Western diet and its effect on motivation, learning and memory

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made. The content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

The HPLC experiment presented in Chapter 4 was performed by Associate Professor Claire Parish at the Florey Neuroscience Institute, Melbourne Australia. Basal Fos immunohistochemistry also presented in Chapter 4 was performed by Sepideh Kosari at RMIT University. The delayed spatial win-shift in radial arm maze behavioural test in Chapter 6 was performed in conjunction with fellow PhD candidate Simone DeLuca at RMIT University.

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Kosari S., Badoer E., **Nguyen JCD.**, Killcross AS., & Jenkins TA. Effect of western and high fat diets on memory and cholinergic measures in the rat. **Behavioural Brain Research**. 2012, 1;235(1):98-103.

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Ali SF., **Nguyen JCD.,** Jenkins TA, & Woodman OL. Acute tocomin treatment improves endothelium-dependent relaxation in aortae from diabetic and western diet fed rats. Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists Annual Meeting, Melbourne, 2013.

Nguyen JCD., Bongiorno D., Killcross AS., & Jenkins TA. Effect of western diet consumption on spatial alternation and brain serotonin measures in the rat. Australian Neuroscience Society Annual Meeting, Melbourne, 2013.

Nguyen JCD., & Jenkins TA. The effect on sociality, anxiety, locomotor activity and hippocampal neurons in rats. Australian Neuroscience Society Annual Meeting, Gold Coast, 2012.

Nguyen JCD., & Jenkins T.A. Changes in behavioural and hippocampal parvalbumin immunoreactive neurons after exercise in the normal and phencyclidine treated rat. Students of Brain Research (SOBR) Symposium October, Melbourne 2011.

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Abbreviations

5-HT	Serotonin, 5-hydroxytryptamine
5-HTT	Serotonin transporter
5-HT_{2A}	Serotonin $_{2A}$
5-HT_{2C}	Serotonin $_{2C}$
AD	Alzheimer's disease
ANOVA	Analysis of variance
APDE9	$APP_{swe}/PS1_{dE9}$ double transgenic Alzheimer's mice model
APP	Amyloid-beta precursor protein
ASST	Attention set shifting task
AUC	Area under the curve
Aβ	Amyloid-beta
BBB	Blood–brain barrier
BMI	Body mass index
CA1	Cornu ammonis area 1
CA2/3	Cornu ammonis area 2/3
Cg	Cingulate gyrus
CL	Control litter rats
CON	Control rats
CS	Conditioned stimulus
D2	Dopamine 2 receptor
DA	Dopamine
df	Degrees of freedom
DG	Dentate gyrus
DIO	Obesity prone
DMTP	Delayed matching to position task
DNMTP	Delayed non-matching to position task
DOPAC	Dihydroxyphenylacetic acid
DR	Obesity resistant

DSST	Digit Substitution Symbol test
DWSh	Delayed spatial win-shift in radial arm maze
EDTA	Ethylenediaminetetraacetic acid
GTT	Glucose tolerance test
HF	High fat
HFD	High fat diet
HPC	Hippocampus
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
IL	Interleukin
ILC	Infralimbic cortex
ITI	Inter-trial interval
LPS	Lipopolysaccharide
MCI	Mild cognitive impairment
MESA	Multi-Ethnic Study of Atherosclerosis
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MUFA	Monounsaturated fatty acids
MWM	Morris water maze
NOIL	Novel object in location
NOIP	Novel object in place
NORT	Novel object recognition test
P	Post-natal day
PBS	Phosphate buffered saline
PFC	prefrontal cortex
PrL	Prelimbic cortex
PS	Presenilin
PUFA	Polyunsaturated fatty acids
RAM	Radial arm maze

RAWM	Radial arm water maze
RR	Random ratio
SDMT	Symbol Digit Modalities test
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SL	Small litter rats
TBST	Tris-buffered saline-Tween
TMT	Trail making test
TNF	Tumour necrosis factor
sTNF-RI	Serum-soluble TNF receptor p55
WCST	Wisconsin Card Sorting test
WD	Western diet
WT	Wild-type

Abstract

In Western society, the prevalence of a hyper-caloric diet consisting of a high consumption of fat and simple sugars has coincided with an exponential rise in diabetes as well as cardiovascular diseases and several types of cancer. While some of these medical comorbidities are themselves associated with adverse cognitive effects, recent studies have also linked the western diet to an increased incidence of Alzheimer's disease and mild cognitive impairment. Moreover, these disorders are considered to be major risk factors for dementia indicating that these metabolic effects have both peripheral and central effects.

Rats that have been a fed high fat diet (HFD) have shown indications to be cognitively impaired compared to those fed a normal chow diet. Research suggests that HFD consumption has a deleterious effect on spatial learning and memory, and this effect consequently may be mediated by damage to the hippocampus. To date however, there is conflicting results regarding the motivational and other types of learning implications after HFD consumption. The primary animal model of obesity used in this thesis is the western diet (WD) model of obesity in rats. This model mimics the 'western' diet typically consumed in developed 'western' countries by feeding rats a WD chow (containing 22% w/w fat) or a control chow diet (containing 6% w/w fat).

Using this model, we explored the ability of 8 weeks of WD consumption to influence changes to Pavlovian & instrumental conditioning as well as motivation. This study used well characterised tests to assess whether learnt feeding behaviour can be affected by WD consumption. The ability for WD consumption to alter motivational drive in varying states of food deprivation was also investigated. Results from this study found that rats fed a WD for 8 weeks did not affect Pavlovian conditioning or motivational state. The effect of WD consumption on instrumental conditioning is still indeterminate with conflicting results.

There was no change in instrumental conditioning in rats fed a WD. However, WD fed rats were impaired in progressive ratio instrumental conditioning acquisition. Additionally, WD exposed rats were no different to changes in states of food deprivation compared to control diet counterparts.

A further study investigated whether a period of 12 weeks WD consumption can affect spatial working and reference memory. No changes in spatial working or reference memory were observed in WD rats. Due to the assumed role of c-Fos, an immediate-early gene and corresponding protein, in learning and use as a surrogate marker of neuronal activation, neuronal activation in selected brain regions was evaluated. We demonstrated that WD consumption increased neuronal activation after environmental novelty in the striatum. Other brain regions involved in memory and learning were also investigated with no differences in neuronal activation before and after environmental novelty between control and WD animals.

In a series of experiments, we explored the ability of WD consumption to influence change in neurotransmitters involved in memory and learning. The expression of serotonin (5-HT) receptors 5-HT_{2A}, 5-HT_{2C} and the 5-HT transporter within the striatum was also investigated, as previous studies have shown that serotonin is implicated in feeding behaviour following WD consumption. Both 5-HT_{2C} receptor and 5-HT transporter expression were found to be increased in WD rats. In contrast, 5-HT_{2A} receptor expression was unchanged in the striatum. This suggests that WD consumption has a selective capacity to alter the serotoninergic system. Furthermore due to the well-recognised role of dopamine in cognition, including motivation, reward, punishment and working memory, the modification of dopamine metabolism was evaluated. High performance liquid chromatography analysis found reduced levels of striatal dopamine, with alterations in dopamine metabolism and turnover also evident in the hippocampus after WD consumption. These neurotransmitter changes were also observed

to be independent of any change in cognitive ability. This suggests that WD consumption may instigate dopaminergic and serotoninergic adaptations before cognitive impairment transpire.

The early life nutritional environment was also investigated to ascertain if early life obesity may contribute to cognitive impairment using a neonatal overfeeding rat model of obesity. As early life is a critical window of vulnerability to long-term programming of health, cognitive assessment was performed by utilizing spatial memory function in the Y-maze test and also spatial reference and working memory using the delayed win-shift task (DWSh) in the radial arm maze. Neonatally overfed rats took longer to learn the DWSh task indicating a poorer memory acquisition compared to control. No change of spatial memory in the less cognitively demanding Y-maze test was observed in neonatally overfed rats.

The potential of a synergistic effect of WD consumption in the $APP_{swe}/PS1_{dE9}$ double transgenic Alzheimer's mice model (APDE9) animal model memory and anxiety-like behaviour was assessed. Metabolically, this study identified that APDE9 mice fed a WD showed impaired glucose tolerance but not in wild-type WD mice or ADPE9 mice fed the control diet indicating impaired insulin receptor signalling. Both APDE9 mice fed control or WD showed a spatial memory deficit in the Y-maze when compared to their wild-type counterparts. There was no observed synergistic effect of WD consumption and APDE9 phenotype in the Y-maze. Additionally no change in anxiety-like behaviour was discerned using the open field test and the light/dark preference test.

Findings from this thesis indicate that WD consumption alone does not affect cognition using a variety of behavioural tasks. Whilst central changes in the dopaminergic and serotoninergic system ensue following WD consumption however, whether these changes occur before cognitive impairment is still unclear. The time period in which the obese phenotype transpires

appears to play a factor in cognitive impairment as shown by the results in the neonatal overfeeding study. Additionally we demonstrated that WD consumption does not affect spatial memory but a possible synergistic interplay between the APDE9 mice phenotype and WD consumption may have a deleterious effect of spatial memory. Further work is necessary to elucidate the factors that contribute to the onset of cognitive impairment observed in rat models of obesity.

Chapter 1 - Introduction and background

1.1. Obesity

The incidence of obesity, classified by a body mass index > 30 kg/m^2 (BMI, body mass divided by the square of one's height), is rising steadily throughout the world's population. It has further been established that abdominal obesity, assessed by waist circumference, also predicts obesity-related health risk (Rexrode et al., 1998, WHO, 1999, Zhu et al., 2002) and is a stronger predictor of obesity-related health risks than BMI alone (Janssen et al., 2004, Zhu et al., 2002). The rapid rise of obesity rates has been attributed to unhealthy diets (that is over-consumption of food and beverages with a high content of fats, sugars and salt) and physical inactivity. Figures from the Organisation for Economic Co-operation and Development 2014 Obesity report suggest that worldwide 18% of the adult population are obese (OECD, 2014). More than one in three adults in Mexico, New Zealand and the United States, and more than one in four adults in Australia, Canada, Chile and Hungary were considered obese (OECD, 2014).

1.1.1.Aetiology of obesity

The lack of energy balance that is energy intake exceeding energy expenditure is the foremost cause of obesity. Due to the global shift in diets and greater availability of high calorific foodstuffs, especially those high in fats, and lack of physical activity obesity rates have soared. However, research has shown that other factors can influence the onset of obesity including environmental, genetic, developmental, social demographic and psychological influences.

1.1.2. Consequences of obesity

Obesity can have damaging effects on many organ systems that can contribute to early mortality. In 2009, the standardised mortality rate for obesity in Australia was 1.0 per

100,000 of population, an increase from 0.6 per 100,000 population in 2000 (Statistics, 2013). The US Nurses' Health Study found all-cause mortality rates to increase significantly with increasing BMI (Li et al., 2006) whilst in the Harvard Male Alumni study, a parabolic relationship was found whereby risk of mortality in men was increased at the lowest and highest values of BMI (Lee et al., 1993). Obesity decreases life expectancy by 7 years at the age of 40, similar to what is seen with smoking (Peeters et al., 2003).

Being overweight or obese contributes to significant health impairments with large increases in the risk of cardiovascular disease, type 2 diabetes and cancer (Adams et al., 2006, McGee, 2005). Prospective cohort studies have demonstrated increases in the risk of coronary heart disease with an 8% increase for each unit increase of BMI (Li et al., 2006). Risk of systemic hypertension is approximately 5 times higher in obese individuals (Wolf et al., 1997). Moreover, the incidence of respiratory diseases such as obstructive sleep apnoea, gastrointestinal and musculoskeletal disorders, thromboembolism, stroke and even cancer are increased with obesity (Grundy, 2004, Haslam and James, 2005).

1.2. Paediatric obesity

In 2013, 42 million children under the age of 5 were found to be overweight or obese worldwide, with the incidence becoming more apparent in low and middle income countries (WHO, 2015). The rate of increase in these countries has been observed to be 30% higher than that of developed countries (WHO, 2015). It has been estimated that the incremental lifetime medical cost of an obese child relative to a normal weight child who maintains normal weight throughout adulthood is US$19,000 (Finkelstein et al., 2014).

1.2.1. Consequences of paediatric obesity

Obesity in childhood is strongly associated with obesity in adulthood such that 60-80% of obese children become obese adults (Guo et al., 2002). Paediatric obesity is associated with higher risk of premature death and disability in adulthood and is likely to be a major cause of ill health in adulthood, but also contributes to the likelihood of pronounced illness in childhood (WHO, 2015). Many of the same chronic illnesses and risk factors seen in adult obesity are also observed in children.

A systematic review collated and identified the many consequences of paediatric obesity (Reilly et al., 2003). Consequences include psychological issues such as low self-esteem and behavioural problems (Epstein et al., 1994, Strauss, 2000), higher incidence of asthma (Belamarich et al., 2000), presence of low grade systemic inflammation (Cook et al., 2000, Visser et al., 2001) and a myriad of cardiovascular risk factors like hypertension, type 2 diabetes mellitus, dyslipidaemia, abnormalities in left ventricular mass and/or function; abnormalities in endothelial function; and hyperinsulinemia and/or insulin resistance (Freedman et al., 1999, Iannuzzi et al., 2004, Maffeis et al., 2001, Ostchega et al., 2009, Reilly et al., 2003, Tounian et al., 2001). If comorbidities are present in childhood, and obesity persists into adulthood, then obesity related disease duration and hence prognosis is worsened.

1.3. Metabolic syndrome

Obesity is also a part of metabolic syndrome, characterised by a large waist measurement, excess abdominal visceral adipose tissue, high triglyceride levels, glucose intolerance and hypertension, with many related comorbid conditions (Despres et al., 1990, Messier et al.,

2010, Wildman et al., 2008). Overweight or obese individuals having the same amount of total body fat can also be characterised by different cardiovascular risk factor profiles (Despres et al., 1990). Regional fat distribution, particularly visceral abdominal and intramuscular adipose tissue, can be used to clearly discriminate those with the metabolic syndrome and higher cardiovascular risk profiles (Despres et al., 1990). Sufferers of metabolic syndrome have an increased risk of hyperinsulinemia, glucose intolerance, type 2 diabetes, altered cytokine profile, impaired fibrinolysis, thrombosis, endothelial dysfunction and cardiovascular diseases (Brunzell and Hokanson, 1999, Fujioka et al., 1987, Kissebah and Krakower, 1994, Rebuffe-Scrive et al., 1989). This suggests that the presence of large intra-abdominal fat stores is a critical determinant of obesity-related metabolic complications.

1.3.1. Regional fat deposition

BMI is the most widely used and most practical method of assessing obesity however BMI does not take into account the variation of body fat distribution (Michels et al., 1998). The distribution of body fat is more strongly associated with negative health outcomes than BMI and total adiposity and is therefore a more useful health indicator (Janssen et al., 2004, Lean et al., 1998, Zhu et al., 2002). Regional fat deposition has been documented to vary markedly at any BMI value (Fujioka et al., 1987, Sjostrom et al., 1986). A 13 year longitudinal study showed that in middle aged males, BMI and skinfold thickness showed no association to risk of cardiovascular disease and death (Larsson et al., 1984). However, waist-to hip circumference ratio, an indicator of abdominal adiposity, showed a strong association with cardiovascular disease and death indicating that distribution of fat deposits may be a better predictor of cardiovascular disease and death than total adiposity (Larsson et al., 1984).

1.3.1.1. Visceral and abdominal fat depots

Visceral and abdominal adiposity has been associated with an adverse cardiometabolic profile, including inflammation, insulin resistance, and myocardial dysfunction (Bays, 2011, Britton et al., 2013, Janssen et al., 2004, Shah et al., 2014, Zhu et al., 2002). The Framingham Heart Study found that visceral adiposity was associated with cardiovascular disease after adjusting for clinical risk factors and overall adiposity (Britton et al., 2013). This was corroborated by the Multi-Ethnic Study of Atherosclerosis (MESA) which demonstrated that visceral adiposity is strongly associated with increased cardiometabolic risk, regardless of age, race, or BMI (Shah et al., 2014). Interestingly, the MESA study also found that neither waist circumference nor BMI was associated with visceral fat (Shah et al., 2014).

1.3.1.2. Pericardial and epicardial fat depots

An excessive amount of pericardial or epicardial fat depots have been implicated in the pathogenesis of obesity-related cardiovascular disease (Aslanabadi et al., 2014, Lim and Meigs, 2014). Epicardial fat tissue is located between the outer wall of the myocardium and the visceral layer of pericardium of the heart (Aslanabadi et al., 2014, Lim and Meigs, 2014). Pericardial fat tissue is located anterior to the epicardial fat tissue and is located between visceral and parietal pericardium of the heart (Lim and Meigs, 2014, Sironi et al., 2004). Epicardial fat volume has been reported to correlate with the severity of coronary artery disease due to the proximity to major coronary arteries (Iacobellis et al., 2005, Mazurek et al., 2003). Iacobellis *et al.* found that epicardial fat tissue measured by echocardiography displayed a strong association with waist circumference, diastolic blood pressure, fasting plasma insulin and low-density lipoprotein cholesterol (Iacobellis et al., 2003). Multiple studies have shown that in healthy participants, pericardial fat is independently correlated with BMI, waist circumference, coronary artery calcification and abdominal aortic artery

calcification (Fox et al., 2009, Rosito et al., 2008, Thanassoulis et al., 2010). The loss of elasticity through artery calcification impairs cardiovascular haemodynamics, resulting in a substantial increase in morbidity and mortality (Keelan et al., 2001, Wayhs et al., 2002).

The studies discussed above indicate that pericardial and epicardial fat may be an important mediator of metabolic risk (Aslanabadi et al., 2014, Despres, 2012). Both fat depot sites are correlated with multiple measures of adiposity and cardiovascular disease risk factors, nevertheless visceral and abdominal fat depots are a strong correlate for most metabolic risk factors (Aslanabadi et al., 2014, Despres, 2012, Rosito et al., 2008).

1.4. Relationship between obesity and mild cognitive impairment

Cognition is an intellectual process by which one becomes aware of, perceives, or comprehends, ideas. Cognition includes higher mental processes, such as perception, memory, language, problem solving, and abstract thinking.

Cognitive aging is a normal process where in older adulthood there is a structural and functional change in the brain that results in a deterioration of cognitive ability (Glisky, 2007). Even when controlling for cognitive aging, studies still show a negative correlation between BMI and global cognitive performance (Elias et al., 2005, Hassing et al., 2010, Jeong et al., 2005). A cross-sectional longitudinal study of over 2000 adults supported the linear association between BMI and cognitive function determined by the word-list learning test, which evaluates verbal learning and memory, and digit substitution symbol test (DSST), which assesses attention, response speed and visuomotor coordination. Obese people recalled fewer words from the list in the word-list learning test and took longer to complete DSST relative to normal weight individuals (Cournot et al., 2006). In another study combining ages

from 20 to 82, overweight and obese people exhibited poorer executive function test performance than normal weight adults with no evidence of a BMI x age interaction (Gunstad et al., 2007). These findings were further reinforced by another study that demonstrated that persistent obesity is associated with poor cognition in later midlife with deficits in short term verbal memory and executive function being identified (Sabia et al., 2009a). A prospective study examined the associations of middle age overweight individuals on specific areas of cognition, that being episodic memory, semantic memory and visuospatial ability. Semantic memory and visuospatial ability, but not episodic memory, was found to be impaired in middle aged overweight individuals when compared to normal individuals (Nilsson and Nilsson, 2009).

A growing body of research also indicates that obesity in mid-life is a predictor of mild cognitive impairment (MCI) at old age. MCI is a syndrome defined as cognitive decline greater than expected for an individual's age and education level (Gauthier et al., 2006, Petersen et al., 1999). In fact, 66.2% of individuals suffering MCI have been shown to progress to Alzheimer's disease (AD) compared with 8.3% without MCI in a 4 year follow-up study in the elderly (Kluger et al., 1999). Thus the impact of obesity on cognition appears to accumulate over the adult life course. However, cognitive impairments have not always been observed in obese individuals.

Across studies, different cognitive domains analysed make it difficult to draw absolute comparisons. Nevertheless, impairment of executive function has been dependably identified in obese adults when compared to normal weight counterparts (Cournot et al., 2006, Lokken et al., 2009, Mond et al., 2007, Sabia et al., 2009b).

1.5. Obesity and impairment of cognition

Studies have investigated the association between human obesity and cognitive function and have identified impairments of specific cognitive domains in both children and adults with obesity compared with their non-obese counterparts (**Table 1.1**). As of April 2017 articles were identified through the Medline electronic database using the terms related to obesity (i.e. obesity, body mass index, adiposity) and cognition (i.e. cognition, cognitive domains, executive function, memory). The search was limited to studies of humans and published in English. It is important to note that although the cognitive tests described below are arranged so that only one cognitive domain is stated, many cannot be evaluated in isolation and involve multiple cognitive domains (Lezak et al., 2012, Strauss et al., 2006). For simplicity, the major cognitive domain was reported for each test by globally recognised clinical neuropsychology references (Lezak et al., 2012, Strauss et al., 2006). The most widely used cognitive screening test in the world is the Mini-mental state examination (MMSE), employed to evaluate dementia. Dementia is a progressive global cognitive impairment syndrome; the hallmark feature being the inability to carry out everyday activities as a consequence of diminished cognitive function. After controlling for known confounders, such as education, age, and socioeconomic status, there have been a total of 5 out of 7 studies that have identified an association between obese adults and deficits in global cognition (Benito-León et al., 2013, Gunstad et al., 2010, Kerwin et al., 2010, Kerwin et al., 2011, Kilander et al., 1997). In a population of 70 year-old men, higher BMI was associated with impaired cognitive function (Kilander et al., 1997). Baseline results from the Women's Health Initiative Memory Study of 7,163 older women found that as BMI increased, performance in the modified MMSE (that covers a wider range of difficulty levels and broadens the range of scores from 0-30 to 0-100 compared to the MMSE) was reduced, with each unit of BMI associated with a decrease of 0.988 score (Kerwin et al., 2010). A follow-up study also found

evidence that older women with high waist-to-hip ratio and BMI to be at greater risk of developing cognitive impairment and dementia (Kerwin et al., 2011).

1.5.1. Obesity and executive function

As of April 2017 there were a total of 10 studies listed on Medline that have investigated the impairment of cognitive function in children and 43 studies in adults with conflicting results that met the search criteria. A consistent specific cognitive deficit identified in human associative studies has been executive function impairments in obese individuals. Executive function is an umbrella term encompassing the complex cognitive processing for the temporal organization of behaviour and the use of working memory to plan a sequence of forthcoming responses associated with the monitoring and controlling of thought and goal-directed behaviours (Baddeley, 1986, Posner and Petersen, 1990, Shallice, 1988). Executive function involves multiple components with a variety of theories that endeavour to encapsulate its key components (Desimone and Duncan, 1995, Miller and Cohen, 2001, O'Reilly et al., 1999). An influential and recognised taxonomy of executive function includes: (i) attention and inhibition, by directing attention on appropriate and inhibiting inappropriate information; (ii) task management, organising processes in complex tasks that require switching of attention; (iii) planning, planning of multiple steps to achieve a desired goal or outcome; (iv) performance monitoring, the cognitive and behavioural adjustments through the use of working memory to increase control to meet demands; and (v) temporal coding, coding the time and place of representations in working memory (Smith and Jonides, 1999). These five executive function components are all interrelated and at times the cognitive tests employed to assess executive function may target multiple components. Therefore it is a higher cognitive process that controls and regulates lower level processes (e.g. perception and motor processes) to guide behaviour towards a goal and is mediated primarily by prefrontal cortex

(PFC) (Stuss and Levine, 2002). The PFC, dopaminergic system and serotonergic system have been linked in the regulation of executive function (Funahashi, 2001, Robbins, 2005).

Table 1.1: Association studies between obesity and cognitive function in children and adults

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
Children							
(Bauer et al., 2010)	Not reported	16.1 ± 1.5	Cross-sectional	Normal weight: n= 68 Obese: n= 41	Stroop Colour Word Test	Response inhibition	Sig ($p<0.05$)
(Bauer et al., 2015)	21.6 ± 5.0	7.6 ± 0.4	Cross-sectional	Normal weight: n= 18 Overweight/obese: n= 15	Neuropsychological Assessment of Children Test	Attention	ns
						Cognitive flexibility	ns
						Verbal memory	ns
						Visual memory	ns
(Blanco-Gomez et al., 2015)	Not reported	Range: 6-10	Cross-sectional	Normal weight: n= 316 Overweight/obese: n= 186	Children's Colour Trails Test	Attention	ns
						Cognitive flexibility	Sig ($p=0.02$)
						Response inhibition	Sig ($p<0.05$)
					Five-Digit Test	Response inhibition	Sig ($p=0.04$)
					Symbol Digit Modalities Test	Psychomotor processing	ns

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Cserjési et al., 2007)	27.2 ± 1.8	12.1 ± 0.9	Cross-sectional	Normal weight: n= 12	D2 Attention Endurance Test	Attention	Sig ($p<0.05$)
				Overweight: n= 12	Digit Span Test, Forward & Backwards	Working memory	ns
					Raven's Progressive Matrices	Logical reasoning	ns
					Semantic Verbal Fluency Test	Verbal fluency	ns
					Wisconsin Card Sorting Test	Cognitive flexibility	Sig ($p<0.05$)
(Gentier et al., 2013)	31.6 ± 3.5	9.2 ± 1.5	Cross-sectional	Normal weight: n= 19	Complex Choice Reaction Task	Complex decision making	Sig ($p<0.001$)
				Obese: n= 19	Simple Choice Reaction Task	Simple decision making	Sig ($p<0.004$)
(Gunstad et al., 2008)	24.6 ± 3.7	11.7 ± 3.1	Cross-sectional	Normal weight: n= 330	Digit Span Test, Backwards	Working memory	ns
				Overweight/obese: n= 121	Switching of Attention Test	Cognitive flexibility	ns
					Verbal Recall Test	Verbal memory	ns
(Huang et al., 2015)	Not reported	13 ± 0.6	Cross-sectional	Normal weight: n= 451	Modified Erisksen Flanker Task	Response inhibition	Sig ($p<0.02$)
				Overweight/obese: n= 74			

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Hughes et al., 2015)	Not reported	4.8 ± 0.4	Cross-sectional	Normal weight: n= 99	Delay of Gratification Task	Decision making	ns
				Overweight/obese: n= 88	Flexible Item Selection Task	Cognitive flexibility	ns
					Gift Delay Task	Self-regulation	ns
					Tapping task	Response inhibition	ns
(Verdejo-Garcia et al., 2010)	31.6 ± 7.1	14.3 ± 1.2	Cross-sectional	Normal weight: n= 34	Five-Digit Test	Response inhibition	Sig (p=0.038)
				Overweight/obese: n= 34	Iowa Gambling Task	Decision making	Sig (p=0.03)
					Stroop Colour Word Test	Response inhibition	ns (p=0.07)
					Trail Making Test	Cognitive flexibility	Sig (p=0.003)
					Letter Number Sequencing	Working memory	ns

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Yau et al., 2014)	35.5 ± 5.9	17.6 ± 1.6	Cross-sectional	Normal weight: n= 30 Obese: n= 30	Digit Symbol Substitution Test	Psychomotor processing	ns (p=0.07)
					Digit Vigilance Test	Attention	ns
					Stroop Colour Word Test	Response inhibition	ns
					Trail Making Test	Cognitive flexibility	ns (p=0.08)
					Wide Range Assessment of Memory and Learning	Attention	ns (p=0.06)
						Verbal memory	ns
						Visual memory	ns
						Working memory	ns (p=0.06)
(Wirt et al., 2015)	Not reported	7.1 ± 0.6	Cross-sectional	Normal weight: n= 235 Overweight/obese: n= 26	Go/No Go Task	Response inhibition	Sig (p<0.05)
					KiTAP Test	Cognitive flexibility	Sig (p<0.01)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
Adults							
(Ariza et al., 2012)	38.3 ± 7.6	31.8 ± 6.5	Cross-sectional	Normal weight: n= 42 Obese: n= 42	Letter-Number Sequencing	Working memory	ns (p=0.179)
					Stroop Colour Word Test	Response inhibition	ns (p=0.403)
					Symbol Digit Modalities Test	Psychomotor processing	ns (p=0.215)
					Trail Making Test	Cognitive flexibility	ns (p=0.44)
					Wisconsin Card Sorting Test	Cognitive flexibility	ns (p=0.869)
(Benito-León et al., 2013)	Not reported	75.3 ± 5.8	Cross-sectional	Normal weight: n= 507 Overweight/obese: n= 1,442	MMSE	Global cognition	Sig (p<0.001)
					Trial Making Test, Part A	Psychomotor speed	Sig (p<0.001)
					Six Objects Test	General memory	Sig (p<0.05)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Boeka and Lokken, 2008)	51.8 (35.0-80.0)	41 (20-57)	Cross-sectional	Normative Sample Obese: n= 68	California Verbal Learning Test	Verbal memory	ns
					Rey Complex Figure Test	Nonverbal memory	Sig (p<0.001)
					Trail Making Test	Cognitive flexibility	ns (p=0.07)
					Wisconsin Card Sorting Test	Cognitive flexibility	Sig (p<0.001)
(Brogan et al., 2010)	36.2 ± 5.0	52.1 ± 11.7	Cross-sectional	Normal weight: n= 20 Obese: n= 18	Iowa Gambling Test	Decision making	Sig (p=0.004)
(Brogan et al., 2011)	52.2 ± 10.0	41.5 ± 9.2	Cross-sectional	Normal weight: n= 50 Overweight/obese: n= 42	Iowa Gambling Test	Decision making	Sig (p=0.02)
(Brooks et al., 2013)	33.0 ± 0.3	Range: 70-75	Cross-sectional	Normal weight: n= 97 Obese: n= 59	Trail Making Test	Cognitive flexibility	Sig (p<0.05)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Calvo et al., 2014)	21.2 ± 2.5	36.4 ± 6.2	Cross-sectional	Normal weight: n= 32	Go/No Go Task	Response inhibition	Sig (p<0.01)
				Overweight/obese: n= 30	Running Memory Continuous Performance Task	Working memory	ns
					Standard Continuous Performance Task	Attention	ns
(Cheke et al., 2015)	Not reported	Range: 18-35	Cross-sectional	Normal weight: n= 26 Overweight/obese: n= 24	Treasure Hunt Task	Working memory	Sig (p=0.038)
(Chelune et al., 1986)	Not reported	32.7 ± 7.5	Cross-sectional	Normative Sample	Category Test	Cognitive flexibility	Sig (p, not reported)
				Obese: n= 44	Trail Making Test	Cognitive flexibility	Sig (p, not reported)
(Coppin et al., 2014)	Overweight: 27.6 ± 1.5 Obese: 36.0 ± 6.5	Overweight: 24.9 ± 4.6 Obese: 25.2 ± 4.4	Cross-sectional	Normal weight: n= 16	Conditioned Cue Preference Test	Associative Learning	Sig (p=0.03)
				Overweight: n= 16	Probabilistic Learning Task	Learning	Sig (p=0.019)
(Cournot et al., 2006)	28.4 ± 1.8	44.7 ± 10.6	Prospective	Normal weight: n= 1,334	Adapted Rey Auditory Verbal Learning Test	Verbal memory	Sig (p<0.001)
				Overweight/obese: n= 889	Digit Symbol Substitution Test	Psychomotor processing	Sig (p<0.001)
					Sternberg Test, Subtest	Attention	Sig (p<0.05)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Cserjesi et al., 2009)	34.2 ± 3.8	48.8 ± 11.0	Cross-sectional	Normal weight: n= 30	D2 Attention Endurance Test	Attention	Sig (p<0.05)
				Obese: n= 30	Digit Span Test, Forward	Working memory	ns
					Hayling Sentence Completion Task	Response inhibition	Sig (p<0.05)
					Trail Making Test	Cognitive flexibility	ns
(Danner et al., 2012)	30.8 ± 3.0	44.6 ± 13.4	Cross-sectional	Normal weight: n= 30 Obese: n= 18	Iowa Gambling Test	Decision making	Sig (p=0.012)
(Davis et al., 2004a)	Not reported	28.6 ± 5.6	Cross-sectional	Normal weight: n= 26	Iowa Gambling Test	Decision making	Sig (p<0.05)
				Overweight/obese: n= 15			
(Davis et al., 2010)	38.6 ± 7.1	35.2 ± 6.7	Cross-sectional	Normal weight: n= 71	Delay Discounting Task	Decision making	Sig (p<0.05)
				Overweight/obese: n= 73	Iowa Gambling Test	Decision making	Sig (p=0.019)
(Dregan et al., 2013)	Not reported	66.9	Cross-sectional	Total: n= 9,432	Immediate and Delayed Recall Test	General memory	Sig (p<0.05)
					Letter-Cancellation Test	General executive function	ns

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Elias et al., 2003)	29.8 ± 1.8	65.7 ± 6.9	Prospective	Normal weight males: n= 190 Overweight/obese males: n= 361	Kaplan-Albert Neuropsychological Test Battery	Working memory	Sig (p=0.02)
						Visual memory	Sig (p=0.0004)
						Verbal memory	Sig (p=0.0002)
(Etou et al., 1989)	34.6 ± 1.1	34.2 ± 2.3 (SEM)	Cross-sectional	Normal weight: n= 13 Obese: n= 13	Tap Test	Psychomotor processing	Sig (p<0.01)
					Transfer coordination Test	Psychomotor processing	Sig (p<0.05)
					Transverse Speed Test	Psychomotor processing	Sig (p<0.05)
(Fagundo et al., 2012)	39.8 ± 7.4	40.5 ± 11.1	Cross-sectional	Normal weight: n= 137 Overweight/obese: n= 52	Iowa Gambling Test	Decision making	Sig (p<0.05)
					Stroop Colour Word Test	Response inhibition	Sig (p<0.05)
					Wisconsin Card Sorting Test	Cognitive flexibility	ns

