronic excess sugar consumption, and suggest changed D2 receptor trafficking as a potential mechanism through which the motivation-reward system may be impacted by chronic excess sugar consumption.

Heterogeneity of D2 Receptor Density in the Striatum

Based on previous studies, it was hypothesised that chronic excess sugar consumption would be related to reduced D2 receptor density in the striatum. In contrast, the current study did not show changes in total density of D2 receptors, yet observed different effects of chronic excess sugar consumption on functional D2 receptor density in different levels of the striatum. The sugar group in the PET study had similar levels of functional D2 receptor in all rostro-caudal striatal levels on the final day of treatment, despite varied functional D2 receptor density across the striatal levels in the control group. While no difference in total D2 receptor density was found between sugar and control groups using autoradiography, total D2 receptor density was also found to vary across the rostro-caudal plane, as well as the medial, dorsal and ventral regions. This data supports previous reports of the heterogeneity of D2 receptor density across the striatum in normal animals, and highlights the importance of examining the striatum as three distinct rostro-caudal levels in the two imaging studies. Moreover, the results of the current study suggest that chronic excess sugar consumption

differentially affects specific regions of the striatum, and this may be reflective of the different functional roles of these regions.

The heterogeneity of the striatum is well established in the literature, with research describing differences in structural and topographical organisation (Berendse et al., 1992; Gerfen, 1992; Tassin et al., 1976) and function (Willuhn et al., 2003; Yager et al., 2015) across this region. Dopamine concentration and uptake decreases from the rostral to caudal areas of the striatum (Tassin et al., 1976). The current study found a similar pattern in total D2 receptor density, with a decline in density moving across the rostro-caudal plane. Studies have also correlated the medial, dorsal and ventral regions of striatum with different behavioural outcomes. For example, the dorsolateral region of the striatum has been related to sensorimotor movement and motor learning (Willuhn et al., 2003; Yin, Knowlton, & Balleine, 2006), whereas the dorsomedial striatum is involved in motivation and goal-directed behaviours (Yager et al., 2015). The varying levels of total D2 receptor density across the medial, dorsal and ventral striatal regions of interest in the present study may similarly reflect different functional roles of the striatal subregions.

Functional D2 receptor density across the rostro-caudal levels of striatum was differentially affected by treatment on the final day of treatment and six weeks post treatment. Specifically, functional D2 receptor density of sugar treated animals was higher than control in the most rostral level, equal to control in the mid rostro-caudal level, and lower than control in the most caudal level. By six weeks post treatment, functional D2 receptor density remained constant in the most rostral level (with the control group increasing to be comparable to the sugar group) but decreased in both the mid and caudal levels of striatum to be below control levels. Through use of *in vitro* microdisc and microinjection studies, it has been established that different areas of the striatum moderate signals from particular areas of the frontal cortex and motivation-reward system (Berendse et al., 1992; Willuhn et al., 2003).

However, in the current study it was not possible to correlate these discrete areas of interest with functional D2 receptor changes due to the relatively low resolution of PET imaging. Nevertheless, the different pattern of functional D2 receptor density across the rostro-caudal levels in each treatment group suggests that chronic excess sugar consumption during adolescence may impact on subregions of the motivation-reward system, to promote diverse behavioural outcomes when compared to control-treated subjects.

Longitudinal Variation of Functional D2 Receptor Density

Previous studies have demonstrated that levels of D2 receptors in the striatum are reduced immediately following chronic excess sugar treatment (Bello et al., 2002; Colantuoni et al., 2001; Spangler et al., 2004), and that chronic excess sugar consumption is associated with changes in motivation-reward related behaviours (Kendig et al., 2013; Sharpe et al., 2015). Although motivation-reward related behavioural measures have not yet been assessed longitudinally with regard to sugar intake, memory deficits as an outcome of chronic excess sugar consumption remained following the removal of sugar from the diet (Kendig et al., 2013). Therefore, it was hypothesised that the effects of chronic excess sugar consumption during adolescence would endure into early adulthood, despite the unavailability of sucrose. Conversely, the present study found diverse changes in functional D2 receptor density from the final day of treatment to six weeks post treatment, within the measured levels of striatum. These findings suggest that chronic excess sugar consumption has residual effects which differently impact areas of the striatum and motivation-reward system after the removal of sugar from the diet.