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Fedor and Gunstad, 2013)	Not reported	20.1 ± 1.8	Cross-sectional	Total: n= 323	Immediate Post Concussion Assessment and Cognitive Testing	Response inhibition	ns
						Psychomotor processing	ns
						Verbal memory	Sig (p<0.01)
						Visual memory	Sig (p<0.01)
(Fergenbaum et al., 2009)	Not reported	Range: 18-55	Cross-sectional	Normal weight: n= 28	Clock Drawing Test	General executive function	ns
				Overweight/obese: n= 179	Trail Making Test	Cognitive flexibility	Sig (p=0.0015)
(Galioto Wiedemann et al., 2014)	21.2 ± 2.9	36.4 ± 5.7	Cross-sectional	Normal weight: n= 36	Go/No Go Task	Response inhibition	Sig (p<0.01)
				Obese: n= 36	Running Memory Continuous Performance Task	Working memory	Sig (p=0.04)
					Standard Continuous Performance Task	Attention	Sig (p=0.04)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Gonzales et al., 2010)	34.3 ± 3.5	48.5 ± 8.6	Cross-sectional	Normal weight: n= 9 Overweight: n= 11 Obese: n= 12	California Verbal Learning Test	Verbal memory	ns (p=0.39)
					Digit Span Test	Working memory	ns (p=0.79)
					MMSE	Global cognition	ns (p=0.07)
					Trail Making Test	Cognitive flexibility	ns (p=0.91)
(Gunstad et al., 2006)	Overweight: 26.8 ± 1.3 Obese: 33.9 ± 4.9	Overweight: 43.5 ± 14.4 Obese: 42.1 ± 16.4	Cross-sectional	Normal weight: n= 261 Overweight: n= 159 Obese: n= 66	Verbal Memory Task	Verbal memory	Sig (p<0.05)
(Gunstad et al., 2007)	28.8 ± 3.9	45.4 ± 8.4	Cross-sectional	Normal weight: n= 210 Overweight/obese: n= 198	Austin Maze	Spatial memory	Sig (p<0.01)
					Choice Reaction Task	Attention	Sig (p<0.01)
					Digit Span Test, Forward	Working memory	Sig (p<0.01)
					Spatial Span Test	Visual memory	Sig (p<0.01)
					Stroop Colour Word Test	Response inhibition	Sig (p<0.01)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Gunstad et al., 2010)	Not reported	Range: 19-93	Longitudinal	Total: n= 1,703	California Verbal Learning Test	Verbal memory	ns
					Card Rotation Test	Visuospatial ability	ns
					Digit Span Test, Forward & Backwards	Working memory	Sig ($p<0.05$)
					MMSE	Global cognition	Sig ($p<0.01$)
(Kerwin et al., 2010)	Not reported	69.6 ± 0.1	Cross-sectional	Normal weight: n= 2,263 Overweight/obese: n= 5,298	MMSE	Global cognition	Sig ($p<0.001$)
(Kerwin et al., 2011)	Not reported	Range: 65-80	Prospective	Total: n= 7,163	MMSE	Global cognition	Sig ($p=0.02$)
(Kesse-Guyot et al., 2015)	Not reported	66.1 ± 4.5	Longitudinal	Normal weight: n= 1,687	Cued Recall Test	Episodic memory	ns
				Overweight: n= 928	Digit Span Test, Forward & Backwards	Working memory	Sig ($p=0.04$)
				Obese: n= 202	Trail Making Test	Cognitive flexibility	Sig ($p= 0.04$)
					Verbal Memory Task	Verbal memory	ns

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Kilander et al., 1997)	Not reported	Range: 69-74	Cross-sectional	Normal weight: n= 94 Overweight/obese: n= 381	Claeson-Dahl's Test Digit Span Test, Forward & Backwards MMSE Rey Complex Figure Test Trial Making Test	Verbal memory Working memory Global cognition Nonverbal memory Cognitive flexibility	Composite Cognitive Score: Sig (p=0.009)
(Mobbs et al., 2011)	33.6 ± 6.4	39.3 ± 12.2	Cross-sectional	Normal weight: n= 16 Obese: n= 16	Mental Flexibility Task	Cognitive flexibility	Sig (p<0.05)
(Nederkoorn et al., 2006)	39.0 ± 5.3	40.9 ± 6.6	Cross-sectional	Normal weight: n= 28 Overweight/obese: n= 31	Iowa Gambling Test Stop Signal Task	Decision making Response inhibition	ns (p=0.3) Sig (p<0.05)
(Pignatti et al., 2006)	42.2 ± 6.0	43.4 ± 8.1	Cross-sectional	Normal weight: n= 20 Overweight/obese: n= 20	Iowa Gambling Test	Decision making	Sig (p=0.04)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcome
(Reis et al., 2013)	Not reported	25.2 ± 3.6	Longitudinal	Total: n= 2,932	Digit Symbol Substitution Test	Psychomotor processing	Sig (p=0.04)
					Stroop Colour Word Test	Response inhibition	Sig (p=0.002)
					Rey Auditory Verbal Learning Test	Verbal memory	ns
(Singh-Manoux et al., 2012)	Not reported	Overweight: 50.0 ± 6.0	Retrospective	Normal weight: n= 3,374	Alice Hein 4-I Test	Reasoning	Sig (p<0.001)
		Obese: 49.7 ± 5.8		Overweight: n= 2,445	Free Recall Test	Verbal memory	Sig (p=0.01)
				Obese: n= 582	Phonetic & Semantic Fluency Test	Verbal fluency	Sig (p=0.01)
(Sorensen and Sonne-Holm, 1985)	Not reported	18	Cross-sectional	Normal weight: n= 2,123 Obese: n= 1143	Borge Priens Prove	Intellectual function	Sig (p<0.001)
(Sorensen et al., 1982)	Not reported	Range: 18-21	Cross-sectional	Normal weight: n= 2,719 Obese: n= 1,806	Borge Priens Prove	Intellectual function	Sig (p<0.001)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Stanek et al., 2013)	45.2 ± 6.9	43.5 ± 11.3	Retrospective	Normal weight: n= 580 Obese: n= 152	Austin Maze	Spatial memory	ns
					Choice Reaction Task	Attention	Sig ($p<0.05$)
					Digit Span Test, Forward	Working memory	ns
					Stroop Colour Word Test	Response inhibition	ns
(Volkow et al., 2009)	Not reported	Range: 22-45	Cross-sectional	Total: n= 21	Stroop Colour Word Test	Response inhibition	ns
					Symbol Digit Modalities Test	Psychomotor processing	ns
					Wisconsin Card Sorting Test	Cognitive flexibility	Sig ($p=0.05$)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Walther et al., 2010)	Overweight: 27.6 ± 1.4 Obese: 34.9 ± 3.3	Overweight: 69.9 ± 8.1 Obese: 66.9 ± 9.9	Cross-sectional	Normal weight: n= 53 Overweight: n= 22 Obese: n= 20	MMSE	Global cognition	ns
					Halstead-Reitan, Trials A	Visuomotor speed	ns
					Face Recognition Test Logical Memory Recall Test Verbal Paired Associates I	General memory	Composite memory score: ns
					Mental Arithmetic Test Mental Control Task Wisconsin Card Sorting Test	Executive function	Composite executive function score: Sig ($p<0.05$)
(Weller et al., 2008)	38.4 ± 6.6	19.6 ± 2.9	Cross-sectional	Normal weight: n= 26 Overweight/obese: n= 29	Iowa Gambling Task	Decision making	Sig ($p<0.02$)

Author, year	Obese group: BMI (μ ± SD)	Obese group: Age (μ ± SD)	Study design	Sample size	Cognitive test	Cognitive domain tested	Reported outcomes
(Wright et al., 2016)	Not reported	59.0 ± 10.6	Cross-sectional	Normal weight: n= 49	Alpha Span Test	Working memory	Sig ($p<0.05$)
				Overweight/obese: n= 145	Benton Visual Retention Test	Visual memory	ns
					Rey Auditory Verbal Learning Test	Verbal memory	Sig ($p<0.05$)
					Stroop Colour Word Test	Response inhibition	ns
					Trail Making Test	Cognitive flexibility	ns

1.5.2. Attention deficits in obese children and adults

Attention is the capacity to focus on one or more important stimuli while suppressing awareness of competing distractions. As of April 2017, four studies investigated attention performance in obese children and only a single study reported an impairment of attention. Obese children were found to respond more poorly in the D2 attention endurance test compared to normal weight counterparts in which participants scanned a list of alphabetical characters and cross out all irregular characters (Cserjési et al., 2007). Conversely, three studies did not report any indication of attention deficit in obese children, each using distinct behavioural tests for attention (Bauer et al., 2015, Blanco-Gomez et al., 2015, Yau et al., 2014). The present evidence indicates there is no impairment of attention in obese children.

In contrast, obese adults were observed to have impairment in attention performance (Cournot et al., 2006, Cserjesi et al., 2009, Galioto Wiedemann et al., 2014, Gunstad et al., 2007, Stanek et al., 2013). Cournot *et al.* assessed over 2000 adults, using a component of the Sternberg test to assess attention, and observed that obese adults took longer to scan a list of alphabetic characters to find a specific character than normal weight counterparts (Cournot et al., 2006). Furthermore, two studies used the Choice Reaction task to discern that obese adults have slower reaction times when given a choice between two possible stimuli, with each stimulus requiring a different response (Gunstad et al., 2007, Stanek et al., 2013).

Thus, the evidence suggests that childhood obesity does not alter attention; however obesity may impair attention processes in adulthood.

1.5.3. Cognitive flexibility in obese children and adults

Cognitive flexibility, also known as set-shifting, is the ability to readily and selectively switch between concepts in response to appropriate environmental stimuli. As of the April 2017, six studies examined cognitive flexibility in obese children where a variety of tests were used. Four reported impairments (Blanco-Gomez et al., 2015, Cserjési et al., 2007, Verdejo-Garcia et al., 2010, Wirt et al., 2015) and two reported no effect on cognitive flexibility (Bauer et al., 2015, Yau et al., 2014). One such test is the Trail Making test (TMT) involving two components; TMT-A component entails the participant is to draw lines to connect circled numbers in a numerical sequence as rapidly in possible and in TMT-B the participant is to draw lines to connect circled numbers and letters in an alternating numeric and alphabetic pattern as rapidly as possible (Salthouse, 2011). Obese children took longer to perform this task indicating impairment of cognitive flexibility (Verdejo-Garcia et al., 2010). Results were further corroborated by three studies that reported a strong association between obese children and cognitive flexibility impairment (Blanco-Gomez et al., 2015, Cserjési et al., 2007, Wirt et al., 2015). Two studies did not observe cognitive flexibility impairment in obese children, although sample sizes for these studies were relatively low and as such could have lower statistical power (Bauer et al., 2015, Yau et al., 2014). Yau *et al.* tested obese children in the TMT and did not report an impairment in cognitive flexibility, however the result did approach significance ($p= 0.08$) (Yau et al., 2014). Meanwhile, participants in the Bauer *et al*. study had a mean BMI of 21.6 ± 5.0 and as such are considered overweight and not clinically obese (Bauer et al., 2015).

Obese adults were also shown to have impairments in cognitive flexibility; eight studies reported impairments (Boeka and Lokken, 2008, Brooks et al., 2013, Chelune et al., 1986, Fergenbaum et al., 2009, Kesse-Guyot et al., 2015, Kilander et al., 1997, Mobbs et al., 2011,

Volkow et al., 2009) while six studies found no effect (Ariza et al., 2012, Cserjesi et al., 2009, Fagundo et al., 2012, Gonzales et al., 2010, Gunstad et al., 2008, Wright et al., 2016). A majority of these studies used the TMT, while some used the Wisconsin Card Sorting test (WCST). The WCST requires participants to sort different cards by finding the correct classification through trial and error and experimenter feedback. Classification rules are changed multiple times during the test demanding participants to change their responses to match changes in reinforcement patterns (Berg, 1948). A study performed by Boeka and Lokken assessed several aspects of neuropsychological performance of severely obese adults (BMI $\geq$ 40) (Boeka and Lokken, 2008) and showed that although impairment of cognitive flexibility was not apparent when assessed by TMT, the WCST revealed there was an approximately 50% reduction in test performance in obese adults compared to normative data (Epstein et al., 1994). Poor performance in the WCST was corroborated with a significant inverse association of BMI and WCST performance reported by Volkow *et al.*, who also reported a negative correlation between BMI and brain activity in the PFC (Volkow et al., 2009).

The above summary demonstrates that the most consistent outcome is that cognitive flexibility is compromised in obese children. While in obese adults, eight of fourteen studies indicate a deficit in this domain, so a definitive conclusion cannot be ascertained from the evidence presented thus far.

1.5.4. Decision making in obese children and adults

Decision-making tasks require responding based on a variety of criteria including probabilistic choice and delay associated with rewards. The Iowa gambling task is a paradigm used to measure decision making involving probabilistic choice via uncertainty, monetary rewards and punishments (Bechara et al., 1994). Beneficial performance requires participants

to forego potential large immediate rewards for small longer term rewards to avoid losses. As of April 2017, decision making ability was assessed in three studies of obese children (Gentier et al., 2013, Hughes et al., 2015, Verdejo-Garcia et al., 2010) and nine studies in obese adults (Brogan et al., 2010, Brogan et al., 2011, Danner et al., 2012, Davis et al., 2004a, Davis et al., 2010, Fagundo et al., 2012, Nederkoorn et al., 2006, Pignatti et al., 2006, Weller et al., 2008). Two studies reported poor decision making ability in obese children, where they were more willing to make riskier decisions to obtain large immediate rewards even though in the long term the probability of more punishments increased (Gentier et al., 2013, Verdejo-Garcia et al., 2010). A similar finding was found in obese adults, where eight studies reported a deficiency in decision making (Brogan et al., 2010, Brogan et al., 2011, Danner et al., 2012, Davis et al., 2004a, Davis et al., 2010, Fagundo et al., 2012, Pignatti et al., 2006, Weller et al., 2008). One study in obese children (Hughes et al., 2015) and another in obese adults (Nederkoorn et al., 2006) did not observe an effect of decision making. Both of these studies are possibly limited in their sample population in that there was a lack of multiple ethnicities in the children study, whereas the adult study was performed only in women. In summary, there is considerable confirmation suggesting that obesity negatively affects decision making in both children and adults.

1.5.5. Psychomotor processing in obese children and adults

Psychomotor processing involves cognitive processes that enable sensation, perception and motor actions. Two tasks that are designed to assess psychomotor processing are the DSST and the Symbol Digit Modalities tests (SDMT), an inverse form of the DSST. The DSST requires participants to write digits into an array of empty boxes positioned below symbols according to a coding table indicating the association between digits and symbols. Obesity at

childhood did not affect completion time in both the DSST and SDMT (Blanco-Gomez et al., 2015, Yau et al., 2014).

In contrast, of the seven studies reported in adults, four reported impairments of psychomotor processing in which obese adults performed more poorly (Benito-León et al., 2013, Cournot et al., 2006, Etou et al., 1989, Reis et al., 2013), whilst three did not (Ariza et al., 2012, Fedor and Gunstad, 2013, Volkow et al., 2009). A prospective study of over 2000 participants showed that higher BMI was associated with poorer performance in the DSST in a linear fashion with females performing worse than males (Cournot et al., 2006). A decline in processing speed is recognised as a predictor for cognitive decline in older adults (Eckert, 2011) and age-related differences in total grey matter volume have been related to differences in processing speed (Chee et al., 2009). In summary, the evidence indicates that an association between obesity and slower psychomotor processing appears to be an age-dependent process and is not observed in children.

1.5.6. Response inhibition in obese children and adults

Response inhibition is the inhibition of actions that are inappropriate in the given context, and interferes with goal-directed behaviour (Mostofsky and Simmonds, 2008). It has been reported that obese children as young as 7 years of age have poor response inhibition performance (Wirt et al., 2015).

In contrast, the picture for obese adults is slightly more indistinct with a total of twelve studies performed and seven studies finding an association between obesity and poor response inhibition performance (Calvo et al., 2014, Cserjesi et al., 2009, Fagundo et al., 2012, Galioto Wiedemann et al., 2014, Gunstad et al., 2007, Nederkoorn et al., 2006, Reis et al., 2013). Seven studies used the Stroop Colour Word Test, based on the observation that individuals can read words much faster than they can identify and name colours. The Stroop

Colour Word Test involves the participant presented with words that name colours, but then printed in a colour different from the one being named and asked to identify the printed colour. Three studies demonstrated obese adults performed worse than healthy controls by making more errors (Fagundo et al., 2012, Gunstad et al., 2007, Reis et al., 2013), whilst four studies reported no difference in the Stroop Colour Word Test (Ariza et al., 2012, Stanek et al., 2013, Volkow et al., 2009, Wright et al., 2016). Using other tests such as the Go/No Go test and Hayling Sentence Completion task there was an observed deficit in response inhibition (Calvo et al., 2014, Cserjesi et al., 2009). Five studies did not show any association with adult obesity and response inhibition (Ariza et al., 2012, Fedor and Gunstad, 2013, Stanek et al., 2013, Volkow et al., 2009, Wright et al., 2016). These equivocal findings may be explained by differences in the populations tested. One study was conducted on athletes (Fedor and Gunstad, 2013) while another had a small sample size (n= 3) of obese adults (Volkow et al., 2009). Overall the evidence seems clear that response inhibition deficits are observed in obese children however it is still unclear if this cognitive domain deficit is observed in obese adults.

1.5.7. Memory impairments in obese children and adults

Memory is the term given to the structures and processes involved in the storage and subsequent retrieval of information (Mayes, 2000). This information takes numerous forms including visual, verbal and semantic in description. Memory can be divided into two different classes, being working memory and long-term memory (Mayes, 2000). Working memory is a limited-capacity system for the temporary storage and manipulation of information for complex tasks such as learning and reasoning (Baddeley, 2000). Working memory interacts with short-term memory and long-term memory to coordinate and divide attention between separate tasks (Baddeley, 2000). Long-term memory can be further

subdivided into declarative and non-declarative memory. Declarative memory refers to the capacity for conscious recollection of information such as verbal and visual memory (Squire and Zola, 1996). Non-declarative memory, also known as procedural memory, does not require conscious thought (Squire and Zola, 1996).

As of the April 2017, we could find no studies in children that have found evidence of a link between memory impairments and obesity. Four separate studies examined three different types of memory being verbal, visual and working memory and found no evidence of associations in obese children (Bauer et al., 2015, Cserjési et al., 2007, Gunstad et al., 2008, Verdejo-Garcia et al., 2010, Yau et al., 2014).

The narrative of the link between obesity and memory impairments in adults is diverse. Two studies found what they regard as a "general" memory impairment (Benito-León et al., 2013, Dregan et al., 2013) and investigation of memory impairments of non-verbal (Boeka and Lokken, 2008, Kilander et al., 1997), spatial (Gunstad et al., 2007), verbal (Cournot et al., 2006, Elias et al., 2003, Gunstad et al., 2006, Fedor and Gunstad, 2013, Kilander et al., 1997, Singh-Manoux et al., 2012, Wright et al., 2016), visual (Elias et al., 2003, Fedor and Gunstad, 2013, Gunstad et al., 2007) and working memory (Cheke et al., 2015, Elias et al., 2003, Galioto Wiedemann et al., 2014, Gunstad et al., 2007, Gunstad et al., 2010, Kesse-Guyot et al., 2015, Kilander et al., 1997, Wright et al., 2016) have been reported. However, other studies contradict these reports of memory impairments. One study reported that there was no "general" memory impairment observed in obese women (Walther et al., 2010) and another found no spatial memory deficit in severely obese adults (BMI $\geq$ 40) (Stanek et al., 2013). Six studies reported no verbal memory impairments in a wide spectrum of obese adults (reported mean BMI: 34-52 kg/m^2 and range age: 25-66) (Boeka and Lokken, 2008, Gunstad et al., 2010, Gonzales et al., 2010, Kesse-Guyot et al., 2015, Reis et al., 2013, Wright et al.,

2016). Additionally, five studies reported no difference in working memory ability between obese adults and their normal weight counterparts (Ariza et al., 2012, Calvo et al., 2014, Cserjesi et al., 2009, Gonzales et al., 2010, Stanek et al., 2013).

To date the evidence strongly suggests obesity does not impair memory in childhood. Conversely the link with adult obesity and memory impairment is still unclear with many contradictory reports.

1.6. Obesity and Alzheimer's disease

In AD, the characteristic behavioural symptoms are the progressive decline in cognitive performance with impaired learning and memory (Albert, 1996). These symptoms are sometimes accompanied with delusions, depression, agitation and aggressive behaviour (Victoroff et al., 1996). The neuropathological characteristic of AD is neuronal damage and death in brain regions vital for learning and memory as well as the occurrence of amyloid plaques and neurofibrillary tangles containing tau protein, which are the pathological markers of AD (Morrison and Hof, 1997, Serrano-Pozo et al., 2011), accompanied by microglial activation and astrogliosis (Beach et al., 1989, Itagaki et al., 1989). Pathological progression is somewhat consistent with plaques, tangles, neuronal, and synaptic loss observed first in medial temporal cortical regions such as entorhinal and perirhinal cortex, followed by hippocampus (HPC) and cerebral cortex (National Institute on Aging, 1997).

Recent human studies suggest that metabolic abnormalities (such as obesity, impaired glucose, insulin and increased triglycerides levels and high blood pressure) induced by over consumption of a high fat diet (HFD) is linked to AD (Besser et al., 2014, Gustafson et al., 2012, Solfrizzi et al., 2004, Whitmer et al., 2005). The relative risk of the development of

dementia and AD for obese (BMI $\geq$ 30 kg/m^2) and overweight (BMI= 25–29.9 kg/m^2) individuals in midlife compared to normal weight individuals was 2.04 and 1.64, respectively (Anstey et al., 2011). Epidemiological studies have shown that obesity in middle age increases the risk of developing dementia and AD, irrespective of associated medical conditions such as diabetes or vascular disease (Besser et al., 2014, Gustafson et al., 2012, Solfrizzi et al., 2004, Panza et al., 2010, Whitmer et al., 2005). Whitmer and colleagues reported that being overweight at age 40–45 increased ones risk of developing dementia by 35%, while being obese increased this risk to 74% when compared to normal weight individuals (Whitmer et al., 2005). The link between elderly obesity with dementia and AD is complicated. Several studies have found an age dependent relationship with AD and late-life obesity (Elias et al., 2003, Gustafson et al., 2003, Gustafson et al., 2009), while others have shown no or even negative correlations (Buchman et al., 2005, Fitzpatrick et al., 2009, Luchsinger et al., 2007, Stewart et al., 2005). In a study performed by Buchman *et al.*, declining BMI was associated with an increased incidence of AD. Individuals that experienced weight loss displayed faster clinical progression and individuals with a higher baseline BMI experienced slower clinical progression of AD (Buchman et al., 2005). A possible explanation of the confounding results is that weight loss is strongly associated with AD and occurs before any presentation of cognitive impairment (Buchman et al., 2005, Stewart et al., 2005).

Interestingly, waist circumference, a measure of the accumulation of adipose tissue in the abdomen and the largest depot of adipose tissue in some individuals, was associated with an increased rate of cognitive impairment in non-demented elderly individuals (West and Haan, 2009). However in the same cohort when inspecting the relationship between BMI and cognitive impairment, an inverse relationship was observed (West and Haan, 2009). As suggested previously, BMI may not be the best measure of obesity in humans. In fact a recent

publication examining the BMI of over 40,000 participants in the National Health and Nutrition Examination Survey, suggested that approximately half of overweight and 45% obese individuals were cardiometabolically healthy with no change in blood pressure, triglycerides, cholesterol, blood glucose, insulin resistance and C-reactive protein (Tomiyama et al., 2016). Meanwhile, 30% of individuals with normal BMI were deemed cardiometabolically unhealthy (Tomiyama et al., 2016). Evidence suggests measuring waist circumference or visceral adipose tissue is a better surrogate than BMI. Researchers have also shown that CT-measured visceral adipose tissue was associated with total brain volume independent of BMI and insulin resistance in middle-aged adults (Debette et al., 2010) where lower total brain volume is known to be a powerful predictor of incident dementia (Jack et al., 2005). When we consider the growing population of overweight and obese people worldwide, along with an increasingly aging population, understanding the pathophysiology of obesity on the central nervous system and in particular those brain subregions important in learning, memory and executive function is necessary.

The mechanisms by which obesity influences risk of AD remain to be fully understood. Higher levels of Amyloid beta (Aβ, the main component of amyloid plaques), precursor protein (APP) and tau expression have been reported in hippocampal sections from morbidly obese patients without cognitive impairment, compared to a cohort of non-obese controls (Mrak, 2009). Indeed increased levels of plasma amyloid proteins have been found in a number of studies of obese individuals suggesting a possible mechanism linking midlife obesity with the later development of AD (Jahangiri et al., 2013, Lee et al., 2009).

1.7. Animal models of obesity

Animal models allow for more accurate control of diet and other confounding factors while also being able to study pathology. Animal models can be categorized into either monogenetic, polygenetic, or dietary and the focus of this dissertation, the western diet-induced model of obesity.

1.7.1. Monogenetic models of obesity

Monogenetic models of obesity are described where a single gene has been mutated leading to dysfunction in either a gene or receptor involved in the regulation of energy homeostasis, thus leading to obesity. Generally these models have initially focussed on leptin, a hormone produced in white adipose tissue that can function as a feedback mechanism that suppresses food intake and hence inducing weight loss (Lutz and Woods, 2012).

1.7.1.1. Ob/Ob mice model

A commonly studied mouse model is the *ob/ob* model of obesity in which mice have a spontaneous mutation of the Lep^{ob} gene that inhibits leptin synthesis prematurely (Ingalls et al., 1950). Leptin deficiency leads these mice to display hyperphagia which contributes to severe weight gain, hyperglycaemia, glucose intolerance, hyperinsulinemia and insulin resistance (Drel et al., 2006, Garthwaite et al., 1980, Mayer et al., 1953).

1.7.1.2. Db/Db mice model

The *db/db* mouse resembles the *ob/ob* mouse in phenotype in that it involves leptin dysregulation. However the spontaneous mutation occurs at the receptor site and not the gene. This mutation of the leptin receptor results in a more severe form of hyperglycaemia by

8 weeks of age (Chua et al., 1996, Coleman, 1978, Halaas et al., 1995). Thus this model is mainly used for research on diabetes rather than obesity.

1.7.1.3. Zucker fatty rat model

The leptin deficient Zucker fatty rat is another common monogenetic model of obesity that has an autosomal recessive mutation of the extracellular domain of the leptin receptor in the fatty gene (*fa*) (Bray, 1977, Zucker and Zucker, 1961). This is suggested to cause a processing defect where the receptor is produced, however is retained intracellularly, leading to reduced numbers of leptin receptors on the extracellular surface (Chua et al., 1996). Zucker fatty rats display an obese phenotype at an early stage of life due to hyperphagia and reduced energy expenditure (Cleare et al., 2015). These rats also present with insulin resistance and impaired glucose tolerance, however glucose levels remain normal, resembling the prediabetic condition in humans (Bray, 1977, Zucker and Antoniades, 1972).

1.7.1.4. Other monogenetic models of obesity

Other monogenetic models of obesity include but are not limited to the propiomelanocortin knockout mouse (Huszar et al., 1997, Yaswen et al., 1999), MC4R knockout mouse (Marsh et al., 1999, Ste Marie et al., 2000), agouti related protein overexpression mouse (Graham et al., 1997) and Otsuka Long Evans Tokushima Fatty rat (Kawano et al., 1992, Moran, 2008).

However, we are aware that most cases of obesity are not due to a single gene mutation with the prevalence of leptin receptor mutation in a group of severe, early-onset obese subjects estimated to be approximately 3% (Farooqi et al., 2007). Thus monogenetic models of obesity are not wholly representative of the pathogenesis of human obesity disorder.

1.7.2. Polygenetic models of obesity

Polygenetic models of obesity do not rely solely on a single gene mutation but rather on multiple errors at multiple sites within the genome. These animals become obese through the interaction of multiple genes and environment factors.

1.7.2.1. Obesity prone/obesity resistant rat model

By selectively breeding Sprague Dawley rats that were fed a high-fat diet over many generations until total segregation of obesity-related traits, obesity prone (DIO) and obesity resistant (DR) rats were produced to create the DIO/DR rat model of obesity (Levin et al., 1997). DIO rats are heavier than their DR counterparts, caused primarily by an increase of adipose tissue without the need to expose rats to HFDs (Levin et al., 1997). Furthermore DIO rats are shown to have an increase in plasma triglycerides, insulin and lipids reflecting metabolic syndrome observed in humans (Levin et al., 1997, Madsen et al., 2010, Tkacs and Levin, 2004).

1.7.2.2. Other polygenetic models

Models of obesity have also investigated in other species besides mice and rats including squirrels (Faust and Mrosovsky, 1987), hamsters (Mercer and Tups, 2003), pigs (Clouard et al., 2016, Pond et al., 1985) and primates (Altmann et al., 1993, Schwartz et al., 1993) mainly through the utilization of high fat (HF) feeding. Spontaneous obesity has been known to occur in about 10–15% of rhesus macaque monkeys raised in captivity with aging when maintained on a relatively low fat diet (Schwartz et al., 1993).

1.7.3. Dietary models of obesity

Dietary models of obesity rely on the feeding of diets high in fat and/or carbohydrate content. This dietary manipulation may increase body weight, blood pressure and cholesterol levels, the primary indices of obesity.

1.7.3.1. Cafeteria diet model

Obesity can also be induced in rats when given a choice of various palatable, unhealthy energy-dense food stuffs such as cookies, candy and cakes along with standard chow which is called the cafeteria diet model (Rothwell and Stock, 1979). This intervention promotes voluntary hyperphagia (Martire et al., 2013, Martire et al., 2014), increase of adipose tissue (Cunningham et al., 1983, Llado et al., 1991, Martire et al., 2013, Martire et al., 2014) and glucose and insulin resistance (Cunningham et al., 1983, Rothwell and Stock, 1979).

1.7.3.2. High fat diet-induced model

Like the cafeteria diet, the HFD-induced model of obesity can also induce the obese phenotype in rodents. The term "HFD" is not yet standardised thus studies have used diets with varying levels fats ranging from 30 to 60 kcal% which can result in minor differences in phenotype. Furthermore different sources of fat can also be used that are derived from either animals or plants sources (Buettner et al., 2007). A challenge for this model is that animals may not always display significant weight gain over control diet counterparts (Arvanitidis et al., 2009, Boukouvalas et al., 2008, Francis et al., 2013, Hargrave et al., 2016, Kanoski and Davidson, 2010, Kosari et al., 2012, White et al., 2009a). However most studies using this model do report increases in total fat percentage or indices of such, whilst also promoting hyperglycaemia and whole-body insulin resistance (Ahren et al., 1999, Lingohr et al., 2002, Oakes et al., 1997).

1.7.3.3. Western diet model

The western diet (WD) model of obesity is a subtype of HFD-induced obesity that mimics the so-called 'western' diet by feeding rats a WD chow (containing 22% w/w fat equivalent to 40 kcal% fat) or a control chow diet (containing 6% w/w total fat). The WD was formulated to represent a typical HFD typically consumed in developed 'western' countries. The WD is defined as containing moderately high levels of fat (saturated and trans-fat), simple sugar (sucrose & fructose) and cholesterol and low in essential polyunsaturated fatty acids (Cordain et al., 2005) . We use the nomenclature for this diet, 'WD or Western diet,' as it is used in our published work and throughout the literature (e.g. (Sobey et al., 2015, Argueta and DiPatrizio, 2017, Johnson et al., 2017), and designates to other researchers the type of diet used, while also delineating it from other types of high fat diets, which typically range in fat content from 30-60%. The WD used in this thesis was formulated to be equivalent to the *Harlan Teklad* TD88137 or *Research Diets* Western Diet D12079B that have previously been used to accelerate and enhance hypercholesterolemia and atherosclerotic plaque formation (Ascencio et al., 2004, Febbraio et al., 2000, Kirk et al., 1998, Plump et al., 1992, Yang et al., 2006). This diet has been shown to induce hyperglycaemia (Briaud et al., 2002, Taouis et al., 2002), hypercholesterolemia (Briaud et al., 2002), hyperinsulinemia (Briaud et al., 2002), and glucose and insulin resistance (Chalkley et al., 2002, Gustafson et al., 2002).

1.7.4.Juvenile animal models of obesity

Well characterized animal models for juvenile obesity are generally lacking and most are focused either in utero or post-weaning exposure to HFDs. Monogenetic models of obesity can also be included in this category provided that animals are assessed immediately upon the conclusion of weaning. Relative to conventional animal models of obesity, few studies have

investigated cognitive impairments in juvenile animals and have focused on the mechanisms and consequences of juvenile obesity.

1.7.4.1. In utero HFD exposure model of obesity

Fetal development is dependent on maternal supply of nutrients and as such perturbation of maternal metabolism may predispose offspring to metabolic disease in later life. Oversupply of nutrients during critical periods of fetal development causes an impairment of hypothalamic development which can induce long-lasting metabolic effects (Metges, 2009). This model involves rats being fed a HFD during pregnancy and/or lactation that results in the obese phenotype in the offspring. These offspring have elevated levels of glucose, triglycerides, insulin and adiposity (Armitage et al., 2005, Srinivasan et al., 2006, White et al., 2009b).

1.7.4.2. Neonatal overfeeding model of obesity

Excess nutrition during the critical neonatal period can lead to weight gain throughout the juvenile period and can persist into adulthood (Bulfin et al., 2011, Plagemann, 2006, Sobesky et al., 2014, Spencer and Tilbrook, 2009). By reducing litter size during the weaning period, competition for food is reduced and thus pups are overfed which can predispose rats to develop obesity. Neonatal overfeeding has been shown to cause elevated insulin (Habbout et al., 2013), glucose (Plagemann et al., 1999), triglycerides (Plagemann et al., 1999) and cholesterol levels (Boullu-Ciocca et al., 2005, Spencer, 2012, Stefanidis and Spencer, 2012).

1.7.4.3. Post weaning HFD obesity model

The post-weaning obesity model is similar to HFD-induced animal model except that feeding begins after the cessation of weaning. Post-weaning HFD feeding can amplify the obese phenotype by increasing weight, leptin (Marco et al., 2013), blood pressure (Torrens et al.,

2012), cholesterol (dos Santos Perez et al., 2015, King et al., 2014) and promote glucose-insulin dyshomeostasis (King et al., 2014).

1.8. Cognitive impairment in rat models of obesity

Multiple studies have investigated cognitive impairment in several rat models of obesity (**Table 1.2**). As of April 2017, 49 articles were identified through the Medline electronic database using the terms related to obesity (i.e. obesity, high fat, adiposity) and cognition (i.e. cognition, cognitive domains, executive function, memory). The search was limited to studies performed in rats and were published in English. These studies focussed on various cognitive domains including spatial memory, motivation, working memory and behavioural flexibility with multiple cognitive impairments being identified.