The observed longitudinal variation in functional D2 receptor density suggests that the effects of chronic excess sugar consumption during adolescence are long lasting in areas of

the motivation-reward system. Sugar treated animals showed a maintained reduction of functional D2 receptor density six weeks post treatment in the caudal region of striatum. That is, while the control group showed a decrease in functional D2 receptor density between the two time points, functional D2 receptor density in the sugar group also reduced such that it was lower than the control group both on the final day of treatment and six weeks post treatment. Functional D2 receptor density in the mid rostro-caudal level of striatum again decreased from the final day of treatment to six weeks post treatment, but the control group increased between these times. The reduced functional D2 receptor density observed in the sugar-treated group could be indicative of diminished behavioural inhibition, as previous research has found increased risk taking and reduced inhibition in subjects with lower striatal D2 receptor density (Bello et al., 2002; Cocker et al., 2012; Ghahremani et al., 2012). Likewise, D2 receptor density is lower in the striatum of obese and drug-addicted individuals, who are proposed to have reduced inhibitory control (Johnson & Kenny, 2010; Thanos et al., 2008; Volkow et al., 2008). An alternative explanation is that the decreased functional D2 receptor density in the mid and caudal levels of striatum in sugar treated animals is reflective of reduced stress resilience. D2 receptor density was observed to decrease in rodents exposed to chronic mild stress (Żurawek et al., 2013), and return to normal levels following antidepressant treatment (Song et al., 2015). Similarly, prenatal stress (Said et al., 2015) and social phobia (Schneier et al., 2000) have been linked to reduced D2 receptor density, suggesting a role of D2 receptors in regulating stress and anxiety. However, these explanations are not exclusive, as heightened stress response has the potential to increase sensitivity to immediate reward (Cocker et al., 2012). Nevertheless, the results of the current study suggest that chronic excess sugar consumption during adolescence can have long-lasting effects on functional D2 receptor density in the striatum in early adulthood, and these effects may correspond with negative behavioural outcomes.

Interestingly, in the mid rostro-caudal level there was no significant difference between the sugar and control groups at the final day of treatment, suggesting that chronic excess sugar consumption did not have direct effects on functional D2 receptor density at this level. This indicates that the reduction in functional D2 receptor density in the sugar group at this striatal level at six weeks post treatment may be related to the removal of sugar access, rather than sugar consumption itself. Previous research has linked altered D2 receptor density to craving (Fehr et al., 2008) and reduced D2 receptor density to restricted food access paradigms (Bello et al., 2002; Colantuoni et al., 2001; Johnson & Kenny, 2010; Thanos et al., 2008). The decrease in functional D2 receptor density observed in the mid rostro-caudal striatum, following removal of sucrose solution, in the current study, may be interpreted to signal a desire for the sugar that has been removed from the environment.

Finally, changes in functional D2 receptor density between the sugar and control groups in the most rostral region of striatum on the final day of treatment were no longer present six weeks post treatment, in contrast to the other striatal regions. Interestingly, functional D2 receptor density remained stable between the two scan times in the sugar group, but changed significantly in the control group from the final day of treatment to six weeks post treatment. Assuming that the control group accurately represented normal changes in functional D2 receptor density, this may suggest that chronic excess sugar consumption accelerated normal increases in functional D2 receptor density in the rostral striatum. An increase in functional D2 receptor density is particularly interesting as sugar consumption has previously been related to reduced levels of D2 receptor in the striatum. This may be due to methodological difference, with previous studies having assessed total D2 receptor density in small samples of striatum *in vitro* following administration of a restricted access sugar protocol (e.g. Bello et al., 2002; Colantuoni et al., 2001). However, the current study showed changes to functional D2 receptor density, rather than total D2 receptor density, limiting the

direct comparisons that can be made to previous D2 receptor binding studies. Thus, while these longitudinal outcomes of chronic excess sugar consumption on functional D2 receptor density are interesting in their diversity, they are difficult to interpret without additional information. In particular, it is necessary for future studies to investigate behavioural alterations that may occur with sugar-induced D2 receptor changes. It should also be noted that D2 receptor density has not yet been assessed in adolescence, particularly in relation to chronic excess sugar consumption, further limiting interpretation of the effect of sugar treatment in the rostral striatal region.

Total D2 Receptor Density

It was hypothesised that chronic excess sugar consumption during adolescence would be related to decreased total D2 receptor density in the striatum in early adulthood. Conversely, the current study found no significant difference in total D2 receptor density between the sugar and control groups, in any of the drawn regions of interest of the rostro-caudal levels analysed. This may suggest that expected differences in locomotor activity between the sugar and control groups were not observed in this cohort of animals (as reported by Franklin, 2015) because there was no impact on D2 receptor density.

The absence of a treatment effect on total D2 receptor density is inconsistent with previous studies, which have reported a relationship between sugar consumption and total D2 receptor density in the striatum (Bello et al., 2002; Colantuoni et al., 2001). However, these studies varied significantly from the current study in methodology in a number of other ways that have not previously been compared, such as the use of adolescent rather than adult animals, and employment of higher affinity [^{11}C]raclopride rather than [^{125}I]iodosulpride for receptor binding. Most prominently, all three studies utilised restricted access models of sugar

consumption, which are commonly used in studies investigating addiction-like responses to sugar rather than the effects of chronic excess sugar consumption itself. Although Colantuoni et al. (2001) did include a group with free access to sugar in their initial experiment, this group was not included in receptor imaging due to significant differences in weight gain compared with the control and restricted access groups. The lack of difference in total D2 receptor density between the sugar and control groups in the current study may therefore suggest that the differences previously observed are related to the restricted access paradigm rather than sugar consumption itself. Indeed, Bello et al. (2002) found the strongest D2 receptor effects in the group with restricted access to food (no sugar), in comparison to groups with restricted access to sugar and controls. A study examining the role of D2 receptor in obesity also found that both lean and obese animals with restricted food access had higher total D2 receptor density than animals with no food restriction (Thanos et al., 2008). Since chronic mild stress models often utilise food restriction as a stressor (e.g. Song et al., 2015; Żurawek et al., 2013) and stress has been related to decreased D2 receptor density (Said et al., 2015; Żurawek et al., 2013), studies using a restricted access protocol may have been reporting the effects of stress rather than sugar. Alternatively, it could be argued that the non-significant findings in total D2 receptor density in the current study reflect a reversal of the sugar effects during the 10 day washout period, as previous studies measured D2 receptor markers on the final day of sugar treatment. While these variations were not expected to have significant impact, the lack of previous comparison makes it difficult to accurately interpret the absence of D2 receptor differences in the current study and the effect that each of these variables may have had on the final outcomes observed.

Discrepancy in Functional and Total D2 Receptor Density

The present study utilised both PET and autoradiography techniques to assess distinct measures of D2 receptor density, namely functional and total D2 receptor density. It was hypothesised that chronic excess sugar consumption during adolescence would be related to parallel decreases in both measures of D2 receptor density in the striatum, however changes were observed in functional but not total D2 receptor density in this region. These results suggest that the motivation-reward system may be affected by chronic excess sugar consumption through alterations of D2 receptor trafficking in the striatum.

While a change in total D2 receptor density would have indicated a difference in the amount of D2 receptor being produced, a change in functional D2 receptor density suggests that there is a difference in the amount of D2 receptor being transported from inside the synaptic terminal to the cell membrane. Previous studies have found differences in receptor trafficking relating to variation in sugar and food consumption (Peng, Ziff, & Carr, 2011), however D2 receptor trafficking has not yet been investigated in this context. Nevertheless, the suggestion that chronic excess sugar consumption might alter D2 receptor trafficking is compatible with previous theories. It has been suggested that sugar consumption gives rise to psychological and behavioural outcomes through altered synaptic plasticity (Cisternas et al., 2015; Molteni et al., 2002). A change in dopamine receptor trafficking, such as that suggested by the current study, is one possible contributing mechanism to altered synaptic plasticity. While a lot of research pertains to the role of dopamine receptors in synaptic plasticity through moderation of other receptor trafficking, particularly AMPA and adenosine receptors (e.g. Peng et al., 2011; Torvinen et al., 2005), the trafficking of dopamine receptors themselves does not appear to be widely investigated. Nonetheless, given that chronic excess sugar consumption has been related to both altered functional D2 receptor density and

reduced synaptic plasticity, the trafficking of D2 receptor offers an interesting new direction for future research in this area.