Table 1.2: Adult animal models of obesity studies investigating cognitive function

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Alzoubi et al., 2013b)	Wistar rats	5 w/w%	25 w/w%	6 weeks	RAWM	Spatial memory	Increased errors in 30 min, 5 h and 24 h probe test.
(Alzoubi et al., 2013a)	Wistar rats	12.1 w/w%	47.7 w/w%	12 weeks	RAWM	Spatial memory	Delayed acquisition of RAWM task and more errors in 30 min, 5h, and 24 h probe test
(Asadbegi et al., 2017)	Wistar rats	5 w/w%	20 w/w%	8 weeks	MWM, passive avoidance task	Spatial memory, Pavlovian conditioning	No reported difference in MWM. Impairment of memory indicated by increased latency to enter and time spent dark compartment 24 hrs after acquisition of passive avoidance task.
(Beilharz et al., 2014)	Sprague Dawley rats	15 kcal%	Cafeteria diet, 45 kcal%	4 weeks	NOIP, NORT	Spatial memory, working memory	Decrease in exploratory ratio in the NOIP task indicating spatial memory deficit but no difference in working memory.
(Beilharz et al., 2016)	Sprague Dawley rats	15 kcal%	Cafeteria diet, 45 kcal%	1 week	NOIP, NORT	Spatial memory, working memory	Decrease in exploratory ratio in the NOIP task indicating spatial memory deficit but no difference in working memory.
(Bocarsly et al., 2015)	Sprague Dawley rats	10 kcal%	45 kcal%	8 weeks	NORT, NOIL, ASST	Working memory, spatial memory, behavioural flexibility	Working memory deficit indicated by reduced discrimination ratio in the NORT. Impaired performance in the NOIL test indicating spatial memory deficit. Impaired behavioural flexibility with impaired performance in the ASST.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Chen et al., 2017)	Sprague Dawley rats	10 kcal%	60 kcal%	4 weeks	MWM with probe test	Spatial memory	Increased latency to find platform. Spent less time in target zone in probe test.
(Davidson et al., 2012)	Sprague Dawley rats	12 kcal%	40 kcal%	4 weeks	Discrimination training	Pavlovian conditioning	HFD impairs performance on a hippocampal-dependent serial feature negative discrimination problem.
(Davidson et al., 2013)	DIO/DR rats	12 kcal%	40 kcal%	12 weeks	Discrimination training	Pavlovian conditioning	HFD impairs performance on a hippocampal-dependent serial feature negative discrimination problem.
(Davis et al., 2008)	Long-Evans rats	14 kcal%	38 kcal%	12 weeks	Instrumental conditioning, breakpoint task	Motivation	Impaired acquisition of instrumental conditioning. Lower motivational breakpoint for sucrose reward.
(Figlewicz et al., 2006)	Sprague Dawley rats	12 kcal%	32 kcal%	5 weeks	Instrumental conditioning	Motivation	Enhanced acquisition for sucrose reward.
(Figlewicz et al., 2013)	Albino rats	13 kcal%	31.8 kcal%	5-8 weeks	Instrumental conditioning, breakpoint task	Motivation	Enhanced acquisition. Higher breakpoint to work for sucrose reward.
(Francis et al., 2013)	Sprague Dawley rats	13 kcal%	39 kcal%	8 weeks	NORT, MWM with 5 day spatial reversal, instrumental conditioning	Working memory, spatial memory, behavioural flexibility, motivation	No change in NORT or MWM acquisition. Impaired spatial reversal learning. Decreased lever press response for sucrose reward.
(Fu et al., 2017)	Sprague Dawley rats	12 kcal%	60 kcal5	24 weeks	NORT, spontaneous alternation in the Y-maze	Working memory, spatial memory	Working memory deficit indicated by reduced discrimination ratio in the NORT. Spontaneous alternation behaviour was impaired.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Gergerlioglu et al., 2016)	Wistar rats	Not reported	35 kcal%	4 weeks	MWM with probe test	Spatial memory	Increased latency to find platform. In probe test, spent less time in target quadrant. Results were independent from weight change.
(Glass et al., 1999)	Zucker fatty rat	n/a	n/a	n/a	Breakpoint task	Motivation	Higher breakpoint for grain reward but not for sucrose reward.
(Greenwood and Winocur, 1996)	Long-Evans rats	4.5 w/w%	20 w/w%	12 weeks	VIDA	Behavioural flexibility	Impaired acquisition of response alternation rules.
(Greenwood and Winocur, 2001)	Long-Evans rats	4.5 w/w%	20 w/w%	12 weeks	VIDA	Behavioural flexibility	Impaired acquisition of response alternation rules.
(Greenwood et al., 1974)	Zucker fatty rat	n/a	n/a	n/a	Instrumental conditioning	Motivation	Increased lever press responses for grain reward.
(Gurung et al., 2016)	DIO/DR rats	n/a	n/a	n/a	MWM with probe test	Spatial memory	No acquisition difference. In probe test, spent less time in target quadrant.
(Hargrave et al., 2016)	DIO/DR	18 kcal%	38 kcal%	1.5-12 weeks	Spontaneous alternation in the Y-maze	Spatial memory	Spontaneous alternation behaviour was impaired at 10 days but not at 40 or 90 days and was independent of weight change.
(Hoane et al., 2011)	Sprague Dawley rats	10 kcal%	45 kcal%	8 weeks	MWM with 15 min spatial reversal	Spatial memory and behavioural flexibility	No acquisition difference. Impaired spatial reversal learning.
(Jen, 1980)	Sprague Dawley rats	Not reported	40 w/w%	12 weeks	Active avoidance task, instrumental conditioning	Pavlovian conditioning, motivation	No reported change in fear conditioning or instrumental conditioning.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Kanoski and Davidson, 2010)	Sprague Dawley rats	13 kcal%	40 kcal%	12 weeks	RAM	Spatial memory	Increased errors after 72 h of diet commencement. After 64 days of HFD and then following 24 h food deprivation, HFD rats made more errors.
(Kanoski et al., 2007)	Sprague Dawley rats	12 kcal%	40 kcal%	12 weeks	Discrimination training with reversal	Behavioural flexibility	No acquisition difference. Impaired reversal learning.
Kanoski et al., 2010 (Kanoski et al., 2010)	Sprague Dawley rats	13 kcal%	40 kcal%	12 weeks	Discrimination training	Pavlovian conditioning	HFD impairs performance on a hippocampal-dependent serial feature negative discrimination problem.
(Komaki et al., 2015)	Wistar rats	Not reported	60.9 kcal%	25 weeks	Passive avoidance task	Pavlovian conditioning	Acquisition of task was delayed. Impairment of memory indicated by increased latency to enter and time spent dark compartment.
(Kosari et al., 2012)	Long-Evans rats	7 w/w%	21 & 60 w/w%	12 weeks	NORT, Y-maze	Working memory, spatial memory	No difference in NORT. Impairment of spatial memory in Y-maze test independent of body weight
(la Fleur et al., 2007)	Wistar rats	Not reported	Not reported	4 weeks	Instrumental conditioning, breakpoint task	Motivation	Impaired instrumental conditioning acquisition. Elevated breakpoint for sucrose pellet.
(Ledreux et al., 2016)	Fisher 344 rats	13 kcal%	36 kcal%	24 weeks	RAWM	Spatial memory	No acquisition differences. Increased errors.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(McNeilly et al., 2011)	Wistar rats	7.4 kcal%	45 kcal%	12 weeks	DMTP, DNMTP, progressive ratio, MWM	Behavioural flexibility, motivation, spatial memory	In DMTP task, lowered number of correct lever presses. In DNMTP task, less accuracy with increased incorrect lever presses. No difference in progressive ratio task or MWM.
(McNeilly et al., 2012)	Wistar rats	7.4 kcal%	45 kcal%	12 weeks	DMTP, DNMTP	Behavioural flexibility	In DMTP task, lower accuracy and total number of lever presses. In DNMTP task, less accuracy with decreased number of total lever presses.
(Molteni et al., 2002)	Fisher 344 rats	13 kcal%	39 kcal%	4-8 weeks	MWM	Spatial memory	At 4 weeks, increased latency to find platform.
(Osborne et al., 2016)	Sprague Dawley rats	13 kcal%	60 kcal%	24 weeks	Contextual fear conditioning	Pavlovian conditioning	Decreased freezing time.
(Pancani et al., 2013)	F344/NIA rats	13 kcal%	42 kcal%	23 weeks	MWM	Spatial memory	No reported difference.
(Pathan et al., 2008)	Sprague Dawley rats	Not reported	58 kcal%	5 weeks	MWM	Spatial memory	Increased latency to find platform.
(Pintana et al., 2016)	Wistar rats	19.8 kcal%	59.3 kcal%	4-12 weeks	MWM with probe test	Spatial memory	At 12 weeks, increased latency to find platform. In probe test, spent less time in target quadrant.
(Pratchayasakul et al., 2015)	Wistar rats	19.7 kcal%	59.3 kcal%	12 weeks	MWM	Spatial memory	At 12 weeks, impaired spatial memory indicated by increased time to find the hidden platform.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Reichelt et al., 2015)	Sprague Dawley rats	12 kcal%	Cafeteria diet, 32 kcal%	8 weeks	Trace fear conditioning	Pavlovian conditioning	Cafeteria rats froze less in context associated with shock indicating encoding impairment.
(Rodriguez-Perdigon et al., 2016)	Wistar rats	13 kcal%	60 kcal%	8 weeks	NORT	Working memory	Lower discrimination ratio indicating impaired working memory.
(Sobesky et al., 2014)	Wistar rats	17 kcal%	60 kcal%	12-24 weeks	Contextual fear conditioning	Pavlovian conditioning	Less freezing after 12 weeks and persistent to 20 weeks of feeding indicating impairment of memory acquisition.
(Stranahan et al., 2008)	Sprague Dawley rats	Not reported	Not reported	24 weeks	MWM	Spatial memory	Increased latency to find platform.
(Tracy et al., 2015)	Long-Evans rats	14 kcal%	40 kcal%	10 weeks	Breakpoint task, CPP task	Motivation	At 6 weeks, decreased breakpoint for sucrose reward. No CPP for sucrose reward observed for HFD rats.
(Vollbrecht et al., 2015)	DIO/DR rats	4.5 w/w%	19.6 w/w%	4 weeks	Instrumental conditioning, breakpoint task	Motivation	More lever press response in instrumental conditioning. Higher breakpoint for grain pellet.
(Winocur and Greenwood, 1999)	Long-Evans rats	4.5 w/w%	20 w/w%	12 weeks	VIDA	Behavioural flexibility	Impaired acquisition of response alternation rules.
(Wang et al., 2016)	Sprague Dawley rats	12 kcal%	40 kcal%	16 weeks	MWM with probe test, NORT	Spatial memory, working memory	Impaired spatial memory indicated by increased latency to escape. In probe test, spent less time in target quadrant. Working memory deficit indicated by reduced discrimination ratio in the NORT.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Woo et al., 2013)	Sprague Dawley rats	Not reported	45 w/w%	13 weeks	MWM	Spatial memory	Increased latency to find platform.
(Woodie and Blythe, 2017)	Sprague Dawley rats	17.1 kcal%	60 kcal%	8 weeks	MWM with probe and reversal tests	Spatial memory, behavioural flexibility	No reported differences.
(Wu et al., 2004)	Sprague Dawley rats	13 kcal%	40 kcal%	8 weeks	MWM with probe test	Spatial memory	Increased latency to find platform. Spent less time in target zone in probe test.

ASST= Attention set shifting task, CPP= Conditioned place preference, DMTP= Delayed matching to position task, DNMTP= Delayed non-matching to position task, HFD= High fat diet, MWM= Morris water maze, NOIL= Novel object in location, NOIP= Novel object in place, RAM= Radial arm maze, RAWM= Radial arm water maze, VIDA= Variable-interval, delayed alternation task.

1.8.1. Spatial learning and memory

Spatial memory is the ability to learn the geographical configuration of environments, to locate objects, to recall previously encountered locations, and to navigate within environments. Multiple lesion studies have shown that the HPC plays an integral role in spatial learning and memory tasks (Broadbent et al., 2004, Morris et al., 1982) by combining spatial information and providing a representation of an animals' current location and heading (Hartley et al., 2014). As of April 2017, of the total 27 studies, HFD exposure was shown not to influence spatial learning in five (Francis et al., 2013, Hoane et al., 2011, Pancani et al., 2013, McNeilly et al., 2011, Woodie and Blythe, 2017). Overall several different behavioural tasks have been used to evaluate spatial learning and memory such as the Y-maze, radial arm water maze (RAWM), novel object in place (NOIP) and novel object in location (NOIL) tasks. By using these tasks, studies have consistently shown that HFD exposure impairs spatial memory and learning (Alzoubi et al., 2013b, Alzoubi et al., 2013a, Beilharz et al., 2014, Beilharz et al., 2016, Bocarsly et al., 2015, Hargrave et al., 2016, Kanoski and Davidson, 2010, Ledreux et al., 2016). Some have even reported spatial memory and learning deficits independent of any increase of body weight compared to control counterparts (Beilharz et al., 2016, Bocarsly et al., 2015, Gergerlioglu et al., 2016, Hargrave et al., 2016) including our study using the Y-maze (Kosari et al., 2012).

Fourteen of HFD studies investigating spatial learning and memory have used the Morris water maze (MWM) behavioural task. The MWM task involves an animal being placed in a circular pool of opaque water from which they progressive learn to escape by finding a hidden platform placed just beneath the surface of the water using surrounding extra-maze spatial cues (Morris, 1984). Generally, these studies differed with respect to fat content (39-60 kcal %) and duration of diet exposure (4-24 weeks); however the results were relatively

consistent. HFD fed rats took longer than control rats to find the hidden platform indicating HFD exposure has a detrimental effect on spatial learning (Asadbegi et al., 2017, Chen et al., 2017, Gergerlioglu et al., 2016, Gurung et al., 2016, Molteni et al., 2002, Pathan et al., 2008, Pratchayasakul et al., 2015, Pintana et al., 2016, Stranahan et al., 2008, Wang et al., 2016, Woo et al., 2013, Wu et al., 2004). To summarise most studies have reported that HFD exposure, using a range of fat content and length of exposure, may have a deleterious effect on spatial learning and memory, and this effect consequently may be mediated by damage to the HPC.

1.8.2. Motivation

Motivation is defined as the process that initiates, guides, and maintains goal-directed behaviours. As of April 2017, a total of ten studies investigated motivational differences in rat models of obesity with most using instrumental conditioning and a subsequent breakpoint task. These tests involve animals being trained in an operant box to press a lever to obtain a food reward, while the subsequent breakpoint task involves pressing a lever for a food reward and as the test progresses, more lever presses are required to obtain the reward.

Three studies reported no impairment of the acquisition in instrumental conditioning (Glass et al., 1999, Jen, 1980, Tracy et al., 2015), while two studies reported a deficit in obese rats (Francis et al., 2013, la Fleur et al., 2007) and three stated excessive lever press response (Figlewicz et al., 2006, Figlewicz et al., 2013, Vollbrecht et al., 2015). Using the breakpoint task, three studies showed that motivational drive to work for a sucrose reward was higher in obese rats, indicated by an elevated breakpoint compared to control (Figlewicz et al., 2006, Figlewicz et al., 2013, la Fleur et al., 2007). For example Figlewicz *et al.*, used a 31.8 kcal% HFD for a period of 5-8 weeks and showed that HFD rats had more lever presses (104 ± 10 vs. 175 ± 25) and obtained more sucrose rewards (8.5 ± 0.4 vs. 10 ± 0.4) compared to control

(Figlewicz et al., 2013). Conversely, three studies reported the opposite outcome with an observed decreased motivational breakpoint for a sucrose reward in 12 week HFD fed obese rats (Davis et al., 2008, Francis et al., 2013, Tracy et al., 2015).

Other studies have indicated that HFD exposure in Sprague-Dawley rats of either 40 w/w% HFD for 12 weeks (Jen, 1980) and Long-Evans rats fed 50 kcal% fat for 10 weeks did not affect acquisition of instrumental conditioning (Tracy et al., 2015). The conflicting literature in conjunction with our own results suggests that HFD consumption singlehandedly does not influence the acquisition of instrumental conditioning and there may be an underlying factor involved that has not yet been identified. The above studies show conflicting results on the effect of HFD exposure and motivation. As such no conclusion can be attained from the current research.

1.8.3. Pavlovian conditioning

Pavlovian conditioning, also known as classical conditioning is considered a reflexive/non-conscious type of motivational learning process (Pavlov, 1927). Pavlovian conditioning is accomplished by pairing a reward (appetitive) or punishment (aversive) with a neutral context (CS, conditioned stimulus) such that the CS alone elicits conditioned response, such as in behavioural tests including discrimination training, fear conditioning and passive avoidance task.

As of the April 2017, a total of ten studies observed both aversive and appetitive Pavlovian conditioning in rat models of obesity with a majority using a discrimination training task (Asadbegi et al., 2017, Davidson et al., 2013, Davidson et al., 2012, Jen, 1980, Kanoski et al., 2007, Kanoski et al., 2010, Komaki et al., 2015, Osborne et al., 2016, Reichelt et al., 2015, Sobesky et al., 2014). The results for aversive Pavlovian conditioning is fairly consistent with four studies showing that the encoding of an aversive memory in obese rats is impaired

denoted by a decrease of freezing once re-exposed to fear conditioning context environment (Osborne et al., 2016, Reichelt et al., 2015, Sobesky et al., 2014) and a lower latency to escape the fear conditioning environment in the passive avoidance task (Komaki et al., 2015).

DIO/DR and Sprague Dawley rats fed a 40 kcal% HFD for 4 weeks underwent both serial feature negative and feature positive Pavlovian appetitive discrimination training (Kanoski et al., 2010). It was observed that both DIO and Sprague Dawley 4 week HFD exposed rats increased their responding to cues that predict non-rewarded trials in the feature negative discrimination task compared to controls demonstrating that obese rats were not able to discriminate between cues that predicted rewarded trials and cues that predicted non-rewarded trials (Davidson et al., 2012). This impairment of learning was also shown to be persistent in DIO rats fed a HFD for 12 weeks (Davidson et al., 2013, Kanoski et al., 2010). In summary, evidence presented above indicates that HFD feeding impairs both aversive and selective appetitive Pavlovian conditioning paradigms.

1.8.4. Working memory

The novel object recognition task (NORT) involves rats exploring two identical objects, then after an inter-trial interval (ITI), a novel and familiar object is presented (Ennaceur and Delacour, 1988). Rats with intact working memory are able to discriminate the novel object and consequently will spend more time interacting with it.

A total of seven studies have scrutinized working memory using the NORT where only three studies observed impairment in working memory (Bocarsly et al., 2015, Fu et al., 2017, Rodriguez-Perdigon et al., 2016). Four studies show no effect of HFD exposure on working memory as rats were able to easily discriminate between novel and familiar objects using a range of fat content and length of exposure (Beilharz et al., 2014, Beilharz et al., 2016, Francis et al., 2013, Kosari et al., 2012). Although the aforementioned HFD studies on

working memory do not converge to give a clear picture, evidence suggests that HFD does not affect working memory in rats.

1.8.5. Behavioural flexibility

Behavioural flexibility refers to an animal's ability to make adaptive changes in their behaviour in response to changes in environment. A common element within behavioural flexibility tests is that behaviour is adaptive where tasks require attention shifting, rule switching or reversal learning. Nine studies investigated behavioural flexibility in HFD exposed rats. All nine studies indicate that there is a deficit of behavioural flexibility after the feeding of a HFD using attention set shifting (Bocarsly et al., 2015), VIDA (Greenwood and Winocur, 1996, Greenwood and Winocur, 2001, Winocur and Greenwood, 1999), DMTP/DNMTP (McNeilly et al., 2011, McNeilly et al., 2012), MWM with probe reversal (Hoane et al., 2011) and discrimination reversal tasks (Kanoski et al., 2007). The VIDA task involves rats inhibiting competing responses to obtain a reward. Greenwood and Winocur demonstrated that male rats fed a diet containing 20 w/w% fat for 3 months displayed a decreased rewarded/non-rewarded ratio, indicating a deficit in behavioural flexibility (Greenwood and Winocur, 1996, Greenwood and Winocur, 2001, Winocur and Greenwood, 1999). The discrimination reversal task is a type of Pavlovian appetitive conditioning that involves the adaptation of behaviour according to changes in stimulus–reward contingencies. HFD exposed rats were able to initially discriminate between the rewarded and non-reward CS's, however once the stimulus-reward contingencies were reversed they were unable to differentiate between the CS's displaying similar response rates (Kanoski et al., 2007). Overall, research suggests that exposure to HFD in adult models of obesity impairs behavioural flexibility.

1.9. Cognitive function in juvenile rat obesity models

Articles were identified through the Medline electronic database using the terms related to obesity (i.e. obesity, high fat, adiposity), cognition (i.e. cognition, cognitive domains, executive function, memory) and adolescence (i.e. adolescence, juvenile). The search was limited to studies of rats and published in English. Twelve studies examined cognitive function in juvenile obesity animal models compared to adult models of obesity using a HFD ranging from 39-60 kcal% mainly focussing on spatial memory (**Table 1.3**).

Table 1.3: Juvenile obesity animal model studies investigating cognitive function

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Boitard et al., 2014)	Wistar rats	2.5 w/w%	24 w/w%	8-12 weeks	MWM with probe and spatial reversal	Spatial memory	Spent less time in target zone in a 4 day but not 2h probe test. Delayed spatial reversal learning.
(Boitard et al., 2015)	Wistar rats	2.5 w/w%	24 w/w%	16 weeks	Conditioned odour aversion, auditory fear conditioning	Pavlovian conditioning	Juvenile but not adult HFD exposure enhanced conditioned odour aversion and fear memory.
(Boitard et al., 2016)	Wistar rats	2.5 w/w%	24 w/w%	24 weeks	MWM with probe test	Spatial memory	No reported difference
(Boukouvalas et al., 2008)	Wistar rats	10 kcal%	45 kcal%	3 weeks	MWM with probe test	Spatial memory	No reported difference.
(Goldbart et al., 2006)	Sprague Dawley rats	13 kcal%	40 kcal%	8 weeks	MWM	Spatial memory	Increased latency to find platform.
(Lepinay et al., 2015)	Wistar rats	12 kcal%	39 kcal%	16 weeks	MWM with probe test	Spatial memory	No acquisition differences. HF offspring with HF dams spent less time in target zone in probe test.
(Marwitz et al., 2015)	Sprague Dawley rats	13 kcal%	41 kcal%	9 weeks	NORT, ASST	Working memory, behavioural flexibility	Working memory deficit indicated by reduced discrimination ratio in the NORT. No reported difference in ASST.
(Murray et al., 2009)	Wistar rats	7.5 w/w%	55 w/w%	1.5 weeks	Random foraging task in RAM	Working memory	Fewer correct arm entries indicating working memory deficit.

Author, year	Species	Control diet (fat content)	HFD (fat content)	Diet length	Behavioural test	Type of behaviour tested	Reported outcomes
(Page et al., 2014)	Sprague Dawley rats	10 kcal%	45 kcal%	12 weeks	MWM with probe test	Spatial memory	CON & HF offspring from HF dams had increased latency to find platform. CON & HF offspring from HF dams spent less time in target zone in probe test.
(Underwood and Thompson, 2016b)	Long-Evans rats	14 kcal%	58 kcal%	12 weeks	Spontaneous alternation in 4 armed RAM	Spatial memory	Reduction of spontaneous alternation behaviour in both males and females.
(Underwood and Thompson, 2016a)	Long-Evans rats	14 kcal%	58 kcal%	12-15 weeks	NOIP	Spatial memory	Decrease in exploratory ratio in the NOIP task indicating spatial memory deficit in both males and females.
(White et al., 2009a)	Long-Evans	10 kcal%	60 kcal%	20 weeks	MWM	Spatial memory	No acquisition differences. Task retention impaired in HF offspring from HF dams.

ASST= Attention set shifting task, COn= control, HF= High fat, HFD= High fat diet, MWM= Morris water maze, NOIP= Novel object in place, NORT= Novel object recognition, RAM= Radial arm maze.

1.9.1. Relationship between juvenile rat models of obesity and cognitive impairments.

Evidence suggests that cognitive impairments are observed in several different juvenile rat models of obesity. Specifically, obese juvenile rats have a spatial learning and memory impairment in the *in utero* HFD exposure (Page et al., 2014, White et al., 2009a) and post-weaning HFD juvenile animal models of obesity (Boitard et al., 2014, Goldbart et al., 2006, Lepinay et al., 2015, White et al., 2009a). As of April 2017, nine studies have studied spatial learning and memory in juvenile models of obesity with the majority of these studies using the MWM task. Obese juvenile rats took longer to find the hidden platform in the MWM (Goldbart et al., 2006, Page et al., 2014) and two studies reported these rats spent less time in the target zone signifying a spatial reference impairments in the MWM probe test (Boitard et al., 2014, Lepinay et al., 2015). Underwood and Thompson extended these findings by revealing that both males and female juvenile obese rats display a spatial memory impairment using a spontaneous alternation task and spatial object recognition task (Underwood and Thompson, 2016b, Underwood and Thompson, 2016a). Two studies reported no such impairment with one study feeding rats a HFD for 3 weeks (Boukouvalas et al., 2008) and the other for 24 weeks.

There has only been one study investigating Pavlovian conditioning that used a post-weaning model of obesity. Boitard *et al.* indicate that 24 w/w% feeding after weaning enhances conditioned odour aversion and conditioned fear memory after 12 weeks but was not apparent after 6 weeks of diet exposure (Boitard et al., 2015).

Two studies reported that juvenile obesity had a detrimental effect on working memory performance (Marwitz et al., 2015, Murray et al., 2009). The working memory deficit was observed as early as 1.5 weeks after HFD exposure (Murray et al., 2009) as well as 9 weeks after exposure (Marwitz et al., 2015). In summary, juvenile obese rats have a deficit of spatial

learning and memory with some indication that Pavlovian conditioning and working memory are also impaired.

1.10. Animal high fat diet models of obesity and Alzheimer's disease

A number of experimental studies have examined markers of AD-related pathology in normal rodents receiving diets high in fat. Mice receiving a HFD had increased expression of APP and APP processing enzyme (Puig et al., 2012, Thirumangalakudi et al., 2008) along with tau phosphorylation (Koga et al., 2014). Moreover in rats fed a HFD followed by streptozotocin injection to induce a model of type 2 diabetes, hippocampal APP-cleaving enzyme and Aβ were found to be present, and raised compared to controls (Zhang et al., 2009).

Similarly, diet-induced obesity has been shown to increase amyloid and tau pathology in transgenic mouse models of AD. In the double-mutant presenilin (PS)-APP model 7 weeks of diet modification resulted in both hypercholesterolemia and significantly increased levels of Aβ peptides in the brain that were strongly correlated with the levels of both plasma and brain total cholesterol (Refolo et al., 2000). Meanwhile, a much longer dietary intervention of 10 month consumption of a HFD (35 kcal%) formula to the triple transgenic (3xTg-AD) mice increased tau, Aβ 40 and 42 levels, suggesting that HFD consumption promotes AD-like neuropathology (Julien et al., 2010).

It is evident that there is a deleterious effect of obesity/HF feeding on cognitive performance. In human clinical studies, obesity has been shown to increase the risk of the development of dementia and AD. Genetic and diet-induced models of obesity further support this link with obese animals displaying deficits in working memory, learning, and memory performance. The exact mechanisms or mediators that underlie the connections between obesity and the risk of cognitive impairment are still unknown but potential avenues of further research

include brain atrophy, disruption in cerebrovascular function, development of AD related pathology, and blood brain barrier (BBB) breakdown.

1.11. Central pathological changes associated with obesity

The negative systemic effects of obesity on cardiovascular and metabolic physiology are well recognised, and it is now clear that the brain is also negatively affected by obesity. Alterations in brain pathology of overweight/obese individuals who are otherwise healthy are supported by preclinical studies, demonstrating the possible underlying mechanisms by which obesity in aging impair higher cerebral function remains wide and varied.

1.11.1. Brain atrophy

Increased adiposity has been correlated with reduced volume in a number of brain regions. In a longitudinal study in a group of female patients born between 1908 and 1922, women with atrophy of the temporal lobe were found to have a higher BMI, with risk of temporal atrophy increased 13-16% per 1 kg/m^2 BMI rise (Gustafson et al., 2004). More recent brain scanning techniques demonstrated that a group of obese individuals (BMI average 39 ± 4.7 kg/m^2) had significantly lower grey matter density in the post-central gyrus, frontal lobe, putamen, and middle frontal gyrus compared to a group of controls with a BMI of 22 ± 2.2 kg/m^2 (Pannacciulli et al., 2006). A further analysis in over 1400 Japanese healthy individuals revealed a significant negative correlation in men, though not in women, between BMI and brain grey matter ratio with temporal, occipital and frontal lobes and the anterior lobe of the cerebellum showing reduced volume with increased BMI (Taki et al., 2008).

The hippocampal formation, a structure essential for learning and memory, is particularly susceptible to aging (Jack et al., 2000, Raji et al., 2009). It is also well recognized that reduced hippocampal volumes predict cognitive decline and dementia in the general

population (Amieva et al., 2005, den Heijer et al., 2010, Elias et al., 2000). As we described previously, a majority of studies have found that obesity in mid-life is associated with an increased risk of developing dementia in later life, and consistent with this there is evidence from the Framingham Offspring Cohort Study of increased rate of hippocampal brain atrophy and executive function decline with mid-life obesity (Debette et al., 2011). This effect of obesity on hippocampal functioning is also found earlier: adolescents with metabolic syndrome showed significantly lower attention and mental flexibility along with smaller hippocampal volumes compared to non-obese children of similar ages (Yau et al., 2012).

Pre-clinical experimental rodent studies have also provided insight into the potential mechanisms underpinning obesity-related cognitive impairment. A number of researchers have reported a reduction in synaptic plasticity in the HPC and cerebral cortex after feeding rodents a diet high in fat (Molteni et al., 2002, Stranahan et al., 2008, Wu et al., 2003), while there is also evidence of neuronal apoptosis and gliosis (Rivera et al., 2013) and a reduction in hippocampal weight (Calvo-Ochoa et al., 2014).

1.11.2. Cerebrovascular

Increasing evidence suggests that the vascular effects of obesity have a key role in the development of vascular cognitive impairment in aged people (Gorelick et al., 2011) by promotion of atherosclerosis in large cerebral arteries and alterations at the level of the cerebral microcirculation (Zlokovic, 2011). Indeed in a recent rodent study, mice fed a HFD displayed disruptions in cerebral vascular function including neurovascular coupling and functioning of arteries (Li et al., 2013, Lynch et al., 2013). Moreover, aging exacerbated obesity-induced decline in microvascular density in the HPC and cerebral cortex which was positively correlated with hippocampal-related cognitive function. Aging also exacerbated the

obesity-induced oxidative stress and impaired cerebral blood flow indicating the possible effects of both aging and obesity and brain vascular integrity (Tucsek et al., 2014b).

1.11.3. Alzheimer's disease related pathology

As stated previously, amyloid plaques and neurofibrillary tangles containing tau protein are the pathological markers of AD (Serrano-Pozo et al., 2011), accompanied by microglia activation and astrogliosis (Beach et al., 1989, Itagaki et al., 1989), in cortical regions and HPC (1997). The mechanisms by which obesity influences risk of AD remain to be fully understood. Higher levels of APP and tau expression were reported in hippocampal sections from morbidly obese patients without cognitive impairment, compared to a cohort of non-obese controls (Mrak, 2009). Indeed increased levels of plasma amyloid proteins have been found in a number of studies of obese individuals (Jahangiri et al., 2013, Lee et al., 2009) suggesting a possible mechanism linking midlife obesity with the later development of AD.

1.11.4. Blood brain barrier

The chemical consequences of a HFD may also influence the brain by disrupting the integrity of the BBB, which has an important role in maintaining a precisely regulated microenvironment within the brain for reliable neuronal signalling (Ballabh et al., 2004). BBB dysfunction is associated with both AD and vascular dementia (Skoog et al., 1998), and can be related to clinical vascular factors (Blennow et al., 1990). In a longitudinal study being overweight or obese in mid-life was correlated with lower BBB integrity almost a quarter of a century later (Gustafson et al., 2007). Further evidence is available from animal studies: rats fed a WD for three months were shown to have a decrease in expression of tight junction proteins in the choroid plexus and BBB (Kanoski et al., 2010). Moreover, WD consumption in rats produces, as a consequence of this BBB dysfunction, an increased permeability of peripheral fluorescent tracer in the HPC (Davidson et al., 2012, Kanoski et al., 2010).

Reduced BBB integrity and increased microgliosis was also observed in the HPC of rats fed a high-saturated-fat and cholesterol diet for 6 months (Freeman and Granholm, 2012) demonstrating that the HPC appears to be particularly vulnerable to diet-induced BBB disruption.

Further support for the relationship between obesity and degeneration of the BBB suggest that high circulating levels of fat impairs active transport of consummatory regulatory hormones such as leptin and ghrelin through the BBB (Banks et al., 2004, Banks et al., 2008), perhaps inhibiting their positive roles in synaptic plasticity via actions in the HPC (Diano et al., 2006, Shanley et al., 2001). Of course it should also be considered that obesity leads to increased circulatory inflammatory markers which in turn gain access to the hypothalamus by increasing BBB permeability and/or via areas that lack an effective BBB.

1.11.5. Systemic and central inflammation

In obesity there is an accumulation of white adipose tissue which is the key site facilitating systemic inflammation (Odegaard and Chawla, 2013). Particularly, both hypertrophied adipocytes and adipose tissue-resident immune cells (primarily lymphocytes and macrophages) contribute to increased circulating levels of proinflammatory cytokines where there is an increase of tumour necrosis factor (TNF)-α, important feeding-related peptides such as leptin and resistin, plasminogen activator inhibitor 1, C-reactive protein and interleukins (IL)-1β and IL-6 in obese individuals (Ouchi et al., 2011, Yudkin et al., 1999, Visser et al., 1999). Higher waist circumference and waist-hip ratio also showed higher C-reactive protein and IL-6 concentrations, with IL-6 positively associated with total body fat (Hermsdorff et al., 2011) suggesting that these measures may be more highly correlated to inflammatory markers than increases in BMI (Hermsdorff et al., 2011, Thewissen et al., 2011). A cross-sectional study of obese women found that T-cell derived cytokines (IL-23

and IL-17) were increased independent of increased abdominal fat and insulin resistance (Sumarac-Dumanovic et al., 2009). This has also been corroborated in a diet-induced obese mice study (Winer et al., 2009). Obesity was also shown to induce the accumulation and activation of macrophages in adipose tissue in both mice and humans (Drake et al., 2011, Weisberg et al., 2003, Xu et al., 2003).

Systemic inflammation can contribute to cognitive decline and dementia. Cytokines, such as IL-1β and IL-6 have been shown to disrupt neural circuits involved in cognition and memory (Gemma and Bickford, 2007, Jankowsky and Patterson, 1999). A recent meta-analysis identified that increased plasma levels of C-reactive protein and IL-6 is associated with an increase of dementia (Koyama et al., 2013). Elevated plasma IL-6 and IL-12 levels were also associated with impaired processing speed and executive function in a group of elderly participants between the ages of 70 and 90 (Trollor et al., 2012).

Peripheral cytokines can act on the brain to induce local production of cytokines (Dantzer et al., 2008). As such, central inflammation is observed after HF feeding and in genetic models of obesity, particularly in the hypothalamus (for review see (Miller and Spencer, 2014)). In *db/db* mice, IL-1β, TNF-α and IL-6 mRNA expression levels in the HPC are increased when compared to wild type controls (Dinel et al., 2011). Moreover, in mice fed a 60% HFD for 20 weeks, raised TNF-α expression was observed in the HPC (Jeon et al., 2012). Juvenile HFD intake did not affect basal expression of pro-inflammatory cytokines in the brain, but potentiated the enhancement of TNF-α expression specifically in the HPC after a peripheral immune challenge with lipopolysaccharide (LPS) (Boitard et al., 2014). Chronic HFD consumption has also been shown to exacerbate LPS-induced cytokine mRNA expression of TNF-α and interferon-γ in the HPC as well as IL-6 and suppressor of cytokine signalling-3 in the hypothalamus (Andre et al., 2014).

1.11.6. Microglia and astrocytes

Microglia, the primary mediators of the central nervous system's immune defence system release pro-inflammatory cytokines, chemokines, nitric oxide, and superoxide species (Loane and Byrnes, 2010). While the relationship between obesity-induced microglia expression within hypothalamic regions in animal models has been well-documented (Miller and Spencer, 2014), new data indicate that brain regions involved in cognition and memory also show exacerbated microglial expression. In the *db/db* mouse, increased levels of microglial activation markers are observed throughout the HPC (Erion et al., 2014). Moreover in aged (24 months) mice, hippocampal microglial activation was shown to be exacerbated by five months HFD treatment (Tucsek et al., 2014a). In addition, treatment of cultured primary microglia with sera derived from these aged obese mice resulted in significantly more pronounced microglia activation and oxidative stress (Tucsek et al., 2014a).

Astrocytes are the most abundant glial cell within the central nervous system and respond to all forms of insults through a process referred to as reactive astrogliosis (Sofroniew and Vinters, 2010). Within the hypothalamus, astrocytes produce cytokines that drive inflammatory responses, however data suggests that central inflammation can extend beyond the hypothalamus in obesity models to affect areas directly related to cognition (Garcia-Caceres et al., 2013). Astrocytes from the CA3 region of HPC showed longer and less abundant projections in HFD mice (Cano et al., 2014). In Zucker fatty rats a similar pathology is observed with a reported significant increase in the number of glial fibrillary acidic protein immunoreactive astrocytes throughout all subfields of the HPC as well as frontal and parietal cortices (Tomassoni et al., 2013).