2.4.3. D2 Receptor Affinity

Apparent D2 receptor affinity of the striatum did not differ between treatment groups, however the current study found a significant main effect of time on apparent D2 receptor affinity in the striatum, with data suggesting that affinity decreased between the end of adolescence and adulthood. This finding is supported by developmental literature, which consistently indicates reductions of dopaminergic activity following adolescence (Casey et al., 2008; Laviola et al., 2003; Spear, 2000; Wahlstrom, White, et al., 2010). Although the functional implications of affinity changes are not clear, the reduction in dopaminergic activity is part of normal developmental progression towards maturity and adulthood (Casey et al., 2008; Laviola et al., 2003; Spear, 2000; Wahlstrom, White, et al., 2010).

It was hypothesised that chronic excess sugar consumption during adolescence would relate to altered apparent D2 receptor affinity, however no treatment effect was observed in the present study. It has previously been noted that the administration of the dopamine antagonist, raclopride, may alter affinity by decreasing the proportion of high affinity state D2 receptors (van Wieringen et al., 2013). Since equal volumes of raclopride were administered to the sugar and control groups, it is unlikely that the effects of raclopride on affinity will have impacted the current study as any affinity decrease in the sugar group should be paralleled in the control group. Reward and addiction processes involve a change in the proportion of receptors in high or low affinity states (e.g. Perreault et al., 2010; Simola, Morelli, & Seeman, 2008), rather than overall apparent affinity as described in the current study. It is therefore possible that sugar consumption could have altered the relative

proportions of high and low affinity state D2 receptors. Affinity state of D2 receptors has most commonly been assessed in relation to psychosis and schizophrenia, with studies suggesting an increase in high affinity state D2 receptors in these disorders (Laruelle, 1998; Perreault et al., 2010; Seeman et al., 2005). Dopamine hypersensitivity, observed in addiction, has been presumed to reflect an increase of D2 receptors in high affinity state (Simola et al., 2008; van Wieringen et al., 2013), but evidence to support this concept is sparse. While it is well described that D2 receptors have both high and low affinity states (van Wieringen, Booij, Shalgunov, Elsinga, & Michel, 2013), a clear method for investigating this phenomena has not been established, with the current methods presenting inconsistent results (van Wieringen et al., 2013). Therefore, the effects of chronic excess sugar consumption on D2 receptor affinity must be re-assessed and interpreted with behavioural measurements in future research, following the establishment of reliable measurement techniques for assessing D2 receptor affinity state.

2.4.4. Implications

The present study demonstrated that chronic excess sugar consumption during adolescence is related to altered functional D2 receptor density across rostro-caudal levels of the striatum in early adulthood. Sugar treatment was observed to have both immediate and residual effects on functional D2 receptor density. In contrast, no residual differences in total D2 receptor density were found, following chronic excess sugar consumption during adolescence. Exploration of these results offers a number of important implications.

The results from the current study offer evidence that chronic excess sugar consumption during adolescence alters functional D2 receptor density in the striatum. Furthermore, differences in functional D2 receptor density remain present in the striatum

following the removal of sugar from the diet. As the striatum moderates many motivation-reward signals, it is reasonable to infer that chronic excess sugar consumption during adolescence is related to lasting alteration of the motivation-reward system. Dopaminergic, and particularly D2 receptor, function has been linked to a number of negative psychological and behavioural symptoms, such as reduced stress resilience (Żurawek et al., 2013). Importantly, where previous studies have utilised restricted access protocols, which may be stressful themselves, the current study observed D2 receptor changes in a non-stressful free access sugar paradigm. This strengthens the evidence indicating that chronic excess sugar consumption is related to D2 receptor changes. Thus, by altering dopaminergic function within the motivation-reward system, chronic excess sugar consumption during adolescence may therefore increase vulnerability to reduced behavioural inhibition and reduced stress resilience.