1.11.7. Dopamine

There are numerous neurotransmitters involved in obesity and cognition; however the focus of this dissertation is on dopamine (DA) and serotonin (5-HT). DA is a neurotransmitter involved in motor system function, cognition, mood, and reward perception via the nigrostriatal pathway (projecting from the substantia nigra pars compacta to the caudate nucleus and putamen), tuberoinfundibular pathway (projecting from the arcuate nucleus to the pituitary gland) and the mesocorticolimbic pathway (also known as the reward pathway, projecting from the ventral tegmental area to the frontal cortex and the limbic system via the nucleus accumbens). It is becoming evident that the peripheral and central mechanisms that control feeding communicate, via the hypothalamus and brainstem, with the brain reward system (Volkow et al., 2013).

In obese individuals, presentation of high-calorie food images triggers an increase of brain activity in the nucleus accumbens, amygdala and HPC, which are areas part of the brain reward pathway (Rothemund et al., 2007, Stoeckel et al., 2008). Functional magnetic resonance imaging studies expanded these findings by demonstrating a blunted striatal dopaminergic response to the receipt of palatable foods in human obese subjects (Green et al., 2011, Stice et al., 2008a, Stice et al., 2008b). Furthermore in obese individuals, there is a reported decrease of striatal dopamine-2 (D_2) receptor availability, reiterating the role of the brain reward system in obesity (de Weijer et al., 2011, Wang et al., 2001).

When a novel food reward is given to rats, DA neurons are activated in the ventral tegmental area, which release DA into the nucleus accumbens (Hajnal and Norgren, 2001). Animal studies have also associated overconsumption of food in obese rats with decreases in striatal D2 receptors (Johnson and Kenny, 2010). DA reuptake was also observed to decrease,

independent of DA transporter protein gene expression, in rats fed a HFD and is thought to be due to interference in DA transporter trafficking or maturation (Petrovich et al., 2007).

Dopamine signalling is mediated by five receptors, termed D1-D5 receptors. Pharmacological manipulations have partially clarified the role of D1 and D2 receptors in the control of various functions. The role of D3, D4 and D5 receptors is still mostly unknown. The D1 receptor is the most widespread and highly expressed dopaminergic receptor (Weiner et al., 1991) and found in the limbic system, hypothalamus and thalamus (Missale et al., 1998). The D2 receptor has been found mainly in the striatum, in the olfactory tubercle, and in the core of nucleus accumbens (Jackson and Westlind-Danielsson, 1994).

Dopamine receptors have an opposing role in locomotor activity with D1 specific agonists administration resulting in increased locomotion whilst D2 specific agonists decrease locomotor activity (Jackson and Westlind-Danielsson, 1994). Pharmacological studies have shown that modulation of the hypothalamus's dopamine receptor activity by D1 and D2-like receptor agonists and antagonists could regulate food intake (Bina and Cincotta, 2000, Fetissov et al., 2002). Both D1 and D2 receptors are involved in reward and reinforcement mechanisms, with agonists at both receptors stimulating and antagonists inhibiting the behaviour (Kornetsky and Esposito, 1981, Self and Stein, 1992). Furthermore both D1 and D2 receptors are involved in the reinforcing properties of different drugs of abuse, with D2 receptors mediating the stimulant drug reinforcement and D1 receptors playing a permissive role (Beninger et al., 1989, Arnsten et al., 1994).

1.11.8. Serotonin

5-HT is a neurotransmitter with wide range of functions including; vascular function, gastrointestinal control, mood, appetite control, and cognition and has also been implicated in obesity. The role of the central serotoninergic system in cognition is modulated by the

activity and function of 5-HT receptors classified into seven groups with a total of 14 known serotonin receptors, which differ in structure, action, and localization. Most of these receptors are G-protein coupled receptors except for the 5HT-3 receptor which is a ligand-gated ion channel. The 5-HT2A and 5HT-2C receptors are of interest due to the overlapping functions on cognition and feeding behaviour.

5HT-2A receptors are located mostly in different parts of the cortex, basal ganglia and less in the hippocampus, where they enhance the release of dopamine, glutamate and GABA, and inhibit the release of noradrenaline (Hoyer et al., 2002, Fink and Gothert, 2007). The observed reduction in the density of 5HT-2A receptor every decade, correlates with cognitive decline (Hasselbalch et al., 2008) and AD (Marner et al., 2012). Administration of TCB-2, an agonist with high affinity to the 5HT-2A receptor, enhanced working memory in rats (Li et al., 2015). Furthermore TCB-2, in a dose-dependent manner, can decrease food consumption in food-deprived mice (Fox et al., 2010).

Within the brain, 5HT-2C receptors have been shown to modulate mesolimbic dopaminergic function, where they exert a tonic inhibitory influence over dopamine neurotransmission (Bubar and Cunningham, 2007) 5HT-2C receptor mRNA and protein are found widely distributed throughout the brain, including the cortex, amygdala, basal ganglia, hippocampus, and thalamus (Clemett et al., 2000, Pasqualetti et al., 1999). 5HT-2C receptors are also expressed in many brain regions involved in regulating food intake, which include the nucleus of the solitary tract, dorsomedial hypothalamus, and the paraventricular hypothalamic nucleus (Berthoud, 2002) which is of particular interest and is explored more deeply in Chapter 4.

Some studies indicate reduced 5-HT levels in obese individuals (James et al.). 5-HT agonism has also been related to weight loss (Bever and Perry, 1997) and women with primarily

abdominal obesity have been associated with low levels of 5-HT metabolites in cerebrospinal fluid (Strombom et al., 1996). Serotonin transporter (5-HTT) binding in cortical and subcortical regions has been negatively correlated to BMI (Erritzoe et al., 2010). Research has also demonstrated a positive correlation between BMI and *in vivo* cerebral 5-HT_{2A} receptor binding (Erritzoe et al., 2009).

Prolonged HFD exposure in animals has been suggested to compromise hippocampal 5-HT homeostasis by reducing basal extracellular 5-HT levels (Zemdegs et al., 2015) and decreasing 5-hydroxyindoleacetic acid (primary 5-HT metabolite) in the HPC (Krishna et al., 2016). In the DIO/DR obesity rat model, higher 5-$HT_{2A/2C}$ binding density in the ventromedial hypothalamic nucleus was observed and was associated with total fat mass (Huang et al., 2004). The effect of obesity on 5-HTT binding appears to be region specific with reported increases in the nucleus accumbens (Huang et al., 2004) and hypothalamus (Levin and Dunn-Meynell, 2002) but a decrease of mRNA levels in the dorsal raphe in HFD exposed rats (Collin et al., 2000).

1.12. Hypothesis and aims

Studies have suggested that there is a link between obesity and impairment of cognitive behaviour in humans. To investigate this association researchers have used animal models of obesity and discovered contrasting results as noted in the literature above. We sought to clarify the literature on the effect of western diet consumption on motivation, learning and memory.

The central hypothesis of this thesis is that impairments of cognitive behaviour such as specific types of memory and learning can be caused by the obesity condition. Furthermore, the cognitive impairments could potentially be mediated through the alteration of the neurotransmitters, DA and 5-HT.

This hypothesis was assessed by the following aims:

Aim 1: Assess Pavlovian & instrumental conditioning, motivation in a WD induced animal model of obesity.

Aim 2: Assess cognitive behaviour and associated basal and neuronal activation in response to novelty using c-Fos immunoreactivity in a WD induced animal model of obesity.

Aim 3: Assess associated changes in DA levels and 5-HT receptor expression immunoreactivity in a WD induced animal model of obesity.

Aim 4: Assess spatial, reference and working memory in an early life overfeeding model of obesity.

Aim 5: Assess memory and anxiety in APDE9 transgenic mice fed a WD.

Chapter 2 - Pavlovian & instrumental conditioning, motivation in a western diet induced animal model of obesity

2.1. Background and rationale

Food intake and body weight regulation is dependent on the ability to balance the inclination to seek out and consume food on some occasions with the capacity to inhibit those responses at other times. Homeostatic energy regulation has been long known to involve the hypothalamus by the adaptation and coordination of metabolic needs to the demands and complexities of the environment. However, it has been purported that hedonic signals can disturb food consumption to heighten food intake and obese individuals perceive palatable foods to be more pleasurable than lean counterparts do (Lowe and Levine, 2005, Yeomans et al., 2004).

Food-related cues (i.e. smell and sight) may overwhelm homeostatic energy mechanisms that lead to exacerbated hedonic feeding thereby causing a positive energy balance and increase of body weight (Corsica and Hood, 2011, King, 2013, Zheng et al., 2009). In human studies, it has been proposed that obesity may modulate the associative properties of food-related cues, evoking cravings for particular foods, thus triggering over-consumption (Cohen et al., 2005, Jastreboff et al., 2013, Meule et al., 2012, Meule et al., 2014). However, these findings have not been replicated in animals. Only one group of researchers has investigated appetitive Pavlovian conditioning in an animal model of obesity. Using DIO/DR rats fed a 40 kcal% HFD for 12 weeks researchers demonstrated that Pavlovian conditioning was not affected (Davidson et al., 2013, Kanoski et al., 2010, Kanoski et al., 2007).

Moreover the literature on the effects of HFD on instrumental conditioning has been conflicting. Performance in the instrumental conditioning task, where food reward delivery is contingent upon the animals' actions, of HFD exposed animals was observed to be enhanced when compared to control diet counterparts (Figlewicz et al., 2006, Figlewicz et al., 2013, Vollbrecht et al., 2015). This suggests that HFD exposed animals enhanced their behaviours

that result in a food reward compared to control diet counterparts. However, these findings are conflicted by other studies that have indicated that HFD exposure does not affect instrumental conditioning performance (Davis and Fox, 2008, Tracy et al., 2015).

It has been established that rats subjected to periods of acute food deprivation increase subsequent consumption of palatable foods (Castellanos et al., 2009, Cameron et al., 2014, Epstein et al., 2003, Wei et al., 2015). The increase of consumption is not solely due to calorific deprivation as food-deprived rats have been shown to increase intake of both caloric sucrose and non-caloric saccharin solutions (Smith and Duffy, 1957). This finding suggests that other factors are involved in the subsequent consumption of palatable foods in a state of food deprivation such as an enhanced hedonic response (Berridge, 1991). By reducing the incentive value of a particular food by pre-feeding to satiety or lithium-induced devaluation, animals decrease their performance responses in Pavlovian and instrumental conditioning (Balleine and Dickinson, 1998, Dickinson et al., 1996, Reichelt et al., 2011). Whereas animals fed a high fat/high sugar diet for 5 weeks showed impairments in outcome devaluation indicating loss of goal-directed control of responding (Furlong et al., 2014). However the effect of increasing the incentive value of a food reward by food deprivation on an animal model of obesity is still unknown.

Given the expanding global burden of HFD consumption and obesity, and an emerging crisis of dementia due to a rapidly aging population, understanding the effects of HFD consumption on cognition are of critical importance. Given conflicting literature on the effect of HFD consumption on instrumental conditioning described above, we sought to clarify the literature by performing these experiments and hypothesized WD consumption may have an effect on instrumental conditioning. Furthermore, appetitive motivational drive, assessed by the breakpoint task, would be impaired and WD fed animals would be more sensitive to changes

to motivation drive in a state of food deprivation compared to control diet counterparts. To investigate this hypothesis we examined instrumental conditioning following WD consumption. Additionally we investigated progressive ratio instrumental conditioning and assessed instrumental extinction under varying states of food deprivation.

2.2. Methods

2.2.1. Animals

The first cohort of male Wistar-hooded rats (n= 32, Laboratory Animal Services, Adelaide, Australia) weighing 206-290 g at the start of the experiment was brought into the RMIT University Animal Facility and was used for Pavlovian and instrumental conditioning, open field and light/dark preference testing. A second cohort of male Wistar-hooded rats (n= 32; 220-266 g) were used to assess progressive ratio instrumental conditioning and instrumental extinction performance under varying states of food deprivation.

Rats were housed at RMIT University animal facility, under a controlled environment (20 ± 1°C) with 12-h light/dark cycle (lights on at 07:00 h) in groups of 4, with food and water *ad libutum* in the home cage. Behavioural tests were performed from 9:00 h to 19:00 h in a dedicated animal behaviour room. All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University Animal Ethics Committee.

2.2.2.Dietary manipulation

Upon delivery, all animals were allowed to acclimatise for at least 1 week before commencement of dietary manipulation. Rats were fed one of two diets (Speciality feeds,

Australia) either a control diet (CON; SF04057) or a western diet (WD; SF00-219) for a period of 8 weeks. The nutritional content of each diet is given in **Table 2.1.**

Table 2.1: Nutritional parameters of CON and WD feed.

	CON (SF04057) (w/w %)	**WD (SF00-219) (w/w %)**
Protein	19	19
Monounsaturated fats	3.43	6.23
Polyunsaturated fats	2.15	0.77
Saturated fats	0.43	13.99
Total fat	6	21
Digestible energy	16.1 MJ/kg	19.4 MJ/kg
Total digestible energy from fat	14 kcal%	40 kcal%
Total digestible energy from protein	21 kcal%	17 kcal%

2.2.3. Food restriction

One week prior to the start of behavioural training, rats were food restricted to 85% of their daily food intake. Food restriction was maintained for the entire duration of behavioural testing. Body weight was monitored twice weekly to ensure rats did not fall below 85% of their free-feeding weight. At the end of the experiment, animals were sacrificed and epididymal adipose tissue was collected and weighed.

2.2.4. Operant box apparatus

The apparatus comprised of 8 operant chambers (Med Associates, USA) individually housed in light and sound attenuating cabinets. Chambers are 30 cm wide x 24 cm deep x 21cm high

and consist of three aluminium walls and ceiling, with a Perspex door serving as the fourth wall. Each chamber comprised of a transparent Perspex back wall, roof and front door, with aluminium left and right-hand walls. The floor consisted of steel bars, aligned perpendicular to the back of the chamber. Each chamber also had a 3 W, 24 V house light that provided illumination inside the enclosed chamber. Grain pellets (45 mg; containing 25 kcal% protein, 10 kcal% fat and 65 kcal% carbohydrates, Bio-Serv, USA) and 0.5 ml of 10 w/v% sucrose solution was delivered into a recessed magazine located at the bottom centre of the right hand wall. Access to the magazine was measured by infrared detectors at the mouth of the recess. Two panel lights were located on either side of the magazine at the top of the right-hand wall. The chambers were fitted with two retractable levers that could be inserted to the left and the right of the magazine. A speaker located to the right of the house light could provide auditory stimuli to the chamber. In addition, a 5 Hz train of clicks produced by a heavy-duty relay placed outside the chamber at the back right corner of the cabinet was used as an auditory stimulus. A computer equipped with Med-PC software (Med Associates, USA) was used to control the experimental procedures and record experimental data.

2.2.5. Pre-training

After 3 days of food restriction, rats were exposed to the pellets by placing a small petri dish containing grain pellets into each cage. The following day, all rats received two 30 min session in the operant chamber to learn to retrieve reinforcers from the magazine recess. A single grain pellet was delivered randomly approximately every 60 s.

2.2.6. Pavlovian conditioning

Rats (n= 16 per group) received 9 sessions of Pavlovian conditioning in the operant box. Two auditory stimuli (tone and clicker) served as CS and were paired with either pellet or 10% sucrose solution delivery (counterbalanced). Three presentations of each stimuli lasting 5 min

each were given in each session in random order interspersed with periods where no stimuli were presented. Reward was delivered into the recessed magazine on a variable interval 90 s during stimuli presentation. An inter-trial period of 90 s was observed after each reward delivery. The number of magazine entries during stimuli presentation and pre-stimulus interval was measured. Each session lasted 30 min.

2.2.7. Instrumental conditioning

Rats received 9 (n= 16 per group) sessions of instrumental training in the operant box. Rats were trained to perform two lever-press responses (left and right) with each reinforced on a random interval 30 schedule. Each lever was trained separately and earned one of two possible outcomes: pellets or 10% sucrose solution (counterbalanced). Both levers were trained in the same session with 6 alternating 5 min lever presentations. The number of lever presses performed and magazine entries was measured. Each session lasted 30 min.

2.2.8. Open field test

The open field test is used to assess the differences in locomotor activity in rats. Locomotor activity was assessed using an open field test chamber (44.5 cm x 44.5 cm x 30.5 cm; Med Associates, USA) with four pairs of photocells spaced evenly along the length of the test chamber to detect horizontal and vertical motor activity. Wistar-hooded rats (n= 16 per group) were placed individually in the test chamber for 10 min to monitor locomotor activity. Test chamber was cleaned with 70% ethanol after each rat to remove foreign odours that can affect explorative behaviours. Total distance travelled and average velocity was measured by Med Associates activity monitor software, version 4.

2.2.9. Light/dark preference test

The light/dark preference test is commonly used to assess anxiety-like phenotypes and validate the pharmacological effects of neuroactive compounds. The test relies on the tendency for rats to explore new environments and to prefer dark environments over brightly illuminated environments. Rats that spend more time in the dark environment indicate elevated anxiety levels. The test took place in the locomotor activity chamber (Med Associates, USA) as described above in Chapter 2.2.8 with a specially designed dark box insert and each session lasted 10 min. Med Associates dark box plastic insert that does not interfere with infrared beams allowed tracking in both light and dark areas. Number of entries and total time spent in either light or dark areas of the box was measured.

2.2.10. Progressive ratio instrumental conditioning

Progressive ratio instrumental conditioning task is a widely utilized behavioural test used to assess motivational drive and reward strength in drug addiction research. In this behavioural test, rats (n= 16 per group) were placed in the operant box and were conditioned to perform lever presses with a grain pellet reward reinforcer. In the first three sessions, the pellets were delivered on a continuous reinforcement schedule, whereas in sessions 4-7, 8-10 and 11-14, they were delivered on a random ratio (RR) 5, RR10, RR20 schedule, respectively. A RR schedule is characterised as the probability of a single lever press delivering a reward. Following training rats underwent a test session, where the response ratio schedule was using the following formula (rounded to the nearest integer): $= [5e^{(n*0.2)}] - 5$ where *n* is equal to the number of food rewards already earned plus 1 (i.e., next reinforcer). Thus, the number of responses required to earn a food reward follow the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 and so on. The final ratio completed is the breakpoint.

2.2.11. Instrumental extinction performance under varying states of food deprivation

This task was used to assess motivational drive and saliency of a food reward under food deprivation. Following the conclusion of progressive ratio instrumental conditioning, rats were placed into an instrumental extinction test, where no rewards were given. Lever press responses and magazine entries were recorded. The rats (n= 16 per group) were allowed 30 min *ad lib* access of control or WD, respectively, prior to the food deprivation period. Food deprivation periods were studied in the same order: 0 h, 6h, 12 h, and 24 hr after the last meal with a staggered start. Each test session lasted 30 min.

2.2.12. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). A p-value of < 0.05 was considered statistically significant. Statistical comparisons were made between groups by repeated measures two-way analysis of variance (ANOVA) for body weights, Pavlovian and instrumental conditioning data using GraphPad Prism version 6.00 (GraphPad Software, USA). Unpaired t-tests were used to compare epididymal adipose tissue weights, basal locomotor activity and light/dark preferences. Further analysis by a post hoc Bonferroni's t-test was performed if a significant effect was detected by the ANOVA.

2.3. Results

2.3.1. Animals

In the first cohort, rats fed a WD were observed to be heavier than rats fed a CON diet (Week 8 CON: 350.7 ± 7.1 g; WD: 372 ± 4.9 g; $F_{(1,261)}= 46.11$ $p< 0.0001$, **Fig. 2.1A**). Both groups showed a similar weight increase over time (time: ($F_{(8,261)}= 84.71$, $p< 0.0001$). Epididymal fat was not different after WD consumption ($p= 0.82$; **Fig. 2.1B**). In the second cohort, rats fed a WD were not significantly heavier than rats fed a CON diet (Week 8 CON: 392.8 ± 7.1 g;

WD: 408.0 ± 5.1 g; group: $F_{(1,30)}$= 1.87 *p*= 0.18; **Fig. 2.2A**). However, WD consumption was shown to increase epididymal adipose tissue with a significant increase in WD rats (CON: 8.66 ± 0.39 g; WD: 13.53 ± 0.75 g, *p*<0.0001; **Fig. 2.2B**).

2.3.2. Locomotor activity and light/dark preference

WD consumption did not affect basal locomotor activity ($p > 0.05$) and anxiety ($p > 0.05$) as indicated by the open field test (**Fig.2.3A**) and light/dark preference test (**Fig. 2.3B**), respectively.

2.3.3. Pavlovian conditioning

2-way ANOVA revealed there was no difference in which both CON ($F_{(1,280)} = 1.43$, $p=0.24$) and WD animals ($F_{(1,300)} < 1$, $p=0.59$) responded to the 10% sucrose solution and grain pellet rewards. Magazine entries steadily increased over training session in both groups at a similar rate (from a mean of 4.6 magazine entries/min in session 1 to 5.2 magazine entries/min in session 9 (group ($F_{(1,29)}= 1.33$, $p= 0.26$); time ($F_{(8,232)}= 2.55$, $p< 0.01$). All rats showed similar proficiency to associate CS presentation with reward delivery by the end of training ($F_{(1,56)}= 45.89$, $p< 0.0001$; **Fig. 2.4A**). Post-hoc analysis shows a marked increase of magazine entries/min in both CON and WD rats compared to baseline in the final Pavlovian conditioning session ($p< 0.001$). The results of Pavlovian conditioning showed that all groups learned to respond significantly more on CS compared to baseline and that the magnitude of this difference did not vary significantly as a function of type of diet or prior dietary restriction condition.

2.3.4. Instrumental conditioning

Lever press responding steadily increased over the course of instrumental training in both groups (**Fig. 2.4B**). An ANOVA applied to this data revealed a significant main effect of Session ($F_{(8,232)}= 90.15$, $p< 0.0001$), but no apparent difference between the groups ($F_{(1,29)}= 1.80$, $p= 0.19$).

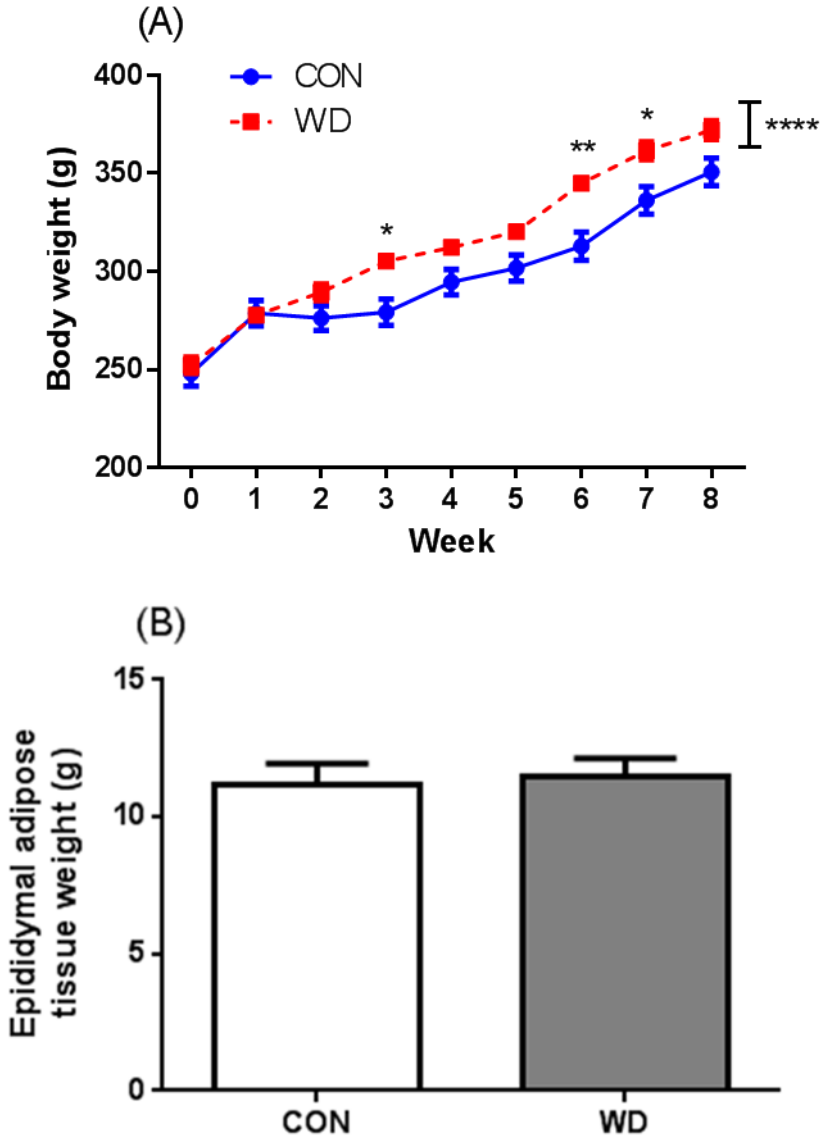

Figure 2.1. Metabolic measures of the first cohort. **(A)** Body weight after consumption of either CON or WD over the 8 week feeding protocol of the **(B)** Epididymal adipose tissue weight in both CON and WD rats. n= 16 per group. Data expressed as mean ± SEM. *p< 0.05, **p< 0.01, ****p<0.0001.

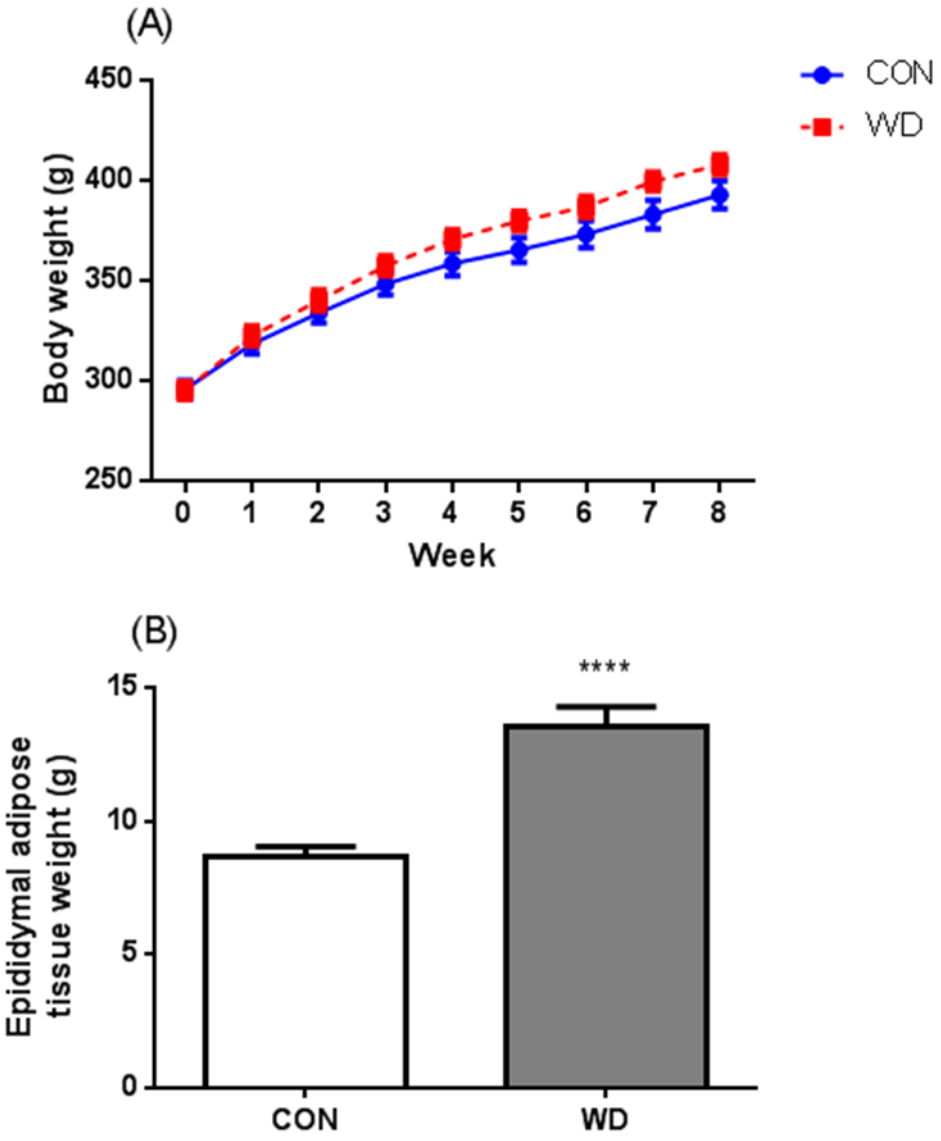

Figure 2.2. Metabolic measures of the second cohort. **(A)** Body weight after consumption of either CON or WD diet over the 8 week feeding protocol of the **(B)** Epididymal adipose tissue mass weight in both CON and WD fed rats. n= 16 per group. Data expressed as mean ± SEM, *p< 0.05, ****p< 0.0001.

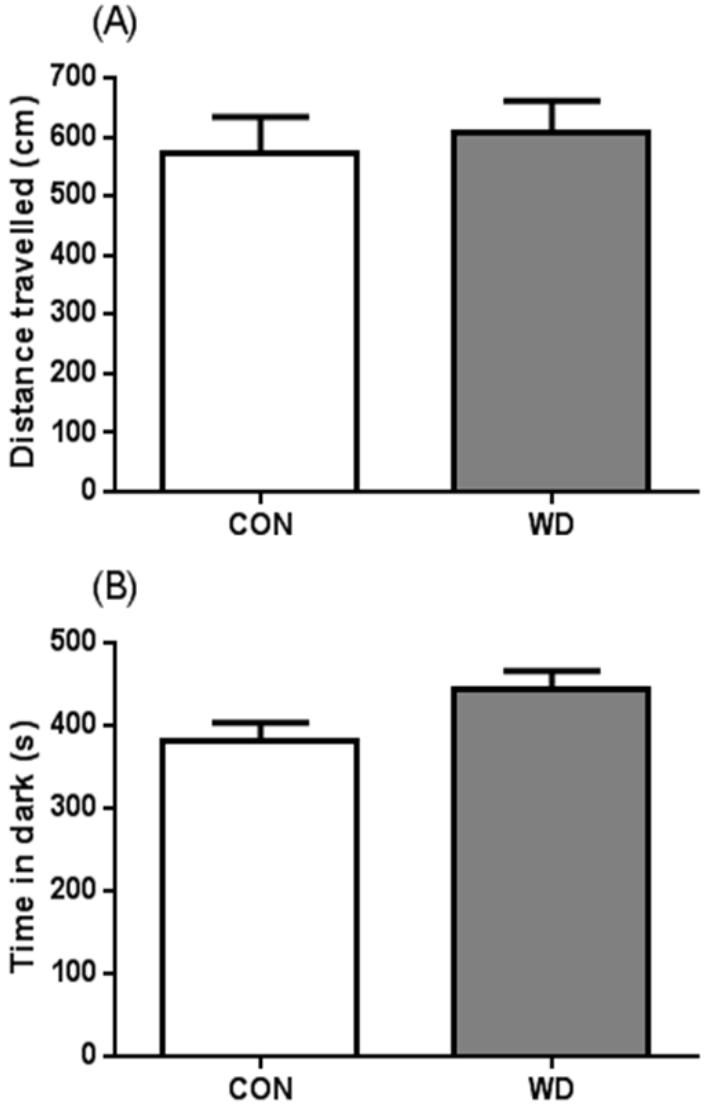

Figure 2.3. Anxiety-like behavioural testing. **(A)** Basal locomotor activity after consumption of CON or WD in open field test. **(B)** Time spent in dark chamber in the light/dark preference test. n= 16 per group. Data expressed as mean ± SEM.

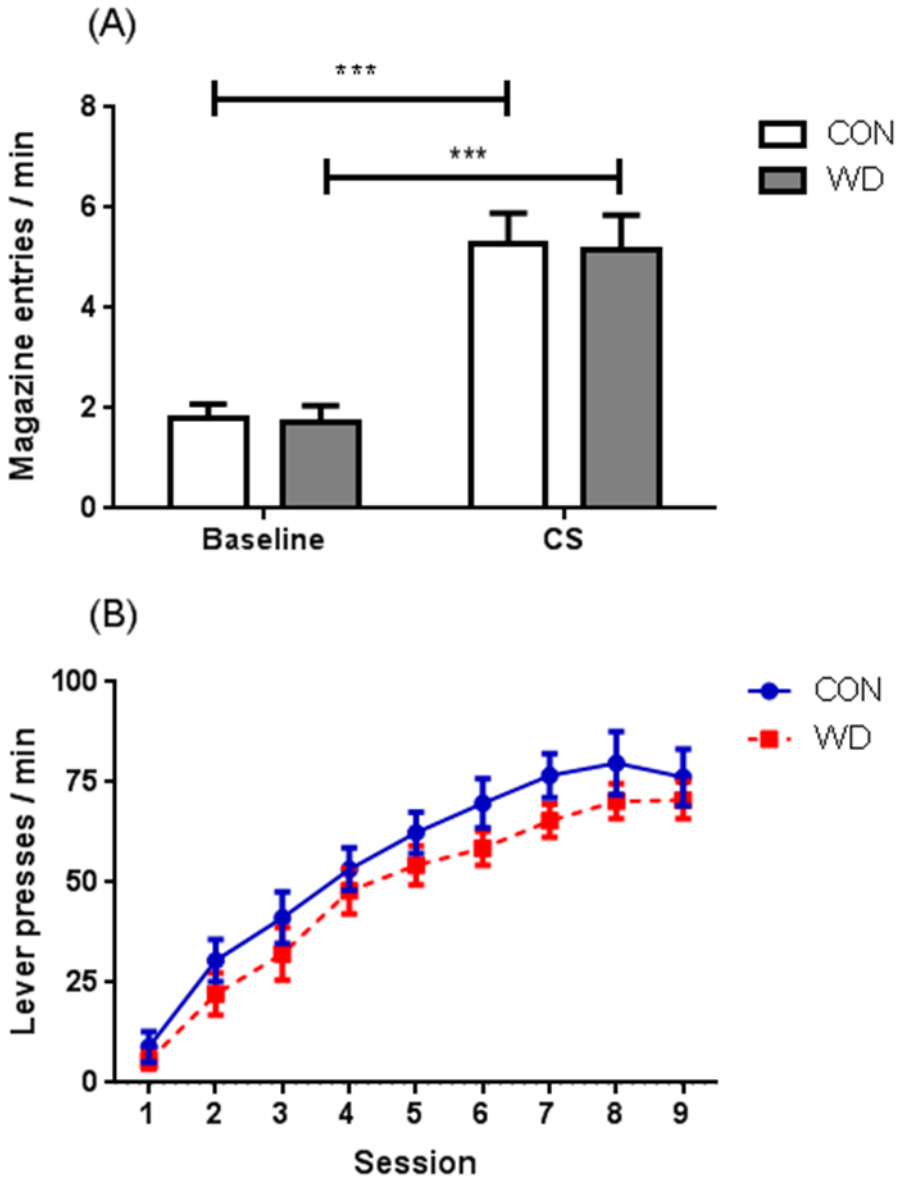

Figure 2.4. Pavlovian conditioning. **(A)** Final Pavlovian conditioning session of CON and WD rats during baseline and CS presentation (magazine entries/min). **(B)** Number of lever presses across instrumental training. n= 16 per group. Data expressed as mean ± SEM, ***p< 0.001.

2.3.5. Progressive ratio instrumental conditioning

Lever press responses increased as training progressed indicating that all rats learnt to associate lever press responses to reward delivery. A significant time effect ($F_{(12,360)}$= 138.40, p< 0.0001) and group x time interaction ($F_{(12,360)}$= 2.73, p= 0.0015) was observed, corresponding to a decrease in lever press responses in WD rats during the end of training (sessions 11 and 13: p< 0.05; **Fig. 2.5A**).