The disparity between functional and total D2 receptor density outcomes following chronic excess sugar consumption, in two cohorts of animals given similar treatment, is also an interesting result. Functional D2 receptor density was altered by sugar treatment, indicating a difference in D2 receptor available for binding, while total D2 receptor density did not differ between sugar and control groups, indicating that the amount of D2 receptor being produced was not affected. Therefore, it is proposed that chronic excess sugar consumption during adolescence modifies D2 receptor trafficking in the striatum. Consequently, this may play a role in the observed reductions in synaptic plasticity following sugar consumption (Cisternas et al., 2015; Molteni et al., 2002) to alter a number of adaptive behaviours.

2.4.5. Limitations and Future Research

The variable results of the current study suggest that the effects of chronic excess sugar consumption on D2 receptors in the striatum are more complex than previously indicated by the literature. Although the novelty of the study has allowed it to provide unique insight into the longitudinal effects of chronic excess sugar consumption during adolescence, technical complications while carrying out the study resulted in a reduced number of subjects included in the final analysis. The low statistical power therefore makes it difficult to determine whether the effects reported are true effects. An absence of similar comparable studies further complicates the interpretation of the results.

The current study found significant differences in functional D2 receptor density, however the behavioural implications of the findings remain unclear as phenotypic effects were not measured. The lack of phenotypic measurements presents a major limitation of the current study. While previous studies have correlated behaviours with particular regions of the striatum, the rostro-caudal levels of striatum measured in the current PET study were determined based on boundaries of a pre-existing brain atlas rather than functionally defined regions. Consequently, it is not possible to use prior knowledge of these regions to extrapolate potential behavioural outcomes based on the direction of D2 receptor changes observed without further refinement of the PET images obtained. Moreover, as this is the first time that functional D2 receptor density in the striatum has been assessed in relation to chronic excess sugar consumption, and total D2 receptor density did not change in accordance with previous findings, it is difficult to interpret the results of the current study with respect to the literature. Similarly, previous human and animal studies have reported significant behavioural correlates to altered D2 receptor density, such as craving (Fehr et al., 2008) and reduced stress resilience (Żurawek et al., 2013). Previous research has also linked chronic excess sugar consumption to deficits in spatial and episodic memory (Cao et al., 2007; Chepulis et al., 2009; Kendig et al.,

2013; Ross et al., 2009), as well as altered reward sensitivity (De Cock et al., 2015; Kendig et al., 2013) and cue learning (Sharpe et al., 2015). However, as yet there are no direct links between chronic excess sugar consumption, altered D2 receptor density in the striatum and behavioural changes. Further research combining the use of behavioural measures and receptor imaging would best elucidate the effects of chronic excess sugar consumption on the striatum and motivation-reward system, providing detail about sugar consumption related behavioural changes in parallel with changes observed in functional D2 receptor density.

Previous studies have found that restricted access to sugar is related to lower total D2 receptor density in the striatum, when compared to controls animals, however the current study found no difference in total D2 receptor density following free access to sugar. While this may suggest that the difference in D2 receptor outcome is related to the different dietary regimens, the current study also diverged from previous studies in a number of other ways. For example, where previous studies have predominantly investigated the effects of chronic excess sugar consumption in adult rodents, the current study examined the effects of chronic excess sugar consumption in adolescent rodents. Research comparing adult and adolescent rodents suggests that adolescents are more vulnerable to the behavioural effects of sugar consumption (Hsu et al., 2014; Kendig et al., 2013), and this may also extend to sugar-related D2 receptor changes in the striatum in the current study. However, it is not unrealistic to suggest that age could be a protective factor against the effects of chronic excess sugar consumption. Indeed, it has previously been implied that children are better able to cope with large amounts of sugar than adults, as the growing brain and body use more energy (Benton, 2008). It is therefore possible that no differences were seen in total D2 receptor density in the current study because adolescents are less prone to some effects of chronic excess sugar consumption. Future research should systematically investigate both the immediate and residual effects of chronic excess sugar consumption in the context of free access versus

restricted access protocols, in adolescent and adult samples, to understand the full repertoire of sugar intake on dopamine systems.

A further limitation of the current study is the use of group animal housing, which meant that the data from an outlying animal in food and fluid intake could not be removed from the dataset. Previous research into the effects of sugar has alternated between housing animals individually (e.g. Bello et al., 2002; Chepulis et al., 2009) or in group housing (e.g. Franklin et al., 2013; Kendig, Boakes et al., 2013). However, studies suggest that the use of individual versus group housing may impact the behaviour and neurological pathways of rodents. For example, individual housing of animals has been related to increased anxiety and reactivity to stimulus (Da Silva, Ferreira, Carobrez, & Morato, 1996; Gentsch, Lichtsteiner, & Feer, 1981). These housing related behavioural changes have the potential to interact with the effects of chronic excess sugar consumption, such that the compounded effects could amplify neurological outcomes. It is therefore important that future studies compare the effects of chronic excess sugar consumption in individually housed and group housed animals, in order to separate the sugar and housing effects.

The current study provides evidence to suggest that chronic excess sugar consumption during adolescence alters functional D2 receptor density in the striatum in early adulthood, however the novelty of the study limits the interpretation of this finding. To better understand the effects of excessive sugar consumption, future studies must longitudinally examine the effects of free access sugar consumption on behavioural and neurological outcomes in the motivation-reward system, in parallel. Furthermore, future research should investigate the effects of chronic excess sugar consumption on D2 receptor trafficking and synaptic plasticity, in attempt to better understand the mechanisms through which chronic excess sugar consumption impacts D2 receptor density.

2.4.6. Conclusion

The current study investigated the effects of chronic excess sugar consumption during adolescence on D2 receptor density in the striatum in early adulthood. Utilisation of both PET and autoradiography imaging techniques allowed for the measurement of both functional and total D2 receptor density at different levels of the striatum, providing a unique insight into D2 receptor changes produced by chronic excess sugar consumption. Differences in functional D2 receptor density, between the sugar and control groups, varied across rostro-caudal levels of striatum, and between the final day of treatment and six weeks post treatment. It is proposed that this variation is reflective of the structural and functional heterogeneity of the striatum. It is further suggested that the distinct differences observed at six weeks post treatment may reflect both the residual effects of chronic excess sugar consumption and removal of access to sugar. The findings of this study provide exciting new directions for future research into chronic excess sugar consumption, presenting rationale for further investigation into the longitudinal effects of free access sugar consumption on behavioural responses in relation to different regions of the motivation-reward system. The study also suggests mechanisms through which the motivation-reward system may be affected by chronic excess sugar consumption, namely, through alteration of dopamine receptor trafficking and synaptic plasticity.

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Appendix A: *Animal Ethics Approval (Project Number: 2015/781)*

THE UNIVERSITY OF SYDNEY

RESEARCH INTEGRITY
Animal Ethics Committee

Tuesday, 14 April 2015

Dr Paul Callaghan
Medical Radiation Sciences; Faculty of Health Sciences
The University of Sydney
Email: pcn@ansto.gov.au

Dear Dr Callaghan

I am pleased to inform you that the University of Sydney Animal Ethics Committee (AEC) has approved your project entitled "**Does high refined sugar intake weaken brain resilience? Analysis of brain dopamine mechanisms using in vivo positron emission tomography (PET)**".

Details of the approval are as follows:

Project Number: **2015/781**

Approval Period: **26/02/2015 – 26/02/2018**

Annual Report Due: **26/02/2016**

Authorised Personnel: **Callaghan Paul; Boh Jessica; Arthur Andrew; Rahardjo Gita; Cornish Jennifer L.**

Documents Approved:

11/02/2015	Application Form	All factors and procedures
11/02/2015	Application Form	Animal monitoring
16/02/2015	Other	External investigator form_Boh, Jessica
16/02/2015	Other	External investigator form_Cornish, Jennifer L.
11/02/2015	Monitoring Sheet	Imaging monitoring sheet
11/02/2015	Monitoring Sheet	Monitoring sheet
11/02/2015	Application Form	Sequence of events
11/02/2015	Not selected	SOP
11/02/2015	Not selected	SOP
11/02/2015	Not selected	SOP 2
11/02/2015	Not selected	SOP 2
11/02/2015	Not selected	SOP 3
11/02/2015	Not selected	SOP 3
11/02/2015	Not selected	SOP 4
11/02/2015	Not selected	SOP 4
11/02/2015	Not selected	SOP 5
11/02/2015	Not selected	SOP 5

Animals Approved:
Please refer to the document at the end of this letter, which details your approved animal usage.
The project is approved for an initial period of **12 months** with approval for up to **(3) years** following receipt of the appropriate report under clauses 2.2.24, 2.2.32 and 2.4.34 of the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

Special Condition/s of Approval Applicable to this Project

You may commence the research without any further review by the AEC, provided the following conditions have been met:

1) **It will be a condition of approval that a collaborative agreement is produced prior to commencement of the project. The University of Sydney Ethics Office will commence the process of the collaborative agreement with ANSTO.**

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THE UNIVERSITY OF SYDNEY

Conditions of Approval

Approval of this project is conditional upon your adherence to the conditions outlined in this letter and your continuing compliance with the Animal Research Act (1985 – Animal Research Regulation 2010) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC 2013).