Motivation to work for a food reward as indicated by the breakpoint task showed no change in total lever presses in the subsequent breakpoint task after WD consumption (p> 0.05; **Fig. 2.5B**). The rate at which rats acquired the food reward in the breakpoint task was also observed not to be different after WD consumption indicating no change in motivational behaviour to perform for a food reward (**Fig. 2.5C**).

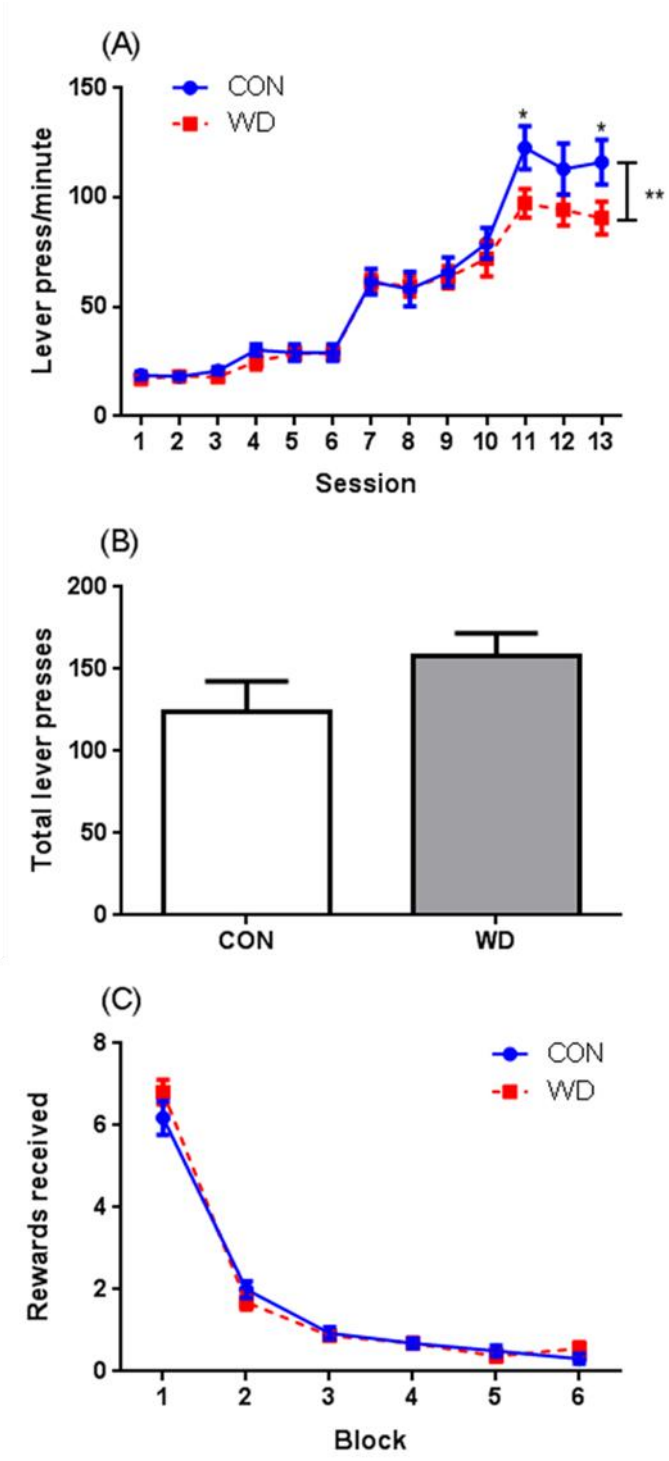

Figure 2.5. Instrumental conditioning. **(A)** Training acquisition using a scaling progressive ratio schedule for CON and WD rats. **(B)** Lever press performance in progressive ratio test session and **(C)** Number of rewards obtained over 5 min blocks of time in progressive ratio test session. n= 16 per group. Data expressed as mean ± SEM, *$p< 0.05$, **$p< 0.01$.

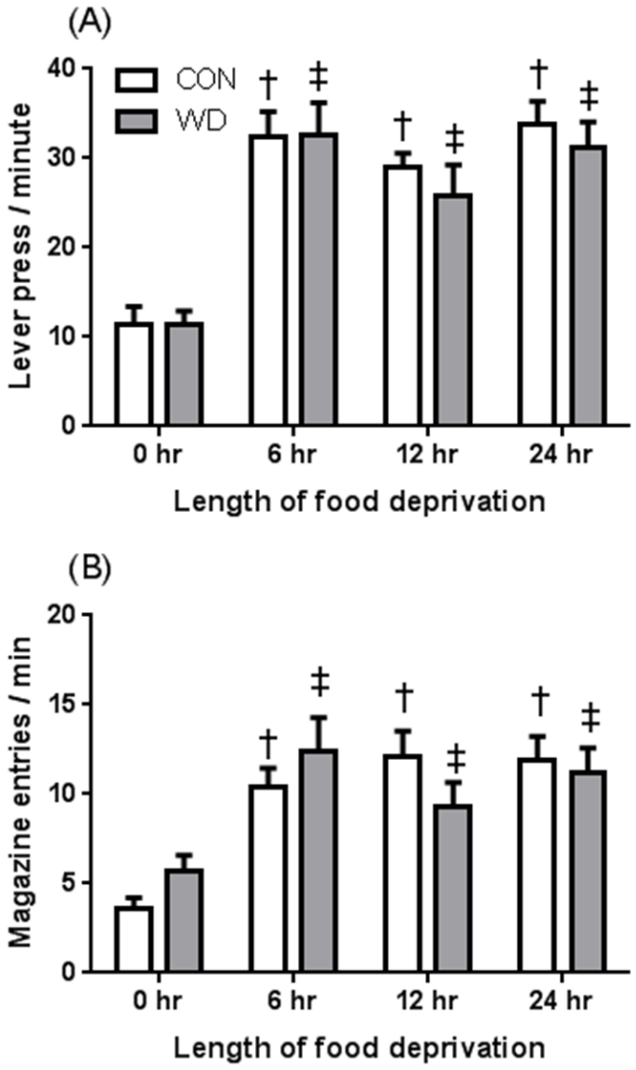

Figure 2.6. Instrumental conditioning response after food deprivation **(A)** Lever press response and **(B)** Magazine entries in an instrumental extinction test after 0, 6, 12 & 24 h food deprivation. n= 16 per group. Data expressed as mean ± SEM, † $p< 0.05$ compared to CON 0 h, ‡ $p< 0.05$ compared to WD 0 h.

2.3.6. Instrumental extinction test following food deprivation

Rats were food deprived for 0, 6, 12, and 24 h with lever press responses and magazine entries recorded in an extinction test where no rewards were delivered to assess instrumental conditioning recall. Following 6, 12, and 24 h food deprivation, both CON and WD rats had a significant increase of lever press response ($F_{(3,120)}$= 28.92, p< 0.0001) and magazine entries ($F_{(3,120)}$= 13.12, p< 0.0001) compared to 0 h baseline. There was no observed difference in all measures recorded in the extinction test between CON and WD (F< 1; **Fig. 2.6A&B**). This indicates that WD consumption did not alter motivation drive in a state of food deprivation to work for a food reward in rats.

2.4. Discussion

Previous research has demonstrated that HFDs can impair learning and memory function, particularly in tasks that rely on the utilization of spatial cues. The present series of experiments was performed to understand how HFDs can impair different types of learning and whether different states of food deprivation can influence appetitive motivation. In the first cohort of rats there was a significant increase of body weight in WD fed rats but no change in epididymal adipose tissue weight. Basal locomotor activity was not affected by WD and anxiety-like behaviour in the light/dark preference test was unaffected. However WD fed rats in the second cohort displayed no change in body weight but displayed a significant increase of epididymal adipose tissue weight compared to controls.

This present study demonstrates that Pavlovian conditioning was not affected by rats being fed a WD for 8 weeks. Both CON and WD fed rats showed similar responses to stimuli which predicted food reward delivery. In a feature negative Pavlovian conditioning task, where the presentation of a single stimulus (i.e. a tone) are rewarded but the presence of the

same stimulus in conjunction with a different stimulus (i.e. light) are not reinforced with a food reward. Animals with hippocampal lesions are unable to learn this feature negative Pavlovian conditioning task (Holland et al., 1999). HFD exposed rats were unable to discriminate between stimuli that predicted food rewards and stimuli that predicted no food reward delivery suggesting that HFD consumption affects the HPC (Davidson et al., 2013, Kanoski et al., 2007, Kanoski et al., 2010). These results suggest that associations with food rewards are not maladaptive and the food related cues does not promote responding in animals fed a WD.

WD was shown not to affect instrumental conditioning acquisition however, when tested in a progressive ratio instrumental conditioning task there was an observed reduced lever press response compared with control diet counterparts. We do not believe that the observed reduction in the lever press response can be explained by a reduction of activity as basal locomotor activity did not differ between cohorts. Rats fed a HF-high sugar diet for 2 weeks (la Fleur et al., 2007) and Long-Evans rats fed a 39 kcal% HFD for 12 weeks (Francis et al., 2013) exhibited impaired instrumental acquisition with less lever press response compared to controls. Further experiments in DIO fed a standard chow diet also showed a similar outcome of impaired instrumental conditioning acquisition (Francis et al., 2013). There have been some reports of enhanced lever press responding during instrumental conditioning in Sprague-Dawley rats fed a 32 kcal% HFD for 5 weeks (Figlewicz et al., 2006), albino rats fed 32 kcal% fat diet 5-8 weeks (Figlewicz et al., 2013) and obese prone rats fed a 20 kcal% HFD for 5 weeks (Vollbrecht et al., 2015). Other studies have indicated that HFD exposure in Sprague-Dawley rats (38 kcal% fat for 16 weeks) (Davis et al., 2008) or Long-Evans rats (50 kcal% fat for 10 weeks) did not affect acquisition of instrumental conditioning (Tracy et al., 2015). These studies in conjunction with our results suggests that HFD consumption alone

does not influence the acquisition of instrumental conditioning which may assist in elucidating previous conflicting research.

Studies have shown that that food motivation is enhanced where HFD exposed rats had an increase of lever presses in a breakpoint task (Figlewicz et al., 2006, Figlewicz et al., 2013, la Fleur et al., 2007). Yet this finding is still disputed as there have been discrepant outcomes with other studies showing a decreased motivational breakpoint for a reward in 12 week HFD fed obese rats (Davis et al., 2008, Francis et al., 2013, Tracy et al., 2015). We found no indication of any appetitive motivational effect of WD consumption as demonstrated by no change of lever presses and number of rewards received in the breakpoint task compared to controls. It is possible that the duration of HFD consumption influences the motivation of animals. Studies where HFD duration occurs for a period of 8–12 weeks have shown reduced motivation for pellet rewards (Davis et al., 2008, Francis et al., 2013, Tracy et al., 2015). In contrast, HFD exposed rats for 4-8 weeks report a significant increase of motivation for pellet rewards (Figlewicz et al., 2013, la Fleur et al., 2007, Vollbrecht et al., 2015). These studies further allude to a time dependent relationship of HFD exposure to motivational drive similar to which is observed for instrumental conditioning performance. A study performed by Tracy et al. (Tracy et al., 2015) showed that 3 weeks of HFD exposure was insufficient to produce a difference in a breakpoint task however, after 6 weeks of HFD exposure a motivational deficit became apparent. Authors suggest that significant weight gain appears to be a contributing factor to motivational deficits in HFD exposed rats but state that weight gain cannot exclusively account for this effect (Tracy et al., 2015). In support of this hypothesis, when HFD exposed rats were significantly heavier they showed a motivational deficit in the breakpoint task (Davis et al., 2008, Tracy et al., 2015). Whereas when HFD exposed rats displayed similar body weight as controls, there was an observed increase of motivational drive in the breakpoint task (Figlewicz et al., 2013, la Fleur et al., 2007, Tracy et al., 2015).

It is also worth noting that, in order to produce robust responding, we food restricted our rats throughout conditioning, which was not the case in studies conducted by La Fleur et al. (la Fleur et al., 2007), Figlewicz et al. (Figlewicz et al., 2013) or Vollbrecht et al. (Vollbrecht et al., 2015), who found increases of breakpoint responding after HFD consumption. Conversely, in food restricted HFD exposed rats, such as in studies performed by Davis et al. (Davis et al., 2008) and Tracy et al. (Tracy et al., 2015), decreases of breakpoint responding was observed. Alternatively Francis et al. (Francis et al., 2013) presented similar results, but these rats were not food restricted. This suggests that food restriction may influence motivation drive when assessed by the breakpoint task.

It is plausible that HFD exposed rats may be less sensitive to changes in states of hunger and satiety than their control counterparts. To examine potential effects of changes in states of hunger and satiety on motivation, in the second experiment we subsequently examined instrumental responding in an extinction test after a period of food deprivation. Rats were tested in extinction (i.e., with no rewards available) in order to probe the effects of WD consumption on previously encoded relationships rather than on new learning. Although there was an increase of magazine entries and lever press responses after more than 6 h of food deprivation compared to satiated rats, we are the first to report that WD consumption did not affect extinction performance when food deprived. However, impaired performance in progressive ratio instrumental conditioning may have confounded potential differential response rates or other performance differences in the extinction test.

The presented results demonstrate that WD consumption for 8 weeks does not affect Pavlovian conditioning. The effect of WD consumption on instrumental conditioning is still indeterminate with conflicting results. There was no change in instrumental conditioning in rats fed a WD. However, WD fed rats was impaired in progressive ratio instrumental

conditioning acquisition. Body weight and adipose tissue increase may possibly be a contributing factor for reported motivational deficits. Additionally, WD exposed rats had no observable changes in motivational state when examined by instrumental conditioning extinction under various food deprivation compared to control diet counterparts.

Chapter 3 - Cognitive behaviour and associated neuronal activation in a western diet induced animal model of obesity

3.1. Background and rationale

Rats fed a HFD have been shown to be cognitively impaired compared to those fed a normal chow diet with much emphasis placed on hippocampal-dependent behavioural tasks (Goldbart et al., 2006, Molteni et al., 2002, Pathan et al., 2008, Stranahan et al., 2008, Wu et al., 2003, Xia et al., 2015). In the MWM, HF fed animals took longer to learn the location of a submerged platform relative to their control counterparts (Goldbart et al., 2006, Molteni et al., 2002, Pathan et al., 2008, Stranahan et al., 2008, Wu et al., 2003, Xia et al., 2015). These studies used varying levels of fat ranging from 21-58 kcal% and different lengths of diet consumption, with the general consensus that HFDs impair performance in the MWM.

The delayed-win shift (DWSh) version of the radial arm maze (RAM) task is used to assess spatial working and reference memory using the rats' ability to locate and retrieve food efficiently using spatial cues (Floresco et al., 1997, Jarrard, 1993). This task emphasises cognitive flexibility as the rodent acquires, retains and uses trial-unique information like the MWM, perhaps with less stress and a food reward (Seamans et al., 1995). Rodents with lesions to the medial PFC (Taylor et al., 2003), HPC (Jarrard, 1993), and ventral striatum (Floresco et al., 1997, Jarrard, 1993) have impaired performance indicating that these areas are critical in proper functioning of working memory and executive function in this task.

Immediate-early genes such as c-Fos are a class of genes that do not require previous protein synthesis and can influence cell function through the downstream genes that it regulates (Aggleton et al., 2012, Herrera and Robertson, 1996). Activation of the c-Fos immediate-early gene can be used as a surrogate marker of neuronal activation and is assumed to have roles in learning (Herdegen and Leah, 1998, Kaczmarek, 1993, Santin et al., 2003) and long term plasticity (Guzowski, 2002, Tischmeyer and Grimm, 1999). This gene is widely distributed through the brain, and occurrences that increase neuronal activity will produce an

upregulation of c-Fos in selective brain regions (Chaudhuri, 1997, Herrera and Robertson, 1996, Kovacs, 2008). In the majority of neurons, the basal level of c-Fos mRNA, and protein are relatively low. Extracellular signals are required constantly to maintain elevated levels of Fos, mRNA peaks after 30 min whilst Fos protein can be visualised by using immunohistochemistry after 90-120 min exposure to a novel stimulus such as environment (Greenberg and Ziff, 1984, Kruijer et al., 1985, Muller et al., 1984).

So far only one study has examined neuronal activation by using Fos immunoreactivity in HFD fed mice. This focussed on the hypothalamus due to its recognized role in regulation of food intake and energy (Lin and Huang, 1999). Mice were fed a 60 kcal% HFD for 15 weeks and had a 20% increase of body weight and 292% increase in epididymal adipose tissue weight compared to control diet counterparts after feeding (Lin and Huang, 1999). These metabolic changes were accompanied with increased basal Fos immunoreactivity the lateral hypothalamic area, dorsal medial hypothalamic area and perifornical nuclei (Lin and Huang, 1999).

Given that we see a cognitive deficit in our animals after WD consumption (Kosari et al., 2012) we aimed to explore the effects of WD consumption on spatial working and reference memory in the DWSh task on the RAM. Furthermore, we investigated changes in neuronal activation following WD consumption. An additional set of animals ("home-cage controls") allowed us to determine whether the effects of WD consumption on Fos mediated neuronal activation were specific to the novel stimulus. We hypothesised that WD consumption has a deleterious effect on spatial working and reference memory mediated through alterations of Fos neuronal activity in basal and after environmental novelty conditions. To investigate this hypothesis, we investigated spatial working and reference memory using the DWSh task in

the WD induced model of obesity. Additionally we examined basal and neuronal activation in response to novelty using Fos immunohistochemistry following WD consumption.

3.2. Methods

3.2.1. Animals

A cohort of male Wistar-hooded rats (n= 20, Laboratory Animal Services, Adelaide, Australia) weighing between 224-321 g at the start of the experiment were brought into the RMIT University animal house facility and were used for the DWSh task and Fos immunohistochemistry. A second cohort of male Wistar-hooded rats (n= 13; 220-264 g) were used as "home-cage controls" for Fos immunohistochemistry.

Rats were housed at RMIT University animal facility, a controlled environment (20 ± 1°C) with 12-h light/dark cycle (lights on at 07:00 h) in groups of up to 4, with food and water *ad libutum* in the home cage. Behavioural tests were performed from 9:00 h to 19:00 h in a dedicated animal behaviour room. The experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University Animal Ethics Committee.

3.2.2. Dietary manipulation

As 8 week WD consumption was insufficient to operant conditioning we extended the dietary period to 12 weeks for this experiment. All animals were allowed to acclimatise after delivery for at least 1 week before commencement of dietary manipulation. Rats were fed one of two diets: either CON diet (SF04057, Speciality feeds, Australia) or WD (SF00-219, Speciality

feeds, Australia) for a period of 12 weeks. The nutritional content of each diet is as seen in **Table 2.1.**

3.2.3. Food restriction

One week prior to the start of behavioural training, rats were food restricted to 85% of their daily food intake. Food restriction was maintained for the entire duration of behavioural testing. Body weight was monitored twice weekly to ensure rats did not fall below 85% of their free-feeding weight. At the end of the experiment, animals were sacrificed and epididymal adipose tissue was collected and weighed.

3.2.4. Delayed win-shift task in the radial arm maze

DWSh version of the RAM task (Lafayette Instrument Company, USA) was used to assess spatial working and reference memory using the rats' ability to locate and retrieve food efficiently using spatial cues (Floresco et al., 1997, Jarrard, 1993). This task emphasises cognitive flexibility as the rat acquires, retains and uses trial-unique information (Seamans et al., 1995). Rodents with lesions to the medial PFC (Taylor et al., 2003), HPC (Jarrard, 1993), and ventral striatum (Floresco et al., 1997, Jarrard, 1993) have impaired performance with a marked increase of errors in the DWSh task indicating that these areas are critical in proper functioning of working and reference memory function in this task.

Testing was carried out in an eight-arm radial maze, consisting of an octagonal central platform (34 cm diameter) and eight equally spaced radial arms (87 cm long, 10 cm wide). At the end of each arm was a food well (2 cm in diameter and 0.5 cm deep). At the start of each arm was a clear Perspex door that controlled access in and out of the central area. Each door was controlled by computerised control box enabling the experimenter to control access to all

the arms. Salient visual cues of different geometric shapes and contrasting colours were placed around the maze on the walls of the room.

On the first 3 days of testing, rats were habituated to the radial arm maze in two sessions per day lasting 10 min each. After the final habituation session of the day, rats were returned to their home cages and given approximately 20 grain reward pellets to familiarise rats to the novel food reward used for this task. Following habituation, rats underwent a total of 12 training sessions with 2 sessions performed per day consisting of a 5 min training phase, 5 min delay phase where the rat was returned to the home cage and a 5 min test phase. Before the training phase, 4 arms were pseudo-randomly chosen and blocked, with the following rule that no more than 2 adjacent arms could be closed in any trail. The remaining arms that were not blocked were baited with grain reward pellets. The training phase involved the rat being given 5 min to enter and retrieve the grain pellet rewards from all the baited arms. After a 5 min delay where the animal was returned to the home cage, all 8 arms were opened in which the previously blocked arms are baited with grain reward pellets. The rat was then placed back inside the maze and the number of arm entries was recorded.

For analysis purposes, four training/test sessions were grouped into a single block. An arm entry was recorded when the animal fully moved off the central platform into the arm. Two types of errors were recorded: within phase error (working memory error, re-entry of an arm that has been baited and has been visited) and across phase error (reference memory error, entry into a training phase baited arm).

3.2.5. Exposure to novel environment

A smaller cohort of these rats (n= 5–7 per group, 12 weeks CON or WD dietary manipulation) was assessed for activated Fos expression. Rats were placed into a novel arena, in our case a Y-maze (three-arm maze with equal angles between all arms which were 50 cm

long × 17 cm wide × 32 cm high. Rats were allowed to move around this novel environment for 30 min. Rats were then returned to their home cages for 90 min in a dark, quiet room. This manipulation was to reduce exposure to other stimuli that might evoke Fos production. Immediately after this 90 min quiet period rats were deeply anesthetized with pentobarbitone sodium (1 mg/kg) and perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS).

3.2.6. Home cage controls

A further cohort of rats (n= 6 per group), underwent the identical dietary manipulation as the cohort above. The home cage control experiment was not simultaneously run with the main cohort, but was completed within the same animal house. These home cage control rats, remained untouched except for weighing until culled. As with the animals exposed to the novel environment, they were also deeply anesthetized with pentobarbital sodium (1 mg/kg) and perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde in 0.1 M PBS.

3.2.7. Brain preparation

Rats underwent cardiothoracic perfusion to prepare brains for immunohistochemistry. Cardiothoracic perfusion entailed rats firstly being deeply anesthetized with pentobarbital sodium. A lateral incision was made just below the rib cage to expose the pleural cavity and an incision was made through the rib cage up to the collarbone to expose the heart. A 15-gauge blunt perfusion needle was inserted into the left ventricle and firmly held into place. An incision was made to the caudal vena cava to aid drainage of blood and fluids. Blood was flushed out of the body with phosphate buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, 1.8 mM monopotassium phosphate, pH 7.4) via a peristaltic pump at a constant flow rate of 25 µL/min. Once blood

had cleared the body, the peristaltic pump was switched to the fixative agent of 4% paraformaldehyde (Sigma-Aldrich, USA) in 0.1 M PBS. Perfusion was complete when fixative tremors were observed and whole body was rigid. The head was removed with a purpose built rat guillotine. The brain was then removed and postfixed for 4 h in the 4% paraformaldehyde in PBS before placing them in 30% sucrose in PBS solution (4°C) until sectioning.

Following fixing of brains, serial coronal sections (30 µm) were cut on a cryostat (Leica CM1950, Leica Microsystems, Germany) at -16°C and placed in cryoprotectant [30% (w/v) sucrose, 30% (w/v) ethylene glycol, 0.01% (w/v) polyvinyl pyrolididne in 0.1 M PBS (pH 7.4) solution] and stored at -20°C to later undergo immunohistochemistry.

3.2.8. C-Fos immunohistochemistry.

Sections were washed and transferred to 0.3% hydrogen peroxide in 0.1 M PBS containing 0.2% Triton X-100 (PBST) for 10 min to inhibit endogenous peroxidase and then washed several times with PBST. Sections were incubated in PBST containing c-Fos rabbit polyclonal antibody (1:5000; Ab-5; Oncogene Science, UK) for 48 h at 4°C with periodic rotation. Sections were then washed with PBST and incubated in biotinylated goat anti-rabbit secondary antibody (diluted 1:200 in PBST; Vectastain; Vector Laboratories, USA) and 1.5% normal goat serum for 2 h at room temperature on a rotator. Sections were then washed and processed with avidin-biotinylated horseradish peroxidase complex in PBST (Elite Kit; Vector Laboratories, USA) for 1 h at room temperature, again with constant rotation. Sections were washed again in PBST and then in 0.05 M Tris buffer. The reaction was then visualised using 3', 3'–diaminobenzidine intensified with nickel chloride. Sections were mounted and allowed to dry overnight before being dehydrated via graded series of alcohol washes and coverslipped.

3.2.9. Image analysis

Photomicrographs of immunolabelled brain sections were captured at 10x objective using a BX60 microscope (Olympus, Japan) and RTKE SPOT camera (Diagnostic Instruments, USA) interfaced to a PC computer with SPOT imaging software. Counts of stained nuclei were carried out using the public domain Image J program (National Institutes of Health, USA). C-Fos immunohistochemistry were automatically counted using Image J. Images were digitized into grey scale where a threshold, set above the mean value ± SEM. of the background, was applied for background correction. Inside each region, the number of particles above the threshold was automatically calculated. There were no observed rostrocaudal differences in all brain regions analysed.

3.2.10. Regions of interest

A total of 7 regions were analysed with sites selected because they have been implicated previously in memory processes. All of the sites from which it was decided *a priori* to count Fos positive cells are presented. For each brain region analysed, counts were taken from a minimum of seven coronal sections. Cytoarchitectonic subfields within the hippocampal formation consisted of the cornu ammonis area 1 (CA1), cornu ammonis area 2/3 (CA2/3) and dentate gyrus (DG) of the HPC were investigated. Hippocampal counts were taken at interaural 5.28 mm and bregma -3.72 mm in Paxinos and Watson rat brain atlas (Paxinos and Watson, 2009). Fos immunoreactive cells were counted in the prelimbic area (PrL), cingulate cortex (Cg), and infralimbic cortex (ILC) corresponding to interaural 12.00 mm and bregma 3.00 mm (Paxinos and Watson, 2009). The striatum were counted at levels corresponding to interaural 11.04 mm and bregma 2.04 mm (Paxinos and Watson, 2009). The striatum was counted by sampling 3 areas of each section and a single value was obtained by averaging the 3 counts.

3.2.11. Statistical analysis

All data are presented as mean ± SEM. A p-value of < 0.05 was considered statistically significant. Statistical comparisons were made between groups by repeated measures two-way ANOVA for body weights, DWSh performance data using GraphPad Prism. Two-way ANOVA was used for Fos counts in basal and after environmental novelty conditions in the PFC and HPC. Unpaired t-tests assessed epididymal adipose tissue weights, Fos counts in the striatum. Further analysis by a post hoc Bonferroni's t-test was performed if a significant main effect was detected by the ANOVA.

3.3. Results

3.3.1. Animals

Rats fed a WD were observed to be heavier than rats fed a CON diet (Week 12, CON: 415.7 ± 9.8 g; WD: 458.2 ± 11.6 g; **Fig. 3.1A**; ($F_{(1,18)}$= 7.2, p< 0.05)). Both groups showed a similar weight increase over time (time: ($F_{(12,264)}$= 332.7, p< 0.0001) and WD rats increased their body weight at a more pronounced rate than CON, (group x time: $F_{(12,264)}$= 3.5, p< 0.01). Post hoc analysis showed significant body weight differences starting from week 8 and until week 12. WD consumption was shown to increase epididymal adipose tissue with a significant increase in WD rats (CON: 8.2 ± 0.3 g; WD: 11.5 ± 0.6 g, p<0.001). WD consumption also increased epididymal fat mass by approximately 2 fold (p< 0.01).

3.3.2. Training phase performance in the delayed win-shift task

Both groups learnt to complete the DWSh task, had fewer errors and entered more correct arms before an error was recorded as training progressed. Performances of rats in the training

phase of the delayed win-shift radial arm maze procedure are shown in **Figure 3.2**. A repeated measures ANOVA was conducted and revealed that both CON and WD rats entered more arms as training progressed ($F_{(5,90)}$= 29.86, p< 0.0001), however there was no group ($F_{(1,18)}$= 0.63, p= 0.44) nor group x block effect ($F_{(5,90)}$= 1.40, p= 0.23; **Fig. 3.2A**). During training, rats steadily increased the number of correct arm choices over blocks (Block effect: $F_{(5,90)}$= 36.34, p< 0.0001) but no group or group x block effect (Both F-values < 1, **Fig. 3.2B**). As training progressed both CON and WD animals became more proficient in the task as the animals made more correct arm choices before an error was recorded (Block effect: $F_{(5,90)}$= 27.78, p< 0.0001; **Fig. 3.2C**).

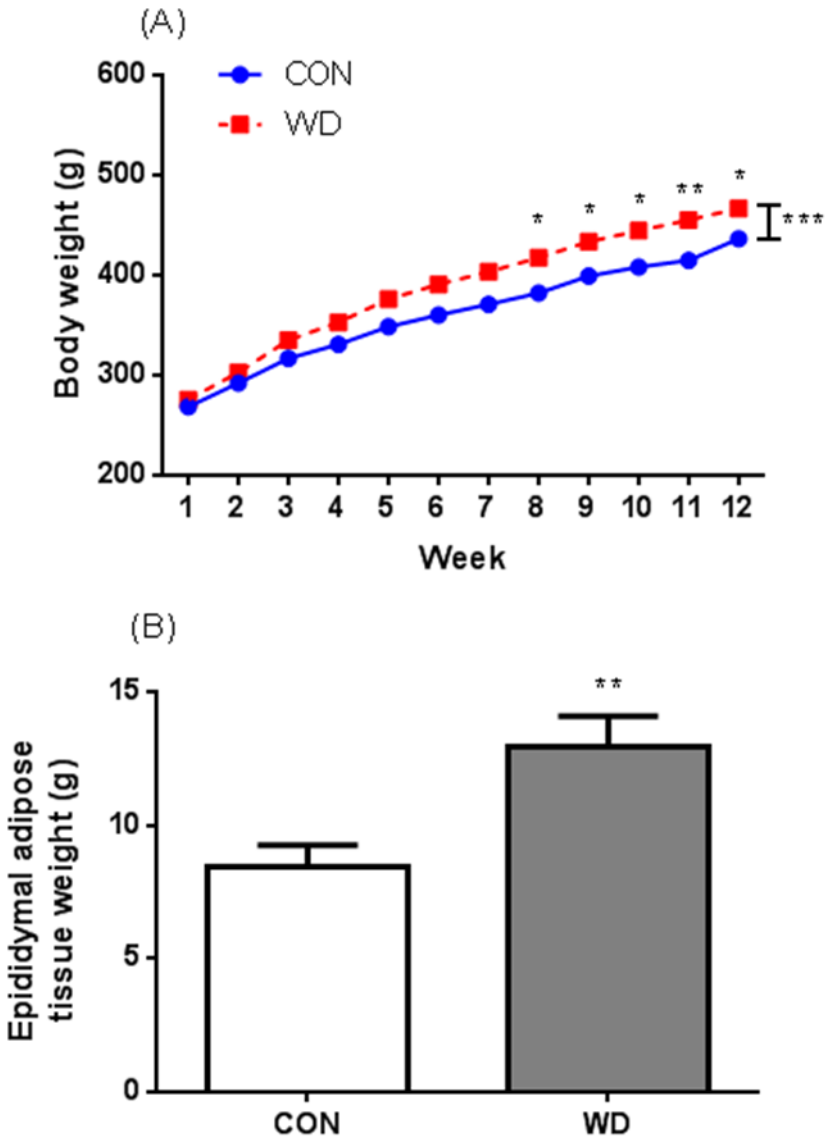

Figure 3.1. Metabolic measures. **(A)** Body weights of rats fed the control or WD for 12 weeks. **(B)** Epididymal adipose tissue weight in both control and WD fed rats. n= 10 per group. Data represented as mean ± SEM, *p< 0.05, **p< 0.01, ***p< 0.001.

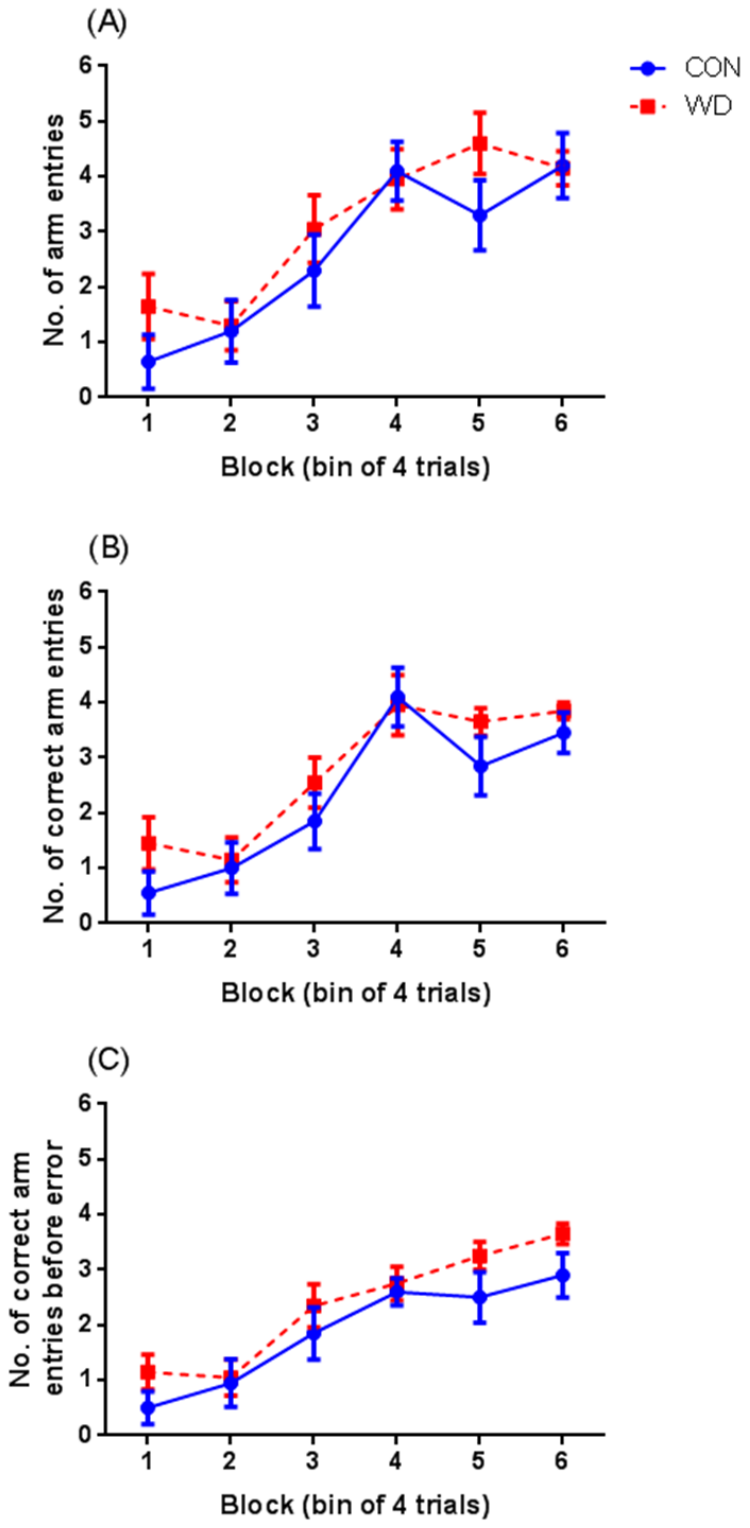

Figure 3.2. Performance in training phase of DWSh task. **(A)** Number of arm entries in each session of training. **(B)** Number of correct arm entries in each session of training. **(C)** Number of correct arm entries before error in each session of training. n= 10 per group. Data expressed as mean ± SEM.

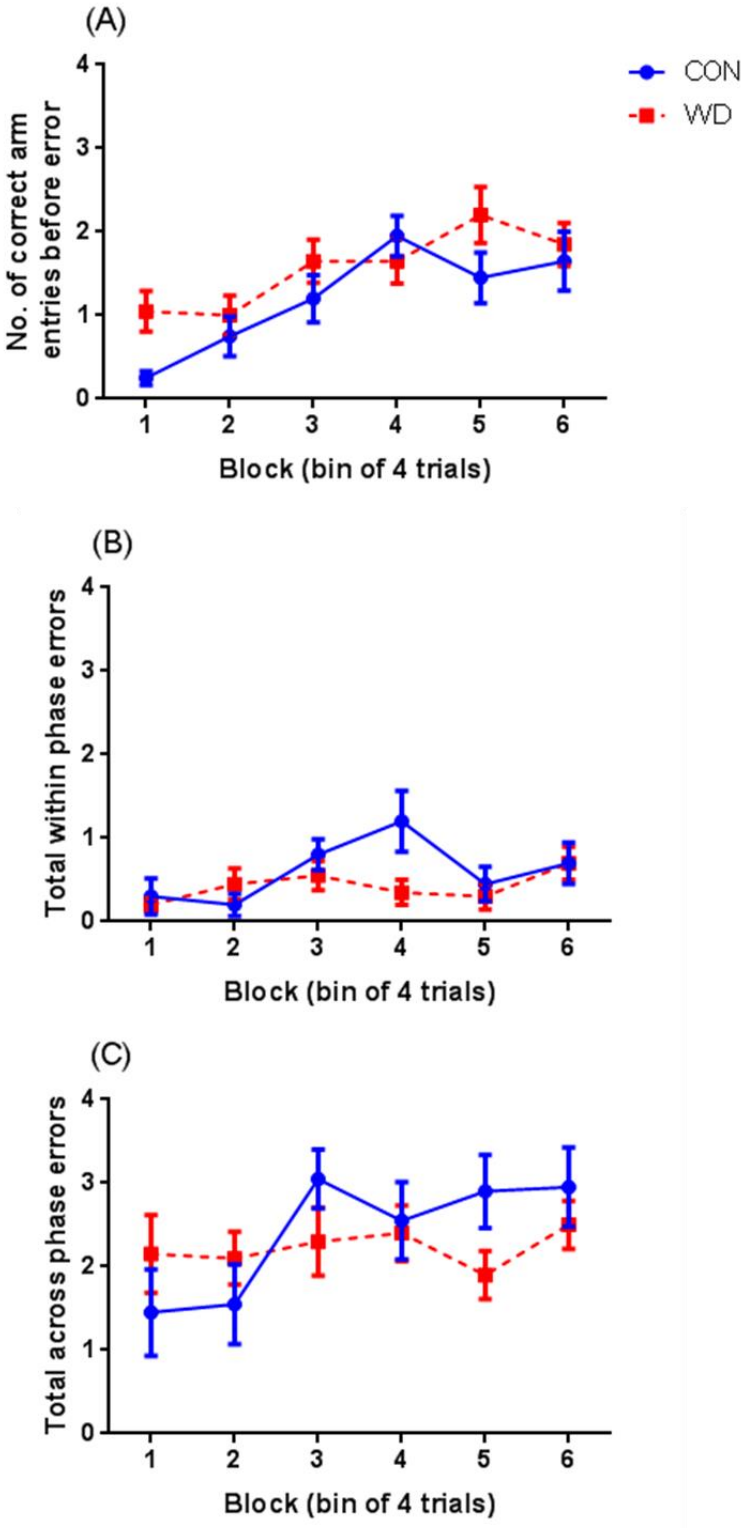

Figure 3.3. Performance in test phase of DWSh task. **(A)** Number of correct arm choices before error in each session of training. **(B)** Total number of within phase errors in each session of training. **(C)** Number of across phase errors in each session of training. n= 10 per group. Data expressed as mean ± SEM.

3.3.3. Test phase performance in the delayed win-shift task

During the test phase, WD animals did not show any evidence of cognitive impairment relative to CON. The number of correct arm choices before an error was made steadily increased as training progressed (Block effect: $F_{(5,90)}= 10.41$, $p< 0.0001$; **Fig. 3.3A**) and there was an overall effect of block to influence total within phase errors ($F_{(5,90)}= 2.79$, $p= 0.02$; **Fig. 3.3B**) but there was no other significant differences in any other measure including group.

3.3.4. Basal neuronal activation

Table 3.1 shows the expression of basal Fos in the regions analysed after 12 week WD consumption. In the PFC subregions a two way ANOVA showed there was no effect of diet group [$F_{(1, 30)}= 0.95$, $p= 0.34$], no effect of sub-region [$F_{(2, 30)}= 2.15$, $p= 0.13$] nor a diet x sub-region effect [$F_{(2, 30)}< 1$, $p= 0.56$] on home cage control Fos expression in the Cg, IL, and PrL sub-regions. Analysis of home cage control Fos in the striatum revealed no significant effect of diet ($p= 0.59$, t= 0.56, df= 10). The initial analysis of the HPC involved separate counts taken across subfields (CA1, CA2/3, and DG). Overall, there was no observed difference in home cage control Fos expression due to diet (group effect: [$F_{(1, 30)}< 0.1$, $p= 0.99$]). There was a significant difference due to hippocampal subfield [$F_{(2, 27)}= 7.06$, $p= 0.003$] however post-hoc analysis revealed no further differences. No diet x hippocampal subfield effect was observed [$F_{(2,27)}< 0.1$, $p= 0.92$].

Table 3.1: Number of positively stained Fos immunoreactive cells of home cage control fed CON or WD.

BRAIN REGION	**CON (n= 6)**	**WD (n= 6)**
Prefrontal cortex		
Cingulate gyrus (Cg)	12.04 ± 2.03	12.83 ± 0.82
Infralimbic cortex (ILC)	17.92 ± 2.77	15.46 ± 2.11
Prelimbic cortex (PrL)	18.88 ± 4.59	16.63 ± 1.86
Striatal area		
Striatum	117.50 ± 17.84	102.50 ± 19.78
Hippocampus		
Cornu Ammonis area 1 (CA1)	61.70 ± 12.86	57.79 ± 19.01
Cornu Ammonis area 2/3 (CA2/3)	209.95 ± 55.71	196.13 ± 48.25
Dentate gyrus (DG)	116.70 ± 22.26	134.25 ± 44.27

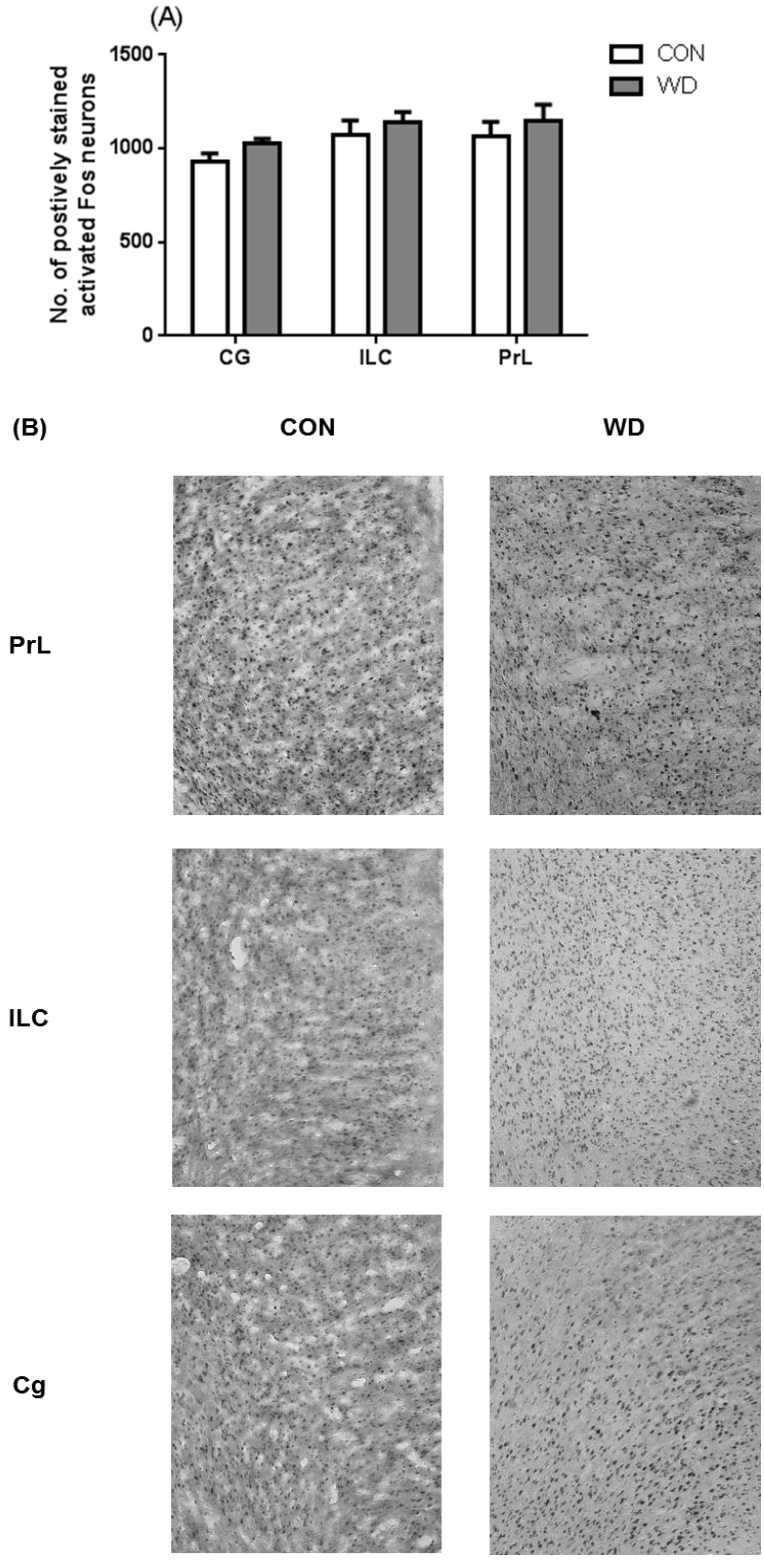

Figure 3.4. Fos immunohistochemistry after environmental novelty. **(A)** Number of positively stained Fos neurons in the PFC. **(B)** Representative photomicrographs of Fos immunoreactivity in the PrL, ILC and Cg of the PFC from rats fed a CON or WD at 100x magnification. n= 5-7 per group. Data expressed as mean ± SEM.

3.3.5. Neuronal activation in response to novelty

Environmental novelty increased neuronal activation in striatum but not PFC or HPC. In the PFC subregions there was no effect of diet ($F_{(1,33)}$= 2.36, p= 0.13; **Fig 3A**), subfield ($F_{(2,33)}$= 2.36, p= 0.09) or diet x subfield effect ($F_{(1233)}$< 1, p= 0.98) on numbers of cells expressing Fos 2 hr after exposure to a novel environment in the Cg, IL and PrL regions group.

Similarly, there were no differences between the diet groups in the HPC subfields (CA1, CA2/3, and DG; diet effect: ($F_{(1,30)}$= 3.67, p= 0.65; **Fig. 3C**) or a diet x subfield: ($F_{(2,33)}$= 2.61, p= 0.09. There was a subfield effect ($F_{(2,33)}$= 41.63, p< 0.0001) which was attributable to a significantly lower amount of activated Fos neurons in the CA1 for both CON and WD groups when compared to the CA2 and DG regions.

In the striatum, however, WD significantly increased novelty-induced neuronal activation compared with control diet (p< 0.05, t= 2.89, df= 10; **Fig. 3B**).

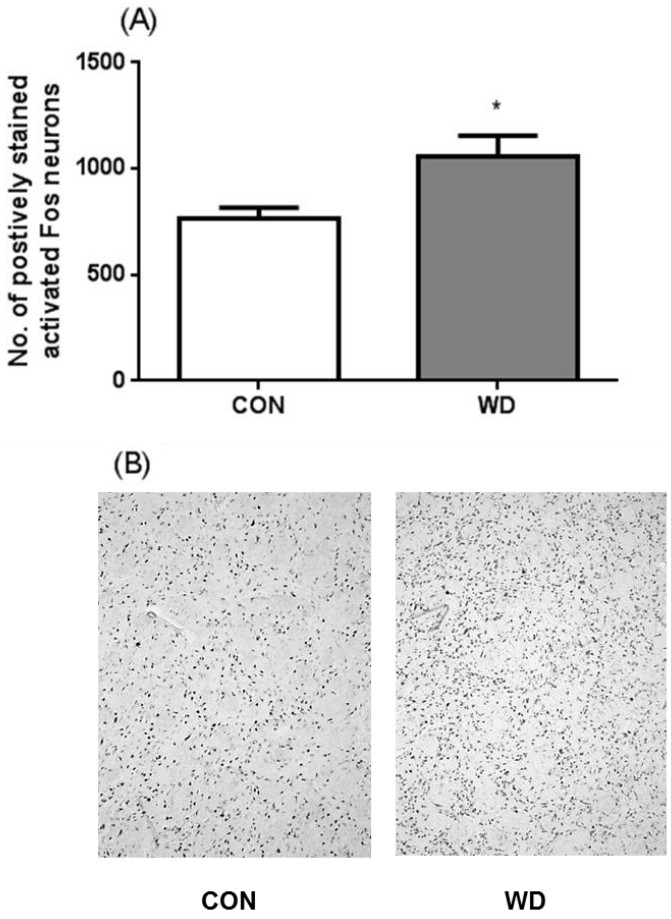

Figure 3.5. Fos immunohistochemistry after environmental novelty. **(A)** Number of positively stained Fos neurons in the striatum. **(B)** Representative photomicrographs of Fos neurons in the striatum in CON and WD fed rats at 100x magnification. n= 6-7 per group. Data expressed as mean ± SEM, *p< 0.05.

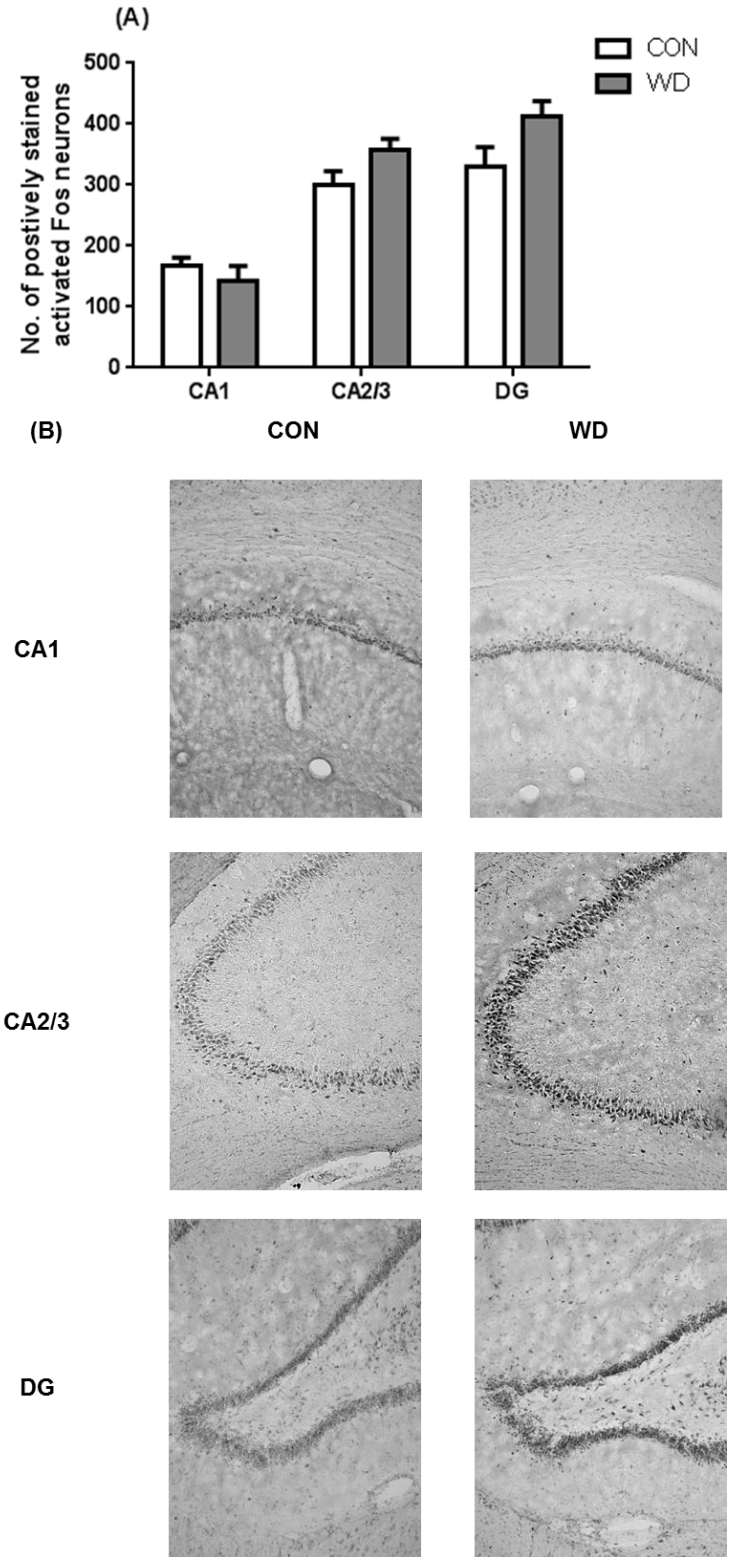

Figure 3.6. Fos immunohistochemistry after environmental novelty. **(A)** Number of positively stained Fos neurons in the HPC **(B)** Representative photomicrographs of positively stained Fos neurons in the CA1, CA2/3 and DG of the HPC in CON and WD fed rats at 100x magnification. n= 6-7 per group. Data expressed as mean ± SEM.

3.4. Discussion

In this study we investigated the effect of WD on cognitive performance using the DWSh in the RAM in adult male Wistar hooded rats. Also using Fos as a marker, this study mapped how WD consumption does not alter the neuronal activation that normally accompanies exposure to a novel environment, except in the striatum. Our findings indicate that WD consumption does not affect learning and performance in the DWSh task, a PFC, hippocampal and striatal-dependent memory task. Basal Fos immunoreactivity was also unchanged with WD consumption in regions of interest. In a further series of animals WD consumption was observed to be associated with an increase in activated Fos expression in the striatum after exposure to a novel environment.

Using the DWSh task we failed to detect any impairment after WD consumption. Similar acquisition rates and spatial memory ability were observed in WD rats as the CON rats. This is in contrast to our previous study with this dietary manipulation where we showed impairment in spatial memory in the Y-maze, a one trial-one test procedure (Kosari et al., 2012). In the MWM, female Fisher 344 rats fed a diet with higher fat (approximately 39 kcal%) and similar sugar content to that in our study for 2 months displayed an impairment of spatial reference memory (Molteni et al., 2002). The radial arm water maze is known for combining the simplicity of results analysis from the RAM with the rapid and strong motivation observed in the MWM without the food deprivation (Alamed et al., 2006). Using the radial arm water maze, Alzhoubi et al. illustrated that male Wistar rats fed a similar WD for 3 months produced an impairment of short and long term spatial memory (Alzoubi et al., 2013a). Researchers have also reported impairments of spatial memory following high fat consumption using other one trial-one test behavioural tasks. Arnold et al. reported spatial memory impairments using the T-maze spontaneous alternation task in C57BL/6J mice fed

45 kcal% fat diet for 8 weeks (Arnold et al., 2014), whilst a 60 kcal% fat diet for 27 weeks, produced an impairment of spatial reference memory in the object location task (Heyward et al., 2012). Of note between these studies, where a memory deficit was observed, and our present one, is a variation in food. Our DWSh paradigm prompts the animals to solve the task using food pellets as a reward, and for both WD and CON animals we used the same "control" grain pellets. A consequence of this is that WD animals, if they solved the task successfully, consumed approximately sixteen 45 mg pellets/day during habituation and testing that were not of WD composition. To address the issue of additional energy intake a, saccharin based reward could be used as it effectively has no food energy. Moreover, throughout the task animals were on food restriction, albeit of their WD or CON food, meaning that for the final 15 days all rats received less of their specific diet than the previous 12 weeks of *ad lib* feeding. These two methodological points may have resulted in our WD animals becoming normalized, and thus attributable to the lack of deficit in this task.

We demonstrate that 12 week WD consumption increases Fos expression after environmental novelty in the striatum in response to a novel environment. Other brain regions involved in memory and learning were also investigated with no comparable differences in Fos expression after environmental novelty in the PFC and HPC between control and WD animals. Much research performed to date has focussed on investigating diet-induced neuronal activation in the hypothalamus due to its well-recognised role in the regulation of food intake and energy homeostasis (Bray et al., 1981). C57BL/6J mice fed a 58 kcal% fat diet for 15 weeks display increased expression of basal Fos in the lateral hypothalamus (Lin and Huang, 1999, Xin et al., 2000), dorsal medial hypothalamus (Lin and Huang, 1999, Xin et al., 2000), and paraventricular nuclei (Wang et al., 1999). A study in female Long-Evans rats fed a 40 kcal% fat diet for 12 weeks also demonstrated an increase of Fos expression after environmental novelty in the hypothalamic paraventricular nuclei induced by

introduction into a novel environment (Ressler et al., 2015). In a further study, acute HFD (21 kcal% fat) consumption in C57BL/6J mice elicited an increase of Fos expression after environmental novelty not only in the lateral hypothalamus but also the ventral tegmental area, nucleus accumbens, and central amygdala (Valdivia et al., 2014), suggesting that acute HFD consumption recruits the mesolimbic system. This finding is corroborated by Del Rio *et al.* who demonstrated that the dorsal medial PFC was selectively increased Fos immunoreactivity after environmental novelty in response to acute HFD consumption (Del Rio et al., 2015).

Whilst the stimulus used to induce activation of Fos expression was the introduction of the animal into a novel environment, our group has not observed any differences of exploration time in novel environments with this model (Kosari et al., 2012). This indicates that the increase of neuronal activation is due to the diet manipulation and not the stimulus, in this case new surroundings. However, it should be considered that there may also be an interaction between the experience of stress and the western diet. In the water maze RAM study (Alzoubi et al., 2013a), the combination of stress and western diet resulted in the strongest impairment in the memory test, suggesting that stress may exacerbate the effect of diet.

The striatum is interconnected to many brain regions that have been implicated in learning, memory and reward such as the PFC, HPC, amygdala and substantia nigra (Bjorklund and Dunnett, 2007, Kelley and Berridge, 2002). These connections are in part through afferent/efferent dopaminergic cell bodies in the mesolimbic, mesocortical and nigrostriatal pathways (see (Bentivoglio and Morelli, 2005) for a comprehensive review). Striatal D2 receptor expression has been previously shown to be decreased after consumption of a HFD in both mice and rat models of diet-induced obesity (Huang et al., 2006, Johnson and Kenny,

2010, van de Giessen et al., 2012). One study also found a clear inverse association between body weight and striatal D2 receptor expression suggested to be due to a down regulation of postsynaptic striatal dopamine-2/3 receptors (Huang et al., 2006, Johnson and Kenny, 2010, van de Giessen et al., 2012).

In conclusion our present results expand on the known relationship between obesity and central effects. We have also extended the previous neuronal activation data largely focused around the hypothalamus to show that WD-manipulation in the rat produces an upregulation of striatal neuronal activation. As this data was collected from a novel environment paradigm of much interest would be to expand this to assess neuronal activation during memory tests where a cognitive deficit is observed. Whether WD manipulation, or indeed fat manipulation, is the ideal model to assess obesity-associated cognitive decline is still contentious; indeed our Fos data is independent of a deficit in memory using the DWSh task. While we have previously shown cognitive deficits along with metabolic changes with this model (Kosari et al., 2012) it is not universal; indeed no deficit was observed in working memory in the novel object recognition task (Kosari et al., 2012), as no deficiency was observed here. It is clear that the biological contribution to obesity in humans involves numerous factors beyond fat, including the more palatable sugar, inactivity, along with broader factors, such as genes and mood, and these are yet to be considered or produced in a single animal model.

In conclusion, WD consumption was not observed to affect spatial working and reference memory using the DWSh task in the RAM. Furthermore, the present study is the first to demonstrate that WD consumption increases Fos expression in the striatum following a novel environment stimulus.

Chapter 4 - Associated changes in serotonin receptor expression and dopamine levels in a western diet induced animal model of obesity

4.1. Background and rationale

There are a multitude of central pathological changes associated with obesity, as discussed in Chapter 1.11. Nonetheless the possible underlying mechanism by which obesity impairs cognitive function is still undetermined. 5-HT and DA are important neurotransmitters for amongst other functions, food intake and cognition. Both 5-HT2_A and 5-HT2_C receptors have been implicated in feeding and cognition. Pharmacological manipulations that increase brain 5-HT levels reduce food intake (Blundell, 1986, Leibowitz et al., 1988) and also impair visual working memory (Jans et al., 2010, Lieben et al., 2004). Conversely depleting 5-HT via tryptophan (precursor to 5-HT) depletion increases food intake (Nonogaki et al., 1998) and impairs both object recognition memory (Jenkins et al., 2010) and fear contextual memory (Uchida et al., 2007). A 5-HT_{2A} receptor agonist injected into the PVN attenuates neuropeptide Y induced hyperphagia (Grignaschi et al., 1996), while injection of the 5-HT_{2C} receptor antagonist, RS-102221, produces hyperphagia (Bonhaus et al., 1997). The serotoninergic system may mediate changes in food intake and/or cognitive state when nutritional state is altered. Zucker fatty rats have depressed hypothalamic 5-HT activity (Routh et al., 1990, Routh et al., 1994) and DIO rats have been shown to have decreased levels of 5-HT in the HPC and brainstem (Kimbrough and Weekley, 1984). 5-HTT regulates the entire serotoninergic system and its receptors by modulation of 5-HT concentration by 5-HT reuptake from the synaptic cleft back into the presynaptic neuron. Of the limited research on 5-HTT expression and obesity, focus has mainly been on the hypothalamus due to its recognised role in the regulation of food intake. Nonetheless the effect of obesity in other brain regions besides the hypothalamus, such as the striatum, is relatively unknown.

DA has a well-recognised role in cognition including motivation, reward, punishment and working memory (Cools, 2008). But recent research has discovered the involvement of DA

with obesity (Volkow et al., 2012, Volkow et al., 2013). It has been postulated that individuals that have a hypo-responsive mesocorticolimbic pathway have an increased risk of the development of obesity (Davis et al., 2004b). Sensitivity to reward has been associated with emotional overeating, preference for HF foods, binge eating and food cravings (Davis et al., 2004b, Davis et al., 2007, Franken and Muris, 2005, Loxton and Dawe, 2001). HFD consumption was shown to decrease brain reward threshold, and this effect was mediated by downregulation of the D2 receptor (Johnson and Kenny, 2010). A HF cafeteria diet was also shown to lower both basal levels of DA and DA release in response to food or amphetamine (Geiger et al., 2009). A consistent finding is the reduction of DA levels in the nucleus accumbens in animal models of obesity including *ob/ob* mice (Fulton et al., 2006), DIO rats (Geiger et al., 2009, Pothos et al., 1998), cafeteria diet model (Geiger et al., 2008) and HFD exposed mice (Carlin et al., 2013). HFD exposure even as short as 5 days has been shown to reduce basal DA levels in the nucleus accumbens (Rada et al., 2010). However, we do not know whether HFD feeding produces alterations of DA levels beyond the nucleus accumbens.

Our group has previously showed that HFDs (22 and 60 w/w %) have a deleterious effect on spatial memory, but do not affect hippocampal acetylcholine measures, suggesting that the brain cholinergic system does not change with this diet or play a major role in the observed spatial memory deficits (Kosari et al., 2012). However, HFD consumption has been shown to affect hippocampal neurons with several experiments finding altered dendritic morphology (Freeman et al., 2011, Granholm et al., 2008) and impaired synaptic plasticity after HFD consumption (Molteni et al., 2002, Stranahan et al., 2008), indicating the HPC is particularly sensitive to dietary manipulation. The PFC (Pasupathy and Miller, 2005, Winocur and Moscovitch, 1990, Xu, 2015) and striatum (Pasupathy and Miller, 2005, Scimeca and Badre,

2012) also play an integral role in learning and memory. However, an assessment of neurotransmitter action in these regions after HFD is lacking.

Considering the previous findings of interactions of HFD feeding with 5-HT and DA, we hypothesized that WD consumption model of obesity would alter 5-HT receptor expression in the striatum and DA levels in the PFC, striatum and HPC. These changes in neurotransmission may also contribute to cognitive impairments observed in obesity models. To investigate this hypothesis we investigated spatial memory by means of the spontaneous alternation behavioural test in the WD induced model of obesity. We also determined 5-HT receptor and 5-HTT expression in striata using western blotting. Additionally we measured DA levels in the PFC, striatum and HPC from WD exposed rats relative to CON.

4.2. Methods

4.2.1. Animals

A group of male Long-Evans rats (n= 12 per group, Monash University, Australia) weighing 255-288 g at the start of experimentation underwent spatial alternation behaviour testing, open field testing and subsequent western blot analysis. A second group of rats (a subset from Chapter 3 cohort) used for brain HPLC analysis. Body weights and epididymal adipose tissue weights previously described in Chapter 3.

Rats were housed at RMIT University animal facility, a controlled environment (20 ± 1°C) with 12-h light/dark cycle (lights on at 07:00 h) in groups of up to 4, with food and water *ad libutum* in the home cage. The experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University

Animal Ethics Committee. At the end of the experiment, animals were sacrificed and epididymal adipose tissue was collected and weighed.

4.2.2. Dietary manipulation

All animals were allowed to acclimatise after delivery for at least 1 week before commencement of dietary manipulation. Rats were fed one of two diets: either CON diet (SF04057, Speciality feeds, Australia) or WD (SF00-219, Speciality feeds, Australia) for a period of 12 weeks. The nutritional content of each diet is as seen in **Table 2.1.**

4.2.3. Spontaneous alternation behaviour in the Y-maze

Spontaneous alternation behaviour is the tendency for rodents to alternate their non-reinforced choices of Y-maze arms and is based upon the tendency for rats to alternate their choices to enter non-reinforced arms of a maze on successive opportunities (Drew et al., 1973). The phenomenon has been ascribed to the operation of a variety of mechanisms including habituation to novelty (Hughes, 2004), foraging strategies (Estes and Schoeffler, 1955) and spatial working memory (Hughes, 2004). Successful spontaneous alternation behaviour is dependent on the HPC (Johnson et al., 1977, Means et al., 1971), septum (Clody and Carlton, 1969, Douglas and Isaacson, 1966), basal forebrain, PFC and dorsal striatum (Lalonde, 2002). After 12 weeks of dietary manipulation rats (n= 12 per group) underwent the spontaneous alternation test in the Y-maze. Spontaneous alternation behaviour is a measure of exploratory behaviour and can be used to assess spatial memory. The Y-maze was a three-arm maze with equal angles between all arms (50 cm long ×17 cm wide × 32 cm high). Rats were placed in the Y-maze for 5 min during which the rats were allowed to freely explore. Activity was videotaped using a Legria FS200 digital video camcorder (Canon, Japan) and later scored. Number of arm entries and the sequence of arm entries was measured. Alternation was determined from successive entries of the three arms on

overlapping triplet sets in which three different arms are entered. Results from this test are expressed as percentage alternation which was calculated by:

$$\frac{\text{Number of alternations x100}}{\text{Total arm entries} - 1.}$$

4.2.4. Open field test

Locomotor activity was assessed as described previously in Section 2.2.8. In brief, rats were placed individually in the test chamber for 10 min to monitor locomotor activity. Total distance travelled and average velocity was measured by Med Associates activity monitor software, version 4.

4.2.5. Sample preparation

Following behavioural testing, rats were killed by 0.5 ml i.p injection of pentobarbital sodium. Brains were snap frozen in iso-pentane cooled to -35°C by dry ice then stored at -80°C. Whole striata, hippocampi and prefrontal cortices were dissected on ice with a scalpel and the Paxinos and Watson rat brain atlas to delineate brain regions (Paxinos and Watson, 2009).

4.2.6. Western blotting

The striata from the first cohort of rats (n= 4-5 per group) underwent western blotting. Tissue was homogenised in lysis buffer (10 mM Tris-hydrochloride, 150 mM sodium chloride and 1% Triton-X100, pH 7.4) with supernatant collected to be used for further analysis. Total protein content of brain samples was determined via bicinchoninic acid protein assay (Thermo Fisher Scientific, USA) with bovine serum albumin as standard. Brain samples were suspended in Laemelli's sample buffer (40% glycerol, 20% 2-mercaptoethanol, 8% sodium dodecyl sulfate, 250 mM Tris, 0.01% Bromophenol blue, pH 6.8) then heated at 95°C for 15

min. Samples were then frozen at -20°C until use. Seventeen ug of protein from brain samples were loaded per lane and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels. The SDS-PAGE gel was subjected to electrophoresis at 110 V for 90 min at room temperature. Separated proteins were transferred onto a nitrocellulose membrane using wet method at 60 V for 2.5 h at 4°C. Nitrocellulose membrane and SDS-PAGE gel were sandwiched between filter blot paper and fibre pads which was then placed into a cassette and immersed in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 7.4). Non-specific binding was blocked by incubating membrane for 1 h in 5% skim milk protein in Tris-buffered saline-Tween (TBST, pH 7.4) for 1 h at room temperature. After blocking, the membrane was incubated in 1:1000 dilutions of either; 5-HT_{2C} (ab32887, Abcam, UK), 5-HT_{2A} (ab16028, Abcam, UK) or 5-HTT (ab1772, Millipore, USA) antibodies in TBST and 1% skim milk, pH 7.4 at 4°C overnight. Primary antibodies were then detected with Alexa-conjugated secondary antibodies (1:1000; Invitrogen, USA) in TBST, pH 7.4 for 1 h. Target protein expression levels were normalised to chicken anti β-actin (1:1000, Abcam, UK) used as a loading control. Blots were visualized using FluoroChemQ MultiImage III (Alpha Innotech, USA), and analysed using Image J Software.

4.2.7. High performance liquid chromatography analysis

Prefrontal cortices, striata and hippocampi were assessed for DA and dihydroxyphenylacetic acid (DOPAC; DA metabolite) levels from home-caged controls described in Chapter 3, n= 5 per group. Samples were homogenised in extraction buffer (4 M perchloric acid, 0.008 M sodium metabisulphate, 0.002 M disodium ethylenediaminetetraacetic acid (EDTA) and MilliQ water to bring volume to 100 ml) and sonicated to rupture vesicular membranes. Samples were then spun at 10,500 g for 5 min, and the supernatant transferred to a fresh tube.

The samples were spun a further two times, to ensure all debris was eliminated. Samples were stored at -80°C until required.