1. The Animal Ethics Committee (AEC) reviews and approves protocols for their compliance with the NSW Animal Research Act (and associated regulations) and the 8th Edition of the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013).

2. This approval is in accordance with your original submission together with any additional information provided as part of the approval process.

3. Any changes to the protocol must be approved by the AEC before continuation of the study. This includes notifying the AEC of any changes to named personnel, source of animals, animal numbers, location of animals and experimental procedures.

4. Investigators should promptly notify the AEC of any unexpected **adverse events** that may impact on the wellbeing of an animal in their care, as per Clause 2.1.5 [v] [d] and 2.4.34 [ii] in the Australian code of practice (NHMRC, 2013). In the event that an unexpected adverse event occurs, please refer to the Animal Ethics website and log into IRMA to complete an Adverse Event form. For further information, please see the AEC Adverse Event Reporting Procedures (GL003) on the Animal Ethics website.

5. In the event an animal dies unexpectedly or requires euthanasia for welfare reasons, an autopsy should be performed by a person with appropriate qualifications and/or experience and the AEC should be notified promptly.

6. Animals must not be euthanised within sight or sound of other animals, in accordance with Clause 3.3.45 [vi] of the Australian code of practice (NHMRC, 2013).

7. Animals should not be housed singly unless otherwise approved by the AEC.

8. All animals must be provided with environmental enrichment appropriate for their species, unless otherwise approved by the AEC.

9. All pens, cages and containers used for holding animals must be clearly identified with chief investigator name, number of animals, DOB if provided and date of arrival, sex and strain.

10. A copy of this approval letter, together with all relevant monitoring records, must be kept in the facility where your animals are housed. These records must be updated regularly as breeding and husbandry events occur and current copies must be maintained in the animal house. Monitoring sheets must contain a section where expected post-operative effects are identified and observations recorded. Where relevant, the pens, cages and container number must be recorded on the monitoring sheet to ensure that affected animals can be easily located. Where electronic breeding records are kept instead of records on cage cards, printed copies of the records should be placed in a folder in the relevant animal house, where they can be inspected by the AEC.

11. Data should be accurately recorded in a durable, indexed and retrievable form that complies with relevant legislation, policy and guidelines. Following completion of the study all data including consent forms must be retained in a secure location, such as a locked filing cabinet, at the University of Sydney for a period of at least seven (7) years.

12. The AEC will make regular announced inspections of all animal facilities and/or specific research protocols. The Animal Welfare Veterinarian will be conducting unannounced inspections of all animal facilities and/or specific research protocols.

THE UNIVERSITY OF SYDNEY

13. All new investigators must successfully complete the Introduction to Animal Research (ITAR) course.

Please do not hesitate to contact the Research Integrity (Animal Ethics) Office at animal.ethics@sydney.edu.au should you require further information or clarification.

Yours sincerely

Professor David Allen
Chair
Animal Ethics Committee

The AEC is constituted and operates in accordance with the NSW Animal Research Act (1985) and its associated Regulations, the 8th Edition Australian code for the care and use of animals for scientific purposes (NHMRC, 2013) and the Australian Code for the Responsible Conduct of Research (2007). All personnel named on the protocol should be conversant with these documents.