For high performance liquid chromatography analysis (HPLC) analysis, 40 µl of sample was transferred to a HPLC recovery vial. Standards for DA and DOPAC were made in the same extraction buffer used for sample preparation. The mobile phase was composed of 70 mM monopotassium phosphate, 0.5 mM EDTA disodium salt, 8 mM octane sulfonic acid sodium salt, 170 ml HPLC grade methanol, to a final volume of 1000 ml and pH 4.2. The flow rate was 500 µl/min with reverse phase C18 columns. HPLC analysis was conducted on PFC, striatal and HPC samples from rats fed either CON diet or WD for 12 weeks for total (intracellular and extracellular) DA and DOPAC levels. HPLC analysis was conducted on PFC, striatal and HPC samples from rats fed either CON diet or WD for 12 weeks for total (intracellular and extracellular) DA and DOPAC levels. Standards of known concentrations for dopamine and DOPAC were used to quantify and identify the peaks on the chromatographs.

4.2.8. Statistical analysis

All data are presented as mean ± SEM. A p-value of < 0.05 was considered statistically significant. Statistical comparisons were made between groups by repeated measures two-way ANOVA for body weights using GraphPad Prism. Two-way ANOVA was used for comparing HPLC data while unpaired t-tests assessed all other data. Further analysis by post hoc Bonferroni's t-test was performed if a significant effect was detected by the ANOVA.

4.3. Results

4.3.1. Animals

In the first cohort, rats fed a WD were observed to be heavier than rats fed a CON diet (Week 12 CON: 345.5 ± 5.6 g; WD: 365.8 ± 6.7 g; **Fig. 4.1A**; ($F_{(1,22)}$= 4.0, p< 0.05)). WD rats increased their body weight at a more pronounced rate than CON, (group x time: $F_{(12,264)}$= 6.0, p< 0.0001). Both groups showed a similar weight increase over time (time: ($F_{(12,264)}$= 362.2, p< 0.0001). Post hoc analysis showed significant body weight differences starting from week 10 and until week 12. WD consumption was shown to increase epididymal adipose tissue weight with a significant increase in WD rats (CON: 7.1 ± 0.3 g; WD: 9.5 ± 0.4 g, p< 0.0001; **Fig. 4.1B**). No effect of WD on basal locomotor activity was observed (p> 0.05).

Body weights and epididymal fat tissue content have been previously described in Chapter 3 for the second cohort of rats. Briefly, WD consumption caused a significant increase of approximately 40 g in body weight and 3 g of epididymal fat mass content compared to controls **(Fig. 3.1).**

4.3.2. Spontaneous alternation task

WD consumption did not affect spontaneous alternation behaviour with similar number of arm entries (CON: 18.8 ± 1.5, WD: 19.2 ± 1.9, p= 0.84, t= 0.20, df= 22; **Fig. 4.2A**) and percentage alternation (CON: 25.6 ± 1.9 %, WD: 25.6 ± 1.7 %, p= 0.98, t= 0.03, df= 22; **Fig. 4.2B**).

4.3.3. Western blot analysis

WD exposure was observed to increase 5-HT_{2C} receptor by 33% (CON: 24.2 ± 2.3%, WD: 38.2 ± 6.3%, p= 0.0375; **Fig. 4.3A**) compared to controls. 5-HT_{2A} receptor expression was unchanged by WD consumption (CON: 69.9 ± 15.9%, WD: 93.2 ± 12.6%, p= 0.31; **Fig. 4.3B**), whilst 5-HTT expression was increased by 18% (CON: 62.4 ± 1.6%, WD: 73.9 ± 2.5%, p= 0.0081; **Fig. 4.3C**).

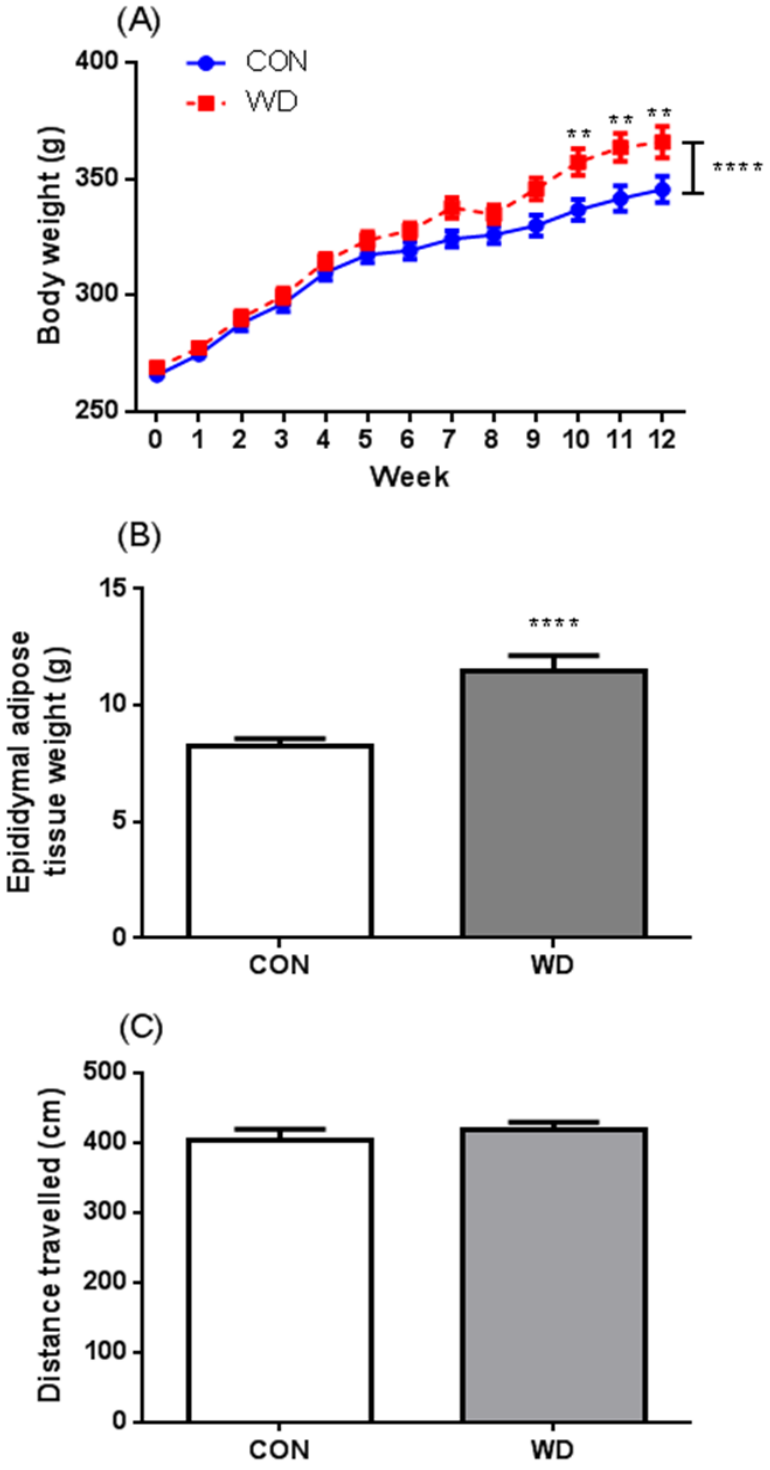

Figure 4.1. Metabolic measures. **(A)** Body weights of rats fed the control or WD for 12 weeks. **(B)** Epididymal adipose tissue mass content in both control and WD fed rats. **(C)** Basal locomotor activity. n= 12 per group. Data represented as mean ± SEM, **p< 0.01, ***p< 0.001.

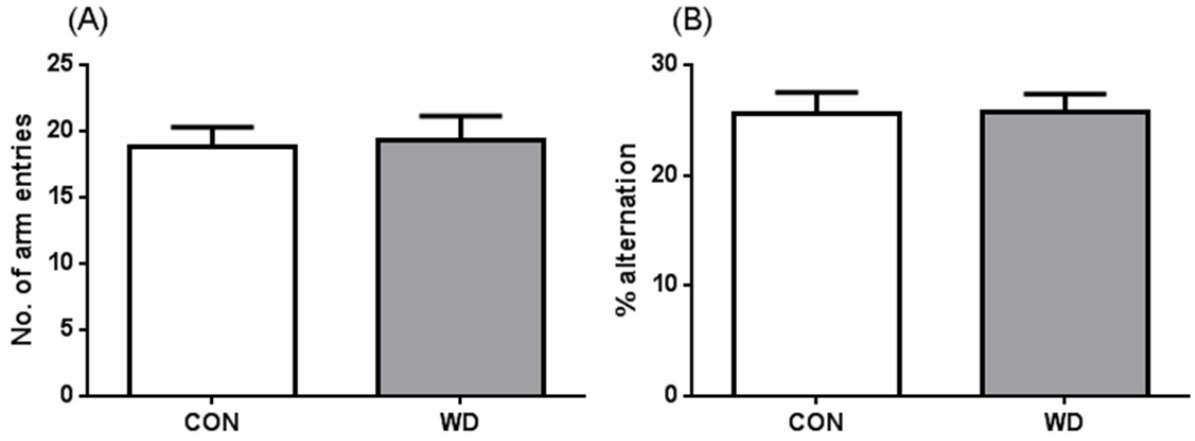

Figure 4.2. Spontaneous alternation behaviour in the Y-maze. **(A)** Total number of arm entries, **(B)** Percentage of arm alternation in CON and WD rats. n= 12 per group. Data expressed as mean ± SEM.

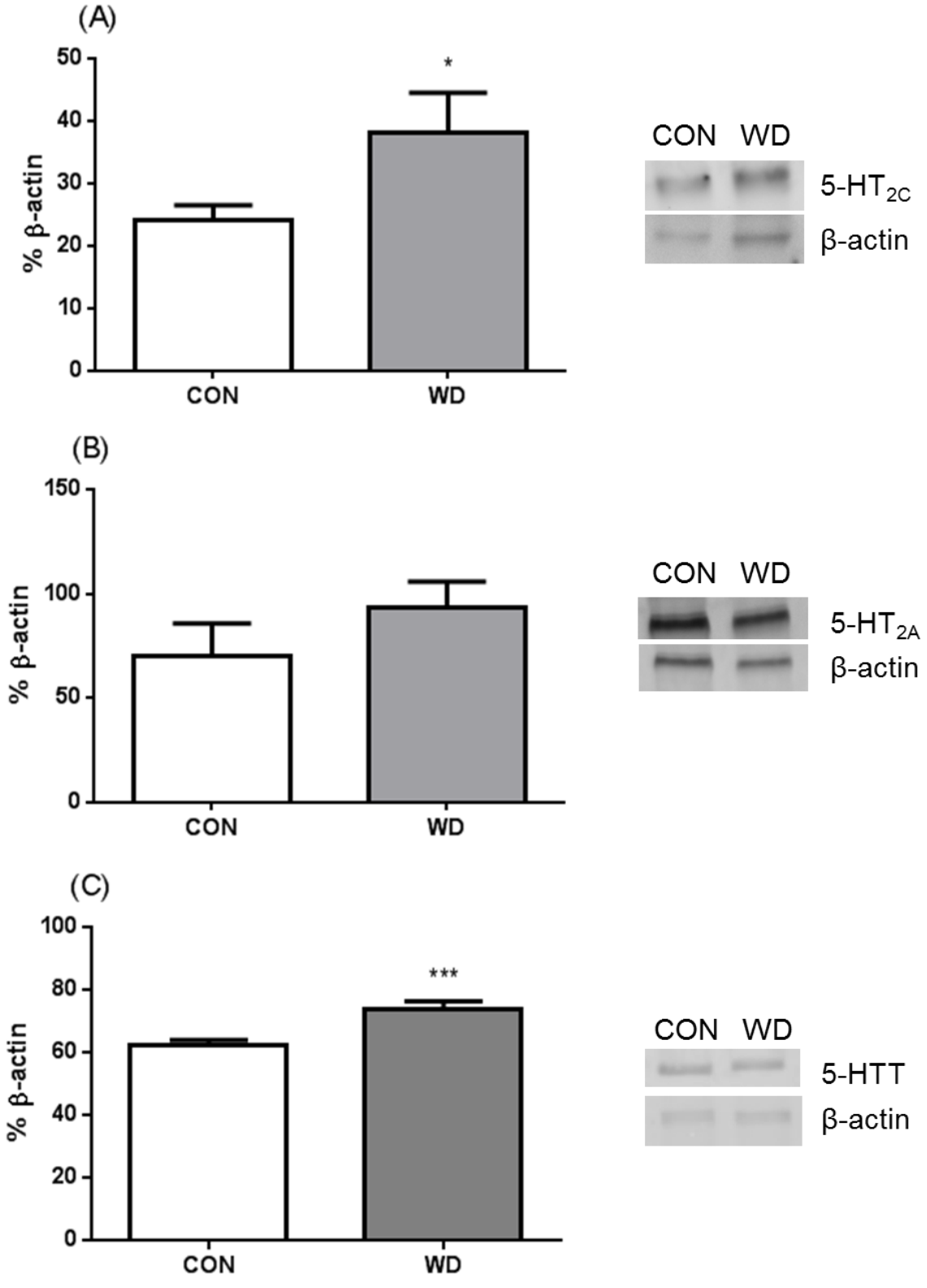

Figure 4.3. Representative western blots. **(A)** 5-HT$_{2C}$ expression, **(B)** 5-HT$_{2A}$ receptor expression, **(C)** 5-HTT expression in the striatum of CON and WD rats normalised to β-actin. n= 4-5 per group. Data represented as mean ± SEM. * $p< 0.05$, *** $p< 0.001$.

4.3.4. High performance liquid chromatography

In the PFC, WD consumption did not alter neurotransmitter levels (DA CON 34.01 ± 14.66 pmol/mg vs. WD 30.17 ± 3.66 pmol/mg and DOPAC CON 25.97 ± 11.24 pmol/mg vs. WD 19.43 ± 3.55 pmol/mg, $F_{(1,16)} < 1$, $p > 0.05$; **Fig. 4.4A**). No group or group x neurotransmitter effect was observed (all $F < 1$). DA turnover was also found not to be different between diet groups (CON 0.75 ± 0.06 vs. WD 0.65 ± 0.11, $p > 0.05$, t= 0.55, df= 8; Fig. **4.4D**).

WD feeding was observed to change DA and DOPAC levels in the striatum relative to CON with a significant effect of group [$F_{(1, 16)} = 10.63$, $p = 0.0049$], and differing amounts of neurotransmitter [$F_{(1, 16)} = 112.1$, $p < 0.0001$] which was attributable to a significantly higher amount of DA than DOPAC (DA CON 1651.59 ± 85.61 pmol/mg vs. WD 1370.88 ± 99.45 pmol/mg; DOPAC (CON 822.54 ± 52.02 pmol/mg vs. WD 615.23 ± 49.83 pmol/mg). No group x neurotransmitter interaction ($F < 1$) was observed. *Post-hoc* analysis showed a marked reduction in DA levels in the striatum relative to CON ($p < 0.05$; **Fig. 4.4B**), but not DOPAC levels ($p > 0.05$). The DA turnover rate in the striatum was not seen to be influenced by WD consumption (CON 0.50 ± 0.02 vs. WD 0.46 ± 0.05, $p > 0.05$, t= 2.88, df= 8; **Fig. 4.4E**).

In the HPC no differences were observed in neurotransmitter levels [$F_{(1, 16)} = 4.3$, $p = 0.06$], nor group x neurotransmitter ($F < 1$), while an overall group effect was observed [$F_{(1, 16)} = 4.9$, $p = 0.04$]. However, with *post-hoc* analysis no individual differences were seen with DA (CON 13.22 ± 3.81 pmol/mg vs. WD 9.51 ± 1.34 pmol/mg, $p > 0.05$) or DOPAC (CON 9.88 ± 2.81 pmol/mg vs. WD 2.68 ± 0.27 pmol/mg, $p > 0.05$) levels compared to CON (**Fig. 4.4C**). WD animals did have significantly reduced DA turnover relative to control (CON 0.78 ± 0.14 vs. WD 0.32 ± 0.07, $p < 0.05$, t= 2.55, df= 8; **Fig. 4.4F**).

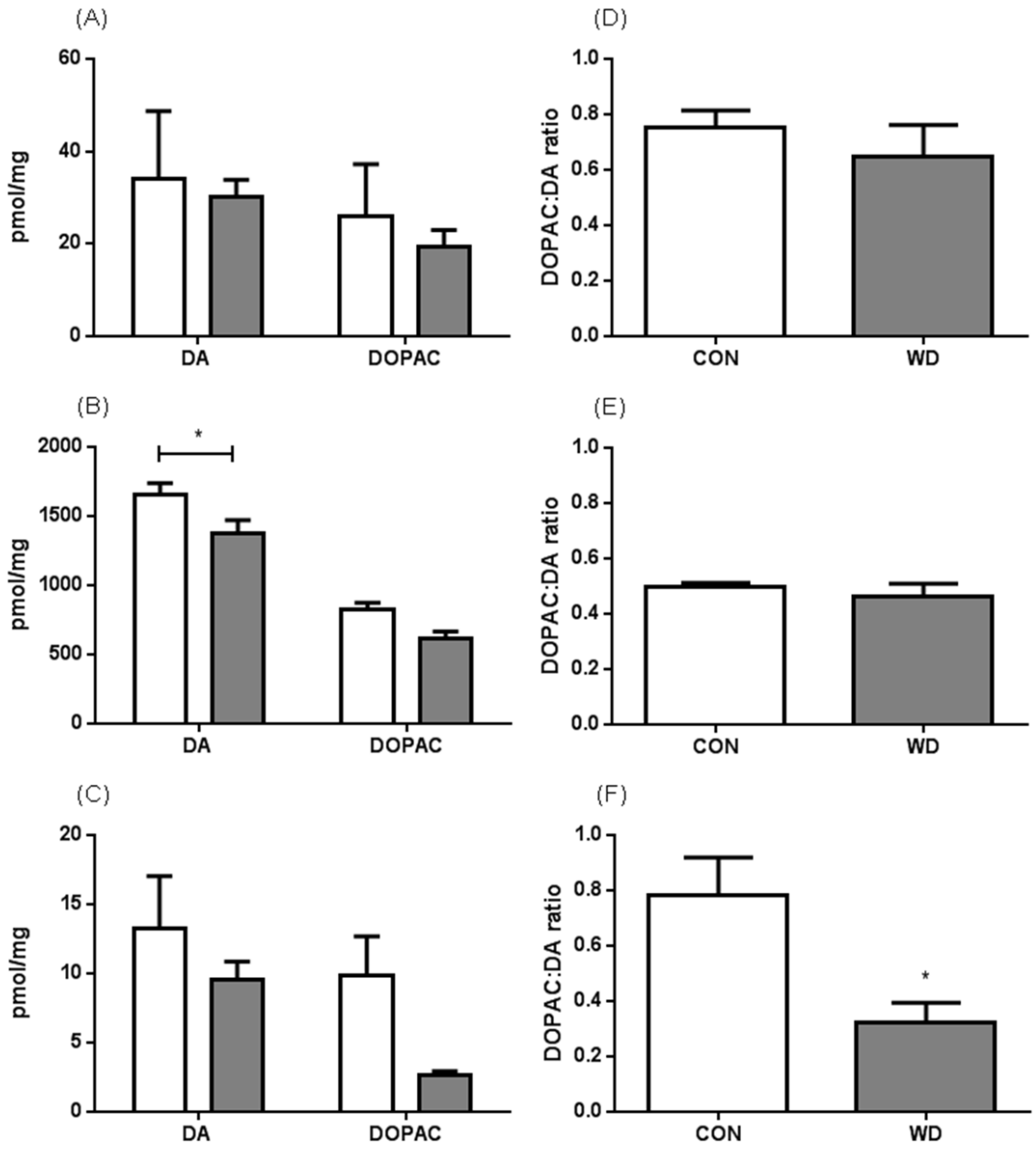

Figure 4.4. HPLC analysis in rats fed a WD compared to CON. **(A)** DA and DOPAC levels in the PFC. **(B)** DA and DOPAC levels in the striatum. **(C)** DA and DOPAC levels in the HPC. **(D-F)** HPLC analysis of DOPAC to DA ratio in the PFC, striatum and HPC, respectively. n= 5 per group. Data represented as mean ± SEM. **p*< 0.05.

4.4. Discussion

Here we have shown that WD consumption increases 5-HT_{2C} and 5-HTT but not 5-HT_{2A} receptor expression levels in the striatum. In addition decreased DA level in the striatum and a reduction of DA turnover in the HPC was observed in WD animals. These neurotransmitter changes were also observed to be independent of any change in spontaneous alternation behaviour.

We show that WD exposure increased striatal levels of 5-HT_{2C} receptor expression by 14%, but not 5-HT_{2A} receptor expression, as measured by western blot. A small number of studies have investigated the effect of HFDs on 5-HT_{2A} and 5-HT_{2C} receptor expression. An autoradiography study using a non-specific ligand, [125I]DOI targeting both 5-$HT_{2A/2C}$ receptors, in mice fed a 40 kcal% fat diet for 20 weeks reported no change in dorsal striatum 5-$HT_{2A/2C}$ binding density, but significantly higher receptor levels in hypothalamic areas such as the anterior olfactory nucleus and ventromedial hypothalamic nucleus (Huang et al., 2004). Meanwhile the same group of researchers using autoradiography demonstrated that rats fed beef tallow, a source of saturated fat, for 8 weeks had a reduction in both 5-HT_{2A} and 5-HT_{2C} receptor binding density in the dorsal striatum compared to control counterparts using [^{3}H]ketanserin and [^{3}H]mesulergine as binding ligands respectively (du Bois et al., 2006).

Pharmacological agonism of 5-HT_{2C} and 5-HT_{2A} receptors have been reported to decrease food intake (Fox et al., 2010, Martin et al., 1998, Schreiber and De Vry, 2002). Moreover, although 5-HT_{2C} and 5-HT_{2A} receptors are of close homology, 5-HT_{2C} knockout mice display lifelong hyperphagia that develops into late-onset obesity (Nonogaki et al., 2003), while 5-HT_{2A} knockout mice demonstrate no change in food intake or body weight (Weisstaub et al., 2006). This indicates that while they are structurally similar, these receptors are functionally distinctive. In addition to its effect on food consumption, 5-HT_{2C} and 5-HT_{2A} receptors show

a regional and cellular distribution within the central nervous system in brain areas associated to learning and memory processes (Barnes and Sharp, 1999, Buhot et al., 2000). 5-HT_{2C} and 5-HT_{2A} receptors have been shown to have opposing roles on inhibitory control and impulsivity. Infusion into the nucleus accumbens of a 5-HT_{2A} receptor antagonist decreases impulsivity while infusion of a 5-HT_{2C} receptor antagonist increases impulsivity in a 5-choice serial reaction task (Robinson et al., 2007, Winstanley et al., 2004). Moreover agonists of 5-HT_{2A} receptors enhance Pavlovian memory consolidation and agonists of 5-HT_{2C} receptors impair Pavlovian memory consolidation (Meneses, 2003).

We are the first to show that 12 week WD exposure also increased 5-HTT expression compared to controls. The effect of HFDs on 5-HTT binding appears to be region specific with reported increases in hypothalamus (Koopman et al., 2013, Levin and Dunn-Meynell, 2002, Okuda et al., 2014, Rowland, 1994) but a decrease of mRNA levels in the dorsal raphe (Collin et al., 2000). Huang *et al.* also investigated 5-HTT binding using autoradiography (Huang et al., 2004). Although HFD exposure caused no differences in 5-HTT binding in the striatum, a reduction in the nucleus accumbens and median raphe nuclei was observed (Huang et al., 2004). In the WD group, increased 5-HTT may result in lower 5-HT availability at synapses, and the increase in 5-HT_{2C} receptor expression could be an attempt to compensate for lowered 5-HT levels.

Several rat obesity models have reported reductions of 5-HT and 5-hydroxyindoleacetic acid (primary 5-HT metabolite, 5-HIAA) content in the ventral medial hypothalamus (Shimizu et al., 1994), brainstem (Kimbrough and Weekley, 1984) and also HPC (Krishna et al., 2016, Zemdegs et al., 2015). In the striatum, after 10 day HFD exposure 5-HT content was not changed, however 5-HT release was reduced (York et al., 2010). In addition 4 week 10% saturated fat exposure was shown to reduce 5-HIAA content (Kirac et al., 2009).

Interestingly, short term use of drugs that increase extracellular 5-HT via inhibition of 5-HTT reduce food intake and result in a decrease in body weight in animals (Simansky, 1996) and humans (Heisler et al., 2006, Simansky and Vaidya, 1990). Conversly, long-term use of selective 5-HT reuptake inhibitors has been associated with obesity (Fava et al., 2000, Raeder et al., 2006).

The presented results also demonstrated that WD consumption for 12 weeks causes a dysregulation of the dopaminergic system in both the HPC and striatum, as reflected by a significant decrease of DA levels in the striatum and DA turnover in the HPC in WD rats compared to controls. Our findings parallel data observed by Ma *et al.* who also showed a decrease of DA levels and no change in DA turnover in the striatum of rats fed a 60 kcal% HFD for 13 weeks (Ma et al., 2015). Interestingly, upon first exposure to a HFD, DA release was elicited in the nucleus accumbens and PFC, detected by microdialysis (Bassareo and Di Chiara, 1997). However, repeated exposure to food rewards severely blunted DA release, suggesting a form of habituation (Bassareo and Di Chiara, 1997). Results by Baladi *et al.* using chronoamperometry indicated a decrease in DA turnover in the striatum however this was independent to any observed changes to body weight in HFD rats (Baladi et al., 2015).

In animal models of obesity, a consistent finding is the reduction of DA levels in the nucleus accumbens observed in *ob/ob* mice (Fulton et al., 2006), diet-induced obesity rats (Geiger et al., 2008, Pothos et al., 1998), cafeteria diet model (Geiger et al., 2008) and HFD exposed mice (Carlin et al., 2013). Mice fed a 60 kcal% fat diet for 12 weeks also had a decrease of DA levels and increased DA turnover in the PFC (Carlin et al., 2013). High fat diet exposure, even as short as 5 days, has been shown to reduce basal DA levels in the nucleus accumbens (Rada et al., 2010). The reduction in DA levels at least in the nucleus accumbens is suggested to be due to reduced stimulated DA release and vesicle size (Geiger et al., 2008, Pothos et al.,

1998). Additionally, DA reuptake has been observed to decrease independent of DA transporter protein gene expression in rats fed a HFD, thought to be due to interference in DA transporter trafficking or maturation (Petrovich et al., 2007).

It could be hypothesized that WD consumption alters D2 receptor expression which can lead to the neuroadaptive response to decrease DA levels in the striatum and DA turnover in the HPC. It is known that the D2 receptor plays an inhibitory role in dopaminergic transmission in the mesolimbic dopaminergic system (Nestler, 1994). Previous observations have shown an inverse correlation between adiposity and striatal D2 binding in HF fed mice (Huang et al., 2005), rats (Johnson and Kenny, 2010) and obese humans (Davis and Fox, 2008).

Our lab has previously considered the possibility that the cholinergic system is associated with spatial deficits caused by WD consumption (Kosari et al., 2012). Nonetheless using immunohistochemistry we reported no change in acetylcholinesterase activity, the enzyme responsible for the metabolism of acetylcholine, in the HPC and striatum after WD consumption (Kosari et al., 2012). In this chapter, we show that that WD consumption selectively increases 5-HT_{2C} receptor and 5-HTT expression levels in the striatum, as well as inducing a reduction of DA level in the striatum and DA turnover in the HPC. These findings were independent of any spontaneous alternation behaviour change.

Chapter 5 - Spatial reference and working memory deficits in a neonatal overfeeding model of obesity

5.1. Background and rationale

The early life nutritional environment has been shown to influence body weight and has important repercussions for metabolism and weight regulation. As such, a critical window of significant vulnerability to long-term programming of health and disease occurs in the days to weeks after birth (Spencer et al., 2006, Spencer and Tilbrook, 2011, Spencer, 2012). The neonatal overfeeding model of obesity involves pups suckled in small litters, allowing for greater access to dam's milk, which are observed to be significantly heavier when compared to normal sized control litters by post natal day 7 (Stefanidis and Spencer, 2012). This observed increase in weight is persistent throughout life (Boullu-Ciocca et al., 2005, Morris et al., 2005, Stefanidis and Spencer, 2012). These animals also have altered stress responses (Spencer and Tilbrook, 2009) and exacerbated inflammatory response to an lipopolysaccharide challenge (Calvo et al., 2014, Clarke et al., 2012, Ziko et al., 2014).

Accumulating evidence indicates that obesity stimulates a chronic, low-grade inflammatory response, provoking the recruitment and activation of immune cells (including mast cells, macrophages, and dendritic cells) in metabolic tissues and particularly in adipose tissues, and also induces recruitment and activation of other cells, such as adipocytes, thus reinforcing the inflammatory process (Lumeng and Saltiel, 2011, Sell et al., 2012). Of particular interest is central inflammation in the HPC and hypothalamus that may converge leading to cognitive impairment. HFD consumption in animals elevates the expression of pro-inflammatory cytokines and activation of the pro-inflammatory transcription factor nuclear factor κB in the hypothalamus (De Souza et al., 2005). Whereas in the HPC, increased microglial activation (Lull and Block, 2010), astrogliosis, and elevated TNF-α protein has been observed in mice once fed a HFD (Jeon et al., 2012, Puig et al., 2012).

Research investigating the relationship concerning cognition impairments in juvenile obesity animal models is still in its infancy stage. How the neonatal nutritional environment can alter learning and memory has yet to be determined. Research investigating the relationship concerning cognitive impairments in juvenile obesity animal models is still in its infancy. We aim to test if spatial memory function may be impaired in a single trial-single test behavioural test, the Y-maze, and a multi-trial spatial memory test, the delayed win-shift task in the radial arm maze (DWSh), in adult rats made obese due to neonatal overfeeding. To investigate this hypothesis, we assessed spatial memory function in the Y-maze test and also evaluated spatial reference and working memory using the DWSh task in the neonatal overfeeding model of obesity.

5.2. Methods

5.2.1. Animals

Pregnant Wistar rat dams arrived at RMIT University animal facility from Animal Resources Centre, Australia. A total of 40 offspring rats were used in the first study (n= 20 male, n= 20 female), spatial memory testing in the Y-maze. A second cohort of male offspring rats (n= 10 per group) were used to assess spatial reference and working memory in the DWSh task.

Rats were housed at RMIT University animal facility, a controlled environment (20 ± 1°C) with 12-h light/dark cycle (lights on at 07:00 h), with food and water *ad libutum* in the home cage. The experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University Animal Ethics Committee.

5.2.2. Litter Size Manipulation

On the day of birth (P0), pups were removed from their dams and randomly reallocated to new dams in litters of 12 (control litter, CL) or 4 (small litter, SL). No dam received any of her own pups and each new litter comprised 50% males and 50% females. Excess pups were culled. At P21, animals were weaned into same-sex, same-treatment pairs. This manipulation results in SL pups being significantly heavier by P7 and heavier throughout life (Smith and Spencer, 2012, Spencer and Tilbrook, 2009, Stefanidis and Spencer, 2012). After weaning, the rats were left undisturbed, except for the usual animal husbandry, until experimentation in adulthood (P70).

5.2.3. Spatial memory in the Y-maze

The Y-maze was a three-arm maze with equal angles between all arms (50 cm long ×17 cm wide × 32 cm high). Rats were habituated twice to the maze for 5 min to become familiarised with the testing environment. On the test day, rats were allowed to explore the maze for 10 min, having access to two of the three arms (home, or start arm, and familiar arm). The rat was then returned to its home cage for a 4 h ITI during which the maze was cleaned with 70% ethanol. The rat was then again placed back into the maze, this time having access to all arms for 5 min. Both trial and test phases were recorded using a Legria FS200 digital video camcorder (Canon, Japan) for subsequent behavioural analysis. The number of entries to the novel arm and the time rat spent in each arm was recorded manually by stopwatch.

5.2.4. Food restriction

One week prior to the start of behavioural training, rats were food restricted to 85% of their daily food intake. Body weight was monitored twice weekly to ensure rats did not fall below 85% free-feeding weight. Food restriction was maintained for the entire duration of behavioural testing.

5.2.5. Delayed win-shift task in the radial arm maze

The DWSh test was performed as described in Section 3.2.4. In brief, rats were habituated to the RAM and then underwent a total of 25 training sessions with 2 sessions performed per day consisting of a 5 min training phase, 5 min delay phase where the rat was returned to the home cage and a 5 min test phase.

For analysis purposes, 2 days of testing (4 training sessions) were grouped into a single block. An arm entry was recorded when the animal fully moved off the central platform into the arm. Two types of errors were recorded: within phase error (working memory error, re-entry of an arm that has been baited and has been visited) and across phase error (reference memory error, entry into a training phase baited arm).

5.2.6. Statistical analysis

All data are presented as mean ± SEM. A p-value of < 0.05 was considered statistically significant. Statistical comparisons were made between groups by repeated measures two-way ANOVA for DWSh performance data and two-way ANOVA for Y-maze data.

5.3. Results

5.3.1. Spatial memory in the Y-maze

In the Y-maze, litter size did not affect the number of entries in the novel arm ($F_{(1,36)}= 1.35$, $p= 0.25$, **Fig. 5.1A,** nor was there an observed effect of gender x litter size in the number of entries into the novel arm ($F_{(1,36)}= 0.20$, $p= 0.66$). However, females in both CL and SL were observed to enter the novel arm more than males ($F_{(1,36)}= 12.90$, $p= 0.001$).

The measure of the time spent in the novel arm, there was no effect of litter size ($F_{(1,36)}$= 0.04, p= 0.85 or gender x litter size ($F_{(1,36)}$= 1.06, p= 0.31, **Fig. 5.1B**). Both CL and SL females spent more time in the novel arm compared to their male counterparts ($F_{(1,36)}$= 42.34, p< 0.0001). These results indicate that there was no impairment of spatial memory in either male or female SL rats.

Gender was also observed to affect distance travelled in the Y-maze ($F_{(1,36)}$= 20.44, p< 0.0001; **Fig. 5.1C**), with males moving less than females (p< 0.05). No difference in distance travelled between CL and SL groups for both genders (F< 1).

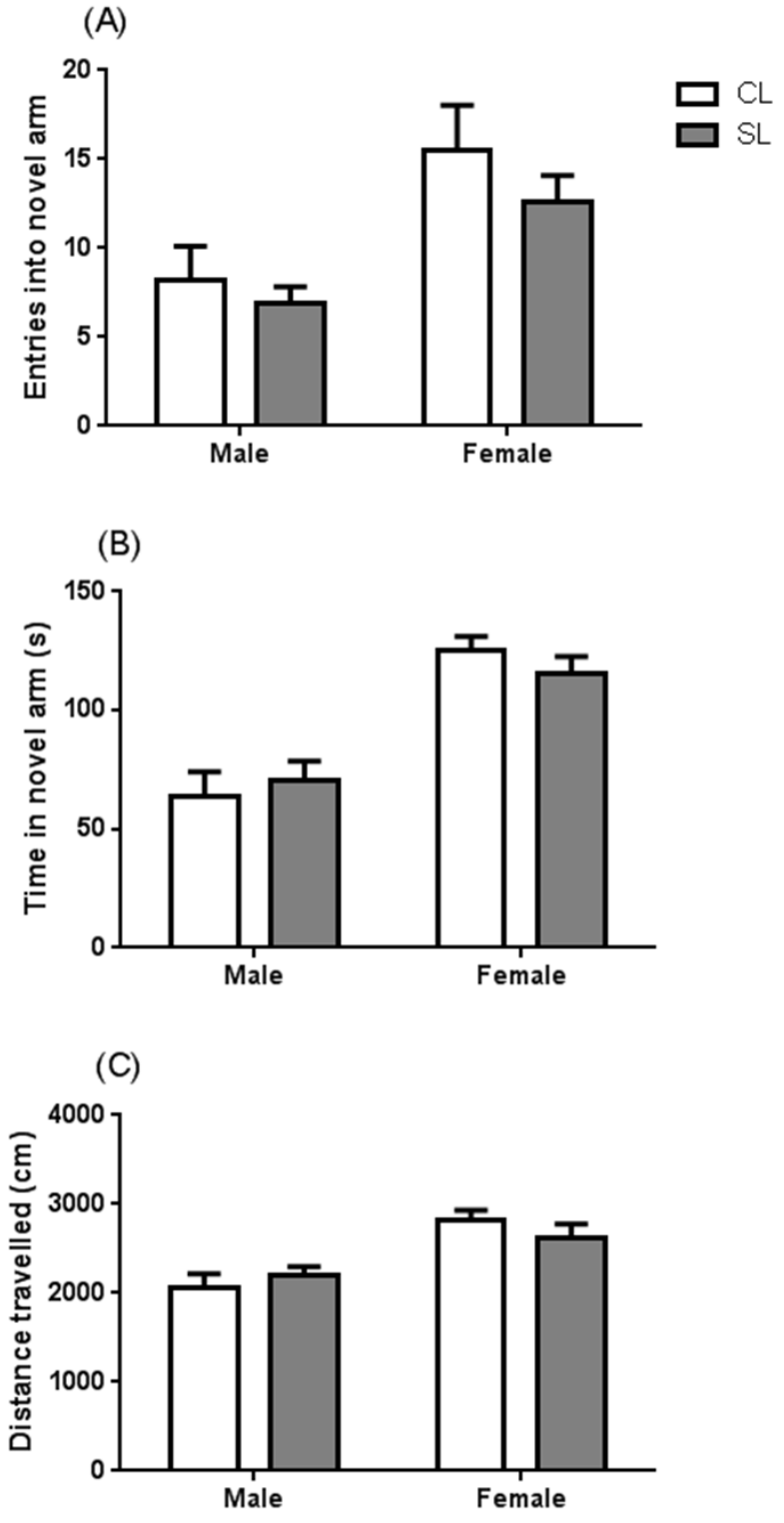

Figure 5.1. Spatial memory test in the Y-maze. **(A)** Number of entries into the novel arm, **(B)** Time spent in the novel arm, **(C)** Total distance travelled in Y-maze behavioural test. n= 10 per group. Data expressed as mean ± SEM, *p< 0.05, *** p< 0.001.

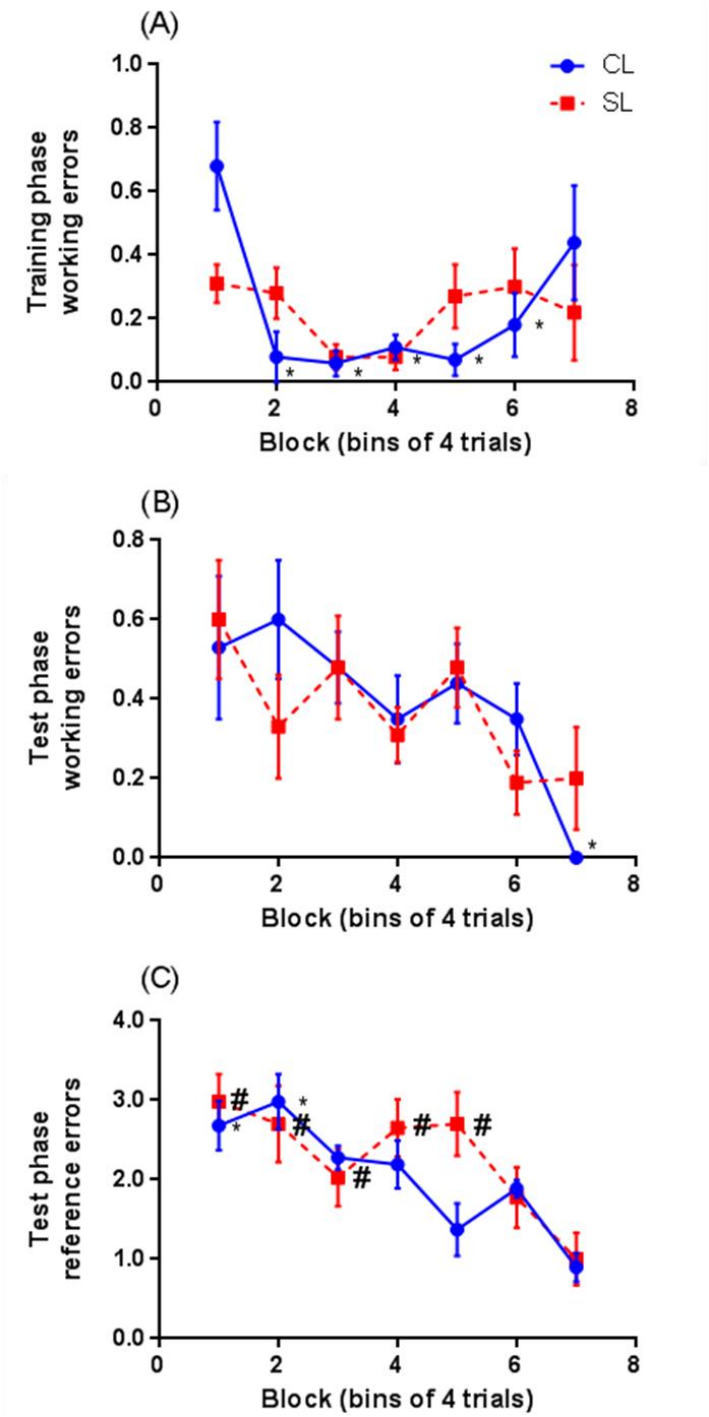

Figure 5.2. Performance in test phase DWSh task. **(A)** Number of correct arm choices before error in each session of training. **(B)** Total number of working errors in each session of training. **(C)** Number of reference errors in each session of training. n= 10 per group. Data expressed as mean ± SEM. A: *p< 0.05 in CL compared with CL block 1. B, C: *p< 0.05 in CL compared with CL block 7; #p< 0.05 in SL compared with SL block 7 (criterion).

5.3.2.Performance in the delayed win-shift task

The CL group commenced the training phase with a significantly greater number of working memory errors but had improved by block 2 (trials 5-8, significant effect of block: $F_{(6,120)}$= 4.46, $p < 0.001$; **Fig. 5.2A**). There was no effect of litter size ($F_{(1,120)}$= 0.014, p= 0.91) nor litter size x block effect ($F_{(6,120)}$= 2.31, p= 0.038).

In the test phase, there was no observed litter size effect ($F_{(1,120)}$= 0.124, p= 0.73) or litter size x block effect ($F_{(6,120)}$= 0.83, p= 0.55). There was a significant improvement in working memory only in the CL, but this was not apparent until block 7 (trial 25, $p< 0.05$). The CL rats also improved significantly more quickly in their reference memory in the test phase (significant effect of block: $F_{(6,120)}$= 7.91, $p< 0.001$; **Fig. 5.2C**). By block 3 (trials 9-12) CL errors were statistically not distinguishable from criterion (block 7; trial 25), whereas the SL rats were still making more errors than at criterion until block 6 (trials 21-24, $p< 0.05$).

5.4. Discussion

In this study we investigated the effect of neonatal overfeeding on the DWSh in the radial arm maze and the Y-maze. Our results demonstrate that neonatally overfed rats had impaired memory in the DWSh task but not in the Y-maze.

The slower acquisition of the DWSh task by the SL animals can be contrasted to studies performed in post-weaning HFD (dietary manipulation beginning at approximately 3 weeks of age) and *in utero* HFD exposure juvenile models of obesity using the MWM to assess spatial learning and memory. A number of studies show that juvenile HFD exposure did not impair learning acquisition in the MWM (Boitard et al., 2014, Goldbart et al., 2006, Lepinay et al., 2015, White et al., 2009a). In subsequent probe trials, HFD animals were shown to have no impairment 2 h post learning (Boitard et al., 2014), but with an increased learning-

probe time of 2-4 days a memory deficit was observed (Boitard et al., 2014, Goldbart et al., 2006, Lepinay et al., 2015, Page et al., 2014). These studies suggest that juvenile HFD exposure has a detrimental effect on spatial memory consolidation and recall. Furthermore, research by Boukouvalas *et al.* indicate that juvenile exposure to 45 kcal% HFD for 3 weeks is insufficient to affect acquisition, consolidation or recall of the MWM task (Boukouvalas et al., 2008), whereas 8 week HFD exposure of similar fat content in juvenile rats impairs spatial memory consolidation and recall (Boitard et al., 2014, Goldbart et al., 2006). These results imply a time dependent influence of juvenile HFD exposure to impair spatial consolidation and recall in the MWM. Transient inactivation of either the HPC or PFC by bilateral injection of lidocaine has been shown to impair performance in the DWSh task (Floresco et al., 1997). This could be indicative of a hippocampal or PFC dysfunction in neonatally overfed rats.

Interestingly using a generational *in utero* model of obesity, HFD-fed dams exhibited no impairment of spatial memory, however their pups took longer to find the hidden platform in the MWM regardless of dietary manipulation denoting a spatial memory acquisition impairment (Page et al., 2014, White et al., 2009a). Pups from pregnant rats fed a control diet did not display any spatial memory deficit (Page et al., 2014, White et al., 2009a). This indicates that maternal diet may also play an influential role in reducing the capacity for spatial memory acquisition.

The contrasting results in juvenile models of obesity between the reported no change in acquisition rate in the MWM and our observed impaired spatial memory acquisition in the DWSh task could potentially be due to these two behavioural tasks burdening motivational and motor systems differentially (Hodges, 1996, Ormerod and Beninger, 2002). The MWM

task is aversively motivated since animals have to swim to escape a pool of water. The DWSh task is appetitively motivated where animals gain food rewards.

In the Y-maze, we show that neonatal overfeeding does not affect spatial acquisition, consolidation or recall in adult males and females. In contrast to our study, one research group has reported impairment of spatial memory following juvenile HFD exposure using similar one trial-one test behavioural tasks. Long-Evans rats fed a HFD consisting of 58 kcal% fat for 12 weeks from weaning reported a spatial memory deficit using the spontaneous alternation task and NOIP behavioural tests (Underwood and Thompson, 2016b, Underwood and Thompson, 2016a). The memory deficit observed in these HFD females was independent of significant weight gain, fasting blood glucose or glucose tolerance change (Underwood and Thompson, 2016a). Authors suggest that young females exposed to HFDs are at larger risk of developing cognitive deficits.

Considering this model our colleagues have previously shown that neonatally overfed rats manifest hypothalamic inflammation, with microgliosis in the paraventricular nucleus of the hypothalamus and an increase in the hypothalamic expression of pro-inflammatory genes (Cai et al., 2014, Ziko et al., 2014). In collaboration, we demonstrated that neonatally overfed rats also exhibited microgliosis in the HPC after 14 days overfeeding and this persisted into adulthood (De Luca et al., 2016). Furthermore, these changes were associated with poor performance in DWSh task and NORT relative to controls (De Luca et al., 2016). This provides evidence that central inflammation may be involved with impairments of spatial memory acquisition observed in neonatally overfed rats.

In this chapter, we show that that neonatally overfed rats did not display impairment in spatial memory when tested in the Y-maze test. However in a behavioural task which requires the constant updating of new information, the DWSh task, neonatally overfed rats took longer to

learn than control litter rats. The possible mechanism by which this occurs may be due to inflammation in the HPC.

Chapter 6 - Memory and anxiety-like measures in an Alzheimer's disease transgenic mice model fed a western diet

6.1. Background and rationale.

Although ageing and genetic predisposition are undoubtedly the predominate risk factors for developing AD, epidemiological and clinical studies also substantiate the association with obesity (Anstey et al., 2011, Besser et al., 2014, Gustafson et al., 2012, Panza et al., 2010, Solfrizzi et al., 2004, Whitmer et al., 2005) and resulting comorbidities, including type 2 diabetes (Barnes and Yaffe, 2011, Biessels et al., 2006, Kloppenborg et al., 2008, Kivipelto et al., 2001, Norton et al., 2014). The protracted period that precedes clinical manifestation of AD constrains the capability to obtain information on subtle changes in brain chemistry. Furthermore the non-existence of definitive AD diagnosis until an autopsy has been conducted has posed a considerable challenge in human studies. As such, transgenic animal models are beneficial for AD research.

The widely used $APP_{swe}/PS1_{dE9}$ (APDE9) double transgenic mouse model of AD expresses a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9). These two mutations are related with familial AD and based on the amyloid hypothesis, which hypothesises an increased production or decreased removal of the APP proteolytic fragment, amyloid β-protein (Aβ), as the primary cause of AD (Hardy and Selkoe, 2002). These mice have been shown to display Aβ plaques in both the HPC and cortex by 4-6 months of age (Jankowsky et al., 2004, Volianskis et al., 2010). Behaviourally these mutant mice were also shown to have impaired passive avoidance behaviour (Phillips et al., 2011). Transient long term potentiation, the molecular basis of memory, has also been shown to be reduced in the HPC of these mice (Volianskis et al., 2010). Furthermore, at 6 months of age, APDE9 mice took longer to learn and made more incorrect decisions in the T-maze indicating a spatial learning and retention impairment (Phillips et al., 2011). Mutant mice tested in the MWM also displayed severely impaired performance with longer latencies

to reach a hidden platform at 8-16 months of age (Butovsky et al., 2006, Lalonde et al., 2005, Malm et al., 2007, O'Leary and Brown, 2009). This performance deficit in spatial learning ability may be present as early as 3 months of age (Gimbel et al., 2010) but others have shown no deficits at 6 months of age (Minkeviciene et al., 2008, Savonenko et al., 2005).

Using the APDE9 transgenic mice model of AD, we hypothesise that visuo-spatial learning and memory is impaired as previously observed. Furthermore, this impairment of visuo-spatial memory would be potentiated by weight gain through WD consumption. To investigate this hypothesis, we determined whether WD consumption altered the metabolic profile, spatial memory and anxiety measures in aged APDE9 transgenic mice.

6.2. Methods

6.2.1. Animals

Forty 12 month old APDE9 transgenic mice with C57BL/6 background were graciously obtained from Associate Professor Siew Yeen Chai from Monash University. Male APDE9 transgenic mice and C57BL/6 wild type controls were bred at Monash University (Clayton, Melbourne, Australia) and transported to RMIT University (Bundoora, Melbourne, Australia) for dietary manipulation and subsequent behavioural assessment.

Mice were group housed at RMIT University animal facility, a controlled environment (20 ± 1°C) with 12-h light/dark cycle (lights on at 07:00 h), with food and water *ad libutum* in the home cage. Behavioural tests were performed from 9:00 h to 19:00 h in a dedicated animal behaviour room. The experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986 and with approval from the RMIT University Animal Ethics Committee.

Seven mice were excluded from analysis as these mice died during the dietary manipulation period. Necropsies completed by the animal welfare officer here at RMIT revealed that mice died from strokes and/or old age. Two mice were excluded in Y-maze data analysis due to failure to move in acquisition and test phases.

6.2.2. Dietary manipulation

All animals were allowed to acclimatise after delivery for at least 1 week before commencement of dietary manipulation. Mice were fed one of two diets: either CON diet (SF04057, Speciality feeds, Australia) or WD (SF00-219, Speciality feeds, Australia) for a period of 8 weeks. The nutritional content of each diet is as seen in **Table 2.1.**

6.2.3. Open field test

Locomotor activity was assessed as described previously in Section 2.2.8. In brief, mice were placed individually in the test chamber for 10 min to monitor locomotor activity. Total distance travelled and average velocity was measured by Med Associates activity monitor software, version 4.

6.2.4. Spatial memory in the Y-maze

The Y-maze test was performed as described in Section 5.2.3. In brief, mice were habituated to the Y-maze and on the test day, mice were allowed to explore the maze for 10 min, having access to two of the three arms (home, or start arm, and familiar arm). The mice was then returned to its home cage for a 1 h ITI during which the maze was cleaned with 70% ethanol. The mice were then again placed back into the maze, this time having access to all arms for 5 min. Both trial and test phases were recorded using a Legria FS200 digital video camcorder (Canon, Japan) for subsequent behavioural analysis. The number of entries to the novel arm and the time mice spent in each arm was recorded manually by stopwatch.

6.2.5. Light/dark preference test

The light/dark preference test was performed as described in Section 2.2.9. In brief, number of entries and total time spent in either light or dark areas of the box was measured during the 10 min test.

6.2.6. Body composition

At 8 weeks post diet, body composition (fat and lean mass, free water, and total water) of all mice was assessed by an EchoMRI™ Whole Body Composition Analyzer (EchoMRI™-900, EchoMRI, USA). Body fat composition was calculated by determining total fat (g) divided by total body weight (g) and expressed as a percentage.

6.2.7. Glucose tolerance test

At the end of the study, mice were fasted overnight, and then a glucose tolerance test (GTT) performed. Blood samples were collected from a single wound made by cutting the tip of the tail. Blood glucose levels were measured before and after an i.p. injection of D-glucose (2 g/kg of a 0.5 g/ml solution) at 5, 10, 20, 30, 45, 60, 90, and 120 min using a commercial glucose testing kit (ACCU-CHEK, Roche Diagnostics, Germany). The trapezoidal rule was used to determine the area under the curve (AUC).

6.2.8. Statistical analysis

All data are presented as mean ± SEM. A p-value of < 0.05 was considered statistically significant. Statistical comparisons were made between groups by repeated measures three-way ANOVA for body weights and glucose levels, two-way ANOVA for all other data. Further analysis by a post hoc Bonferroni's t-test was performed if a significant effect was detected by the ANOVA.

6.3. Results

6.3.1. Animals

There was a significant percentage of weight change over time for all groups (time effect: $F_{(7,91)}$= 7.90, p< 0.001; **Fig 6.1A**). WD exposed mice of both phenotypes also increased their body weight at a more pronounced rate than CON (diet x time: ($F_{(7,91)}$= 22.47, p< 0.001). No effect of time x phenotype ($F_{(7,91)}$= 1, p= 0.44) or time x phenotype x diet effect ($F_{(7,91)}$= 1.64, p= 0.11) was observed. WD exposure was shown to significantly increase fat mass to body weight ratio ($F_{(3,29)}$= 54.61, p< 0.0001; **Fig. 6.1B**). Both WT (p< 0.0001) and APDE9 (p< 0.0001) WD fed mice had higher fat content compared to their control diet counterparts.

6.3.2. Glucose tolerance testing

Fasting blood glucose levels were not found to be different among the groups (WT Con: 6.64 ± 0.63 mmol/l, WT WD: 8.36 ± 0.30 mmol/l, APDE9 Con: 8.88 ± 0.64 mmol/l, APDE9 WD: 8.23 ± 0.56 mmol/l). During the GTT, there was a change of glucose levels over time [time effect: ($F_{(8,216)}$= 37.95, p< 0.001)]. WD exposure over time affected body weight [time x diet effect: ($F_{(8,216)}$= 22.47, p< 0.001)] as well as an interactive effect of diet x phenotype x time effect: ($F_{(8,216)}$= 2.15, p= 0.03). No diet or time x phenotype effect was observed (all F< 1). WD exposure in ADPE9 mice significantly decreased glucose tolerance as evidenced by increased blood glucose level from 30 to 90 min (p< 0.01).

The glucose area under the curve of the animals was affected by diet ($F_{(1,12)}$= 5.91, p= 0.03) and transgenic phenotype ($F_{(1,12)}$= 5.61, p= 0.04) but no diet x transgenic phenotype was observed ($F_{(1,12)}$< 1, p= 0.58). *Post-hoc* analysis shows that APDE9 mice fed a WD had significantly higher glucose AUC than CON WT mice (p< 0.01).

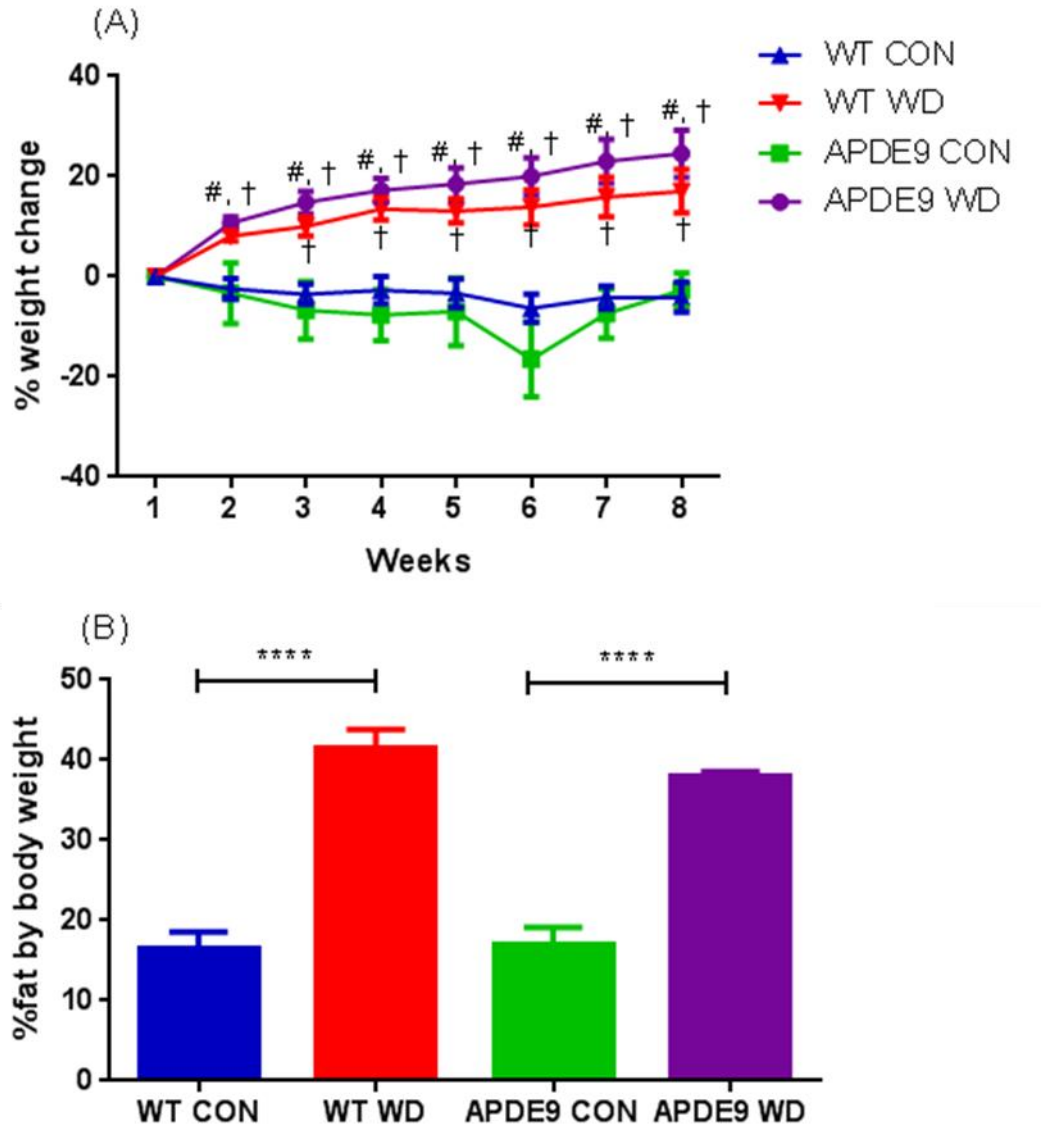

Figure 6.1. Metabolic measures. **(A)** Change in body weights during 8 weeks of feeding of CON or WD in WT and APDE9 mice. **(B)** Percentage fat composition of WT and APDE9 transgenic mice fed a WD for 8 weeks. n= 6-9 per group. Data expressed as mean ± SEM, #$p< 0.05$ to APDE9 CON, †$p< 0.05$ to WT CON, ****$p< 0.0001$.

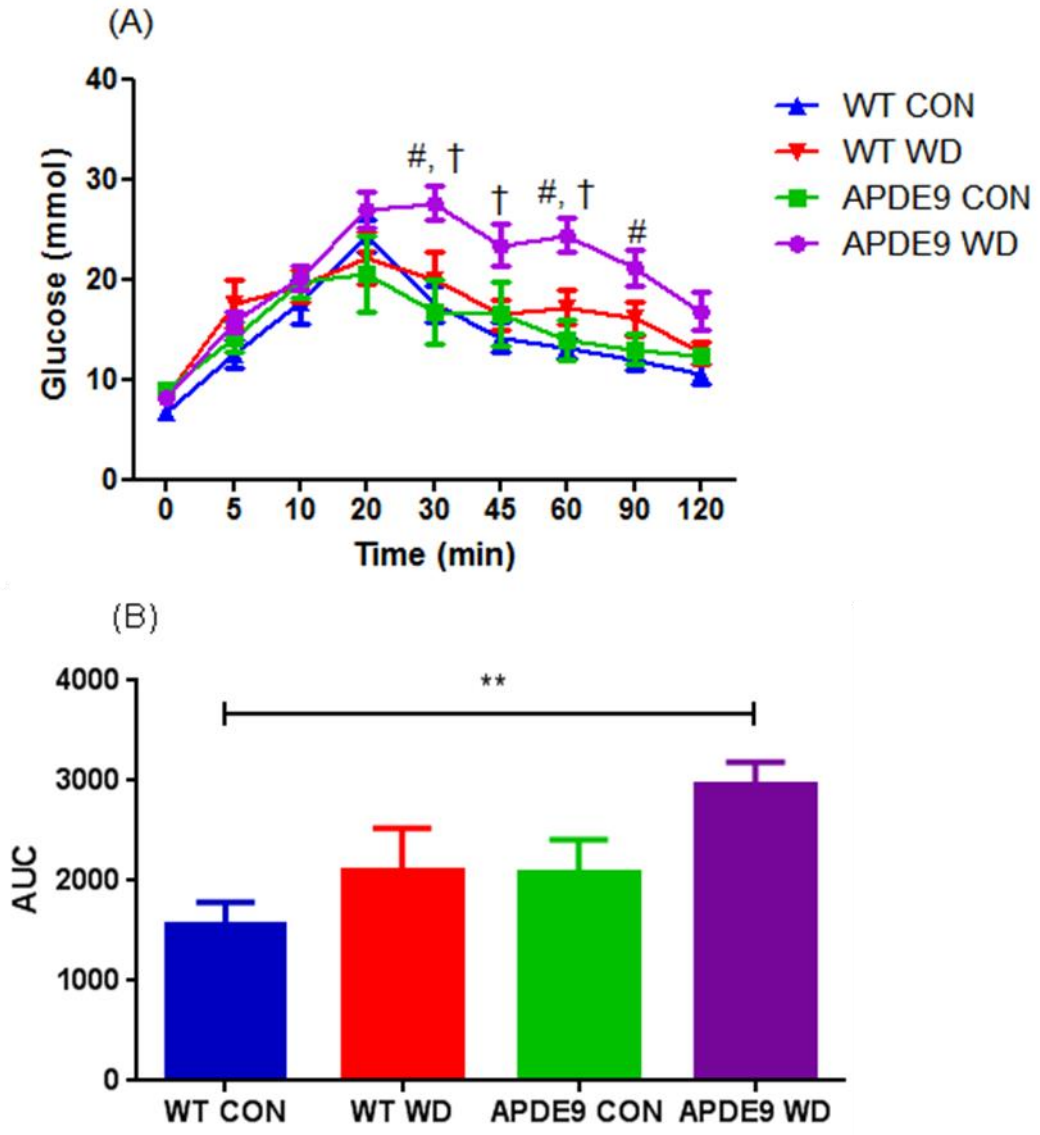

Figure 6.2. Glucose tolerance testing. **(A)** Blood glucose levels during GTT. **(B)** Area under the curve (AUC) of blood glucose profiles. n= 5-9 per group. Data expressed as mean ± SEM, #p< 0.05 compared to APDE9 CON, †p< 0.05 compared to WT CON and WT WD, *** p< 0.001.

6.3.3. Locomotor activity

There was no effect of diet ($F_{(1,29)}$= 2.62, p= 0.12), transgenic phenotype ($F_{(1,29)}$= 4.02, p= 0.06) nor diet x transgenic phenotype ($F_{(1,29)}$< 1, p= 0.80) on distance travelled in the basal locomotor activity test. Velocity during the basal locomotor activity test was also assessed. No effect of diet ($F_{(1,29)}$= 1.71, p= 0.20), transgenic phenotype ($F_{(1,29)}$< 1, p= 0.92) nor diet x transgenic phenotype ($F_{(1,29)}$= 1.85, p= 0.16) on velocity was observed.

Table 6.1: Basal locomotor activity of APDE9 and WT mice fed CON or WD.

	WT CON	**WT WD**	**APDE9 CON**	**APDE9 WD**
Distance (cm)	1254 ± 190	1091 ± 149	1382 ± 86	1298 ± 195
Velocity (cm/s)	61 ± 10	47 ± 9	66 ± 20	54 ± 12

6.3.4. Light/dark preference

WD consumption regardless of transgenic phenotype significantly reduced the number of entries into the light area ($F_{(1,29)}$= 10.12, p= 0.0035, **Table 6.2**). No transgenic phenotype effect ($F_{(1,29)}$= 2.26, p= 0.14) or any post-hoc differences were observed. WD consumption also reduced the amount of time spent in the light area ($F_{(1,29)}$= 7.56, p= 0.01).

Table 6.2: Number of entries and time spent in light area of the light/dark preference test of APDE9 and WT mice fed CON or WD

	WT CON	**WT WD**	**APDE9 CON**	**APDE9 WD**
Light area entries	24.33 ± 2.19	15.88 ± 2.25	32.17 ± 6.74	18.5 ± 2.99
Light time (s)	400.59 ± 17.50	446.95 ± 23.79	360.45 ± 37.19	434.46 ± 20.40

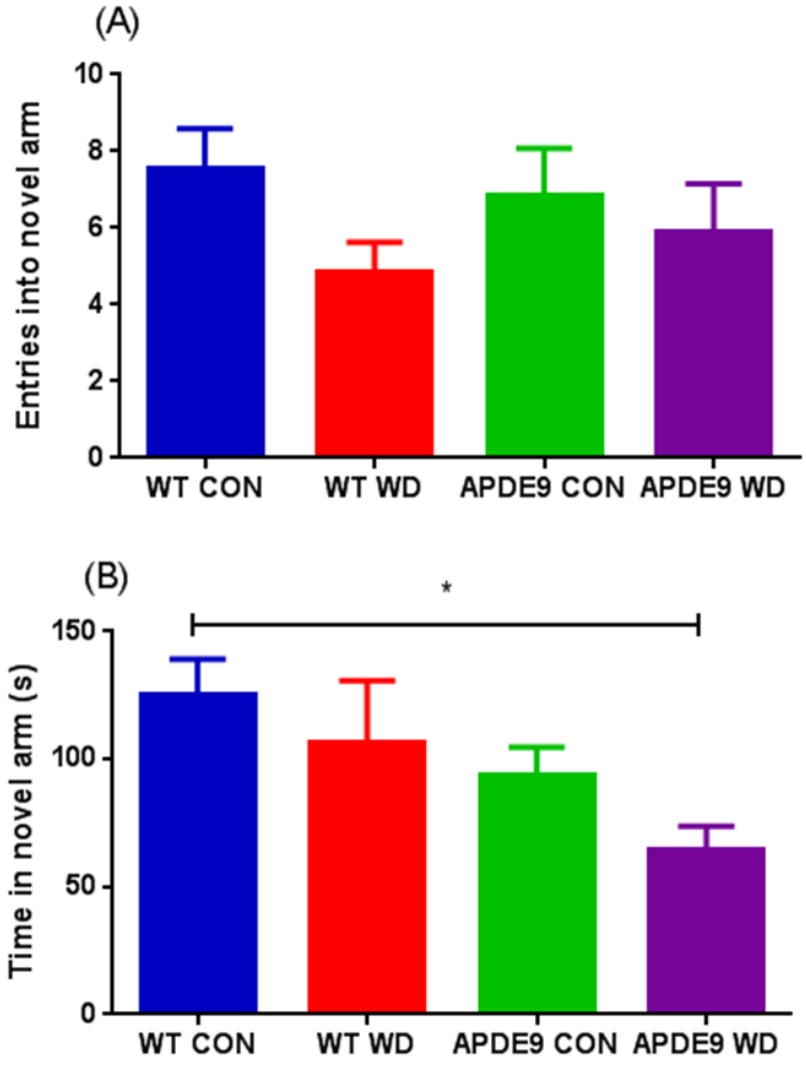

Figure 6.3. Spatial memory test in the Y-maze. **(A)** Number of entries into the novel arm, **(B)** Time spent in the novel arm of APDE9 and WT mice fed CON or WD. n= 6-10 per group. Data expressed as mean ± SEM, *p< 0.05.

6.3.5. Spatial memory in the Y-maze

There was no observed change in the number of entries in the novel arm between groups due to diet ($F_{(1,27)}$= 2.33, p= 0.14), transgenic phenotype ($F_{(1,27)<}$ 1, p= 0.89) or diet x transgenic phenotype effect ($F_{(1,27)}$< 1, p= 0.46; **Fig. 6.3A**). WD consumption also did not affect the time spent in the novel arm ($F_{(1,27)}$= 2.72, p= 0.11) nor was there a diet x transgenic phenotype effect ($F_{(1,27)}$< 1, p= 0.71). However, there was a significant effect of transgenic phenotype alone to affect time spent in the novel arm ($F_{(1,27)}$= 6.42, p= 0.02). *Post-hoc* analysis revealed that WD fed APDE9 mice spend significantly less time in the novel arm in contrast the WT controls (p< 0.05; **Fig. 6.3B**). This suggests that the APDE9 mice had impaired spatial memory compared to WT controls.

6.4. Discussion

In this study we investigated the potential additive effect of WD exposure on metabolics markers and behaviour in the transgenic APDE9 AD mice model. WD exposure significantly caused a 21% increase in WT mice and a 27% body weight increase in APDE9 mice. Total fat content was markedly augmented by approximately 25% and 21% in WT and APDE9 mice, respectively compared to control diet counterparts. Transgenic phenotype and diet were not shown to influence basal locomotor activity with no change in distance travelled and velocity in the open field test. Furthermore, anxiety levels measured by the light/dark preference test revealed no significant differences between WD exposed or APDE9 mice.

In the Y-maze, APDE9 mice that consumed WD spent less time in the novel arm compared to WT CON indicating a spatial memory deficit. WD exposure alone in WT mice was insufficient to produce a spatial memory deficit which suggests an additive effect of APDE9 phenotype and WD exposure to impair spatial memory. Our results are in concordance with a

MWM study where 27 weeks of HFD consumption impaired memory performance compared to APDE9 control diet mice (Ramos-Rodriguez et al., 2014).In subsequent probe trails APDE9 mice, regardless of diet, were shown to have impairment 24 h post learning and with increasing probe time of 72 hr, impairment was more pronounced in HFD exposed APDE9 mice (Ramos-Rodriguez et al., 2014). When we consider the Tg2576 model, a spatial memory deficit was observed in HFD fed Tg2576 mice denoted by a longer escape latency (Winstanley et al., 2004) at an age where Tg2576 mice have yet to develop a spatial memory deficit (King and Arendash, 2002).

It is noteworthy our results show that despite fasting blood glucose levels not being different between all groups, APDE9 WD mice interestingly showed impaired