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RESEARCH INTEGRITY
Animal Ethics Committee

Animals Approved:

Country	State	Invasiveness	Location	Classification one	Classification two	Common/strain name	Applied	Anticipated re-use
Australia	NSW	2. Animal unconscious without recovery	Brain and Mind Research Institute (M02G), Camperdown	Laboratory mammals	Rats	Sprague Dawley rats	24	0

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Appendix B: *Animal Ethics Approval (Project Number: 2011/029-5)*

MACQUARIE UNIVERSITY

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2011/029-5 **Date of Expiry:** 31 July 2014

Full Approval Duration: *01 August 2011 to 31 July 2014 (36 months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry.**

Principal Investigator (PI) :
Dr Jennifer Cornish
Dept of Psychology
Macquarie University NSW 2109
0404 807 175
Jennifer. cornish@mq.edu.au

Associate Investigators (AI):

Jane Franklin	0432 219 402
Kelly Clemens	0415 298 002
Judi Homewood	0413 041 461
Travis Wearne	0404 296 726
Niree Kraushaar	0420 361 933

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Manager, CAF: 9850 7780 / 0428 861 163 and **Animal Welfare Officer**: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Sensitisation to methamphetamine challenge following treatment through adolescence with caffeine, sucrose or their combination

Purpose: 4 – Research: human or animal biology

Aims: To investigate whether a diet high in sucrose and caffeine through adolescence can change gene expression and behavioural responses to methamphetamine exposure in adulthood.

Procedures category: 3 (Minor Conscious Intervention)
All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Sex	Weight	Age	Total	Supplier/Source
Rat	Sprague Dawley	Male	80g	PND 26	120	ARC
Rat	Sprague Dawley	Male	250g	Young adult	120	ARC
Rat	Sprague Dawley	Male	80g	PND 26	8	ARC
Rat	Sprague Dawley	Male	250g	Young adult	8	ARC
				TOTAL	**256**	

Location of research:

Location	*Full street address*
Central Animal House Facility	Building F9A, Research Park Drive, Macquarie University NSW 2109

Conditions of Initial Approval:

1. *AEC meeting16 June 2011: The ARA issued subject to the adolescent animal experiments being conducted concurrently with the adult animal experiments. Please submit an amendment form documenting this change to sections2.2a and 2.2b, detailing any changes to animal numbers per year that might be required because of this alteration to the protocol.*

Amendments approved by the AEC since initial approval:

1. *Approved 11 August 2011: Concurrent experimentation on adolescent and adult rats.*
2. *Approved 8 September 2011:*
   a) *Increase the number of animals to add 8 adolescent and 8 adult rats*
   b) *Add an initial experiment to test the new water delivery system and to identify the peak stimulatory effect for each treatment.*
3. *Executive approved & AEC ratified 16 May 2013: Extension to approval duration by 1 year*

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date:** 15 August 2013

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

Appendix C: *AINSE Notification of Research Award (Award Number: ALNGRA14523)*

AINSE

AINSE is the Australian Institute of Nuclear Science and Engineering

Nomination for a Research Award - 2014

Notification of Research Award

Award No	**ALNGRA14523**
Project Title	**The effect of adolescent sugar consumption on brain resilience**
Investigator	**A/Prof Jennifer Cornish**
Institution	**Macquarie University**
Committee	**Biomedical Science & Biotechnology**
Contact Officer	**Callaghan, Paul**

Facility Costs	Samples/Unit	Month	Cost
Investigations with Imaging: Longitudinal studies with PET/CT and SPECT/CT	5 multiple	July	$2,500.00
Investigations with imaging: Modelling Partial Saturation Approach	4 multiple	September	$2,000.00
Radio-halogen & -carbon probes: Synthesis of fluorine-18, carbon-11, radio-iodine radiotracer cold standards and labelling precursors	16 multiple	July	$8,000.00
Investigations with imaging: Radioreceptor binding and autoradiography (receptor density distribution and G protein coupling functionality)	1 multiple	July	$500.00

ANSTO Costs	Cost
Animals (n=20) and 12 weeks housing costs	$2,000.00

Total Amount Awarded	**$15,000.00**

Tenure of Award

This Award must be taken up between 1/7/2014 and 30/06/2015. This award expires on 30/06/2015. This award will not be carried over past its expiry date of 30/06/2015.

Access Arrangements and Conditions

Please refer to the attached "Conditions and Procedures for AINSE Awards, 2014 Series".

AINSE Ltd | ABN 18 133 225 331
Locked Bag 2001 Kirrawee, DC NSW 2232 Australia
Ph: (02) 9717 3436 | Fax: (02) 9717 3376 | Email: ainse@ainse.edu.au

Appendix D: *Experiment Timeline (Animal Treatment)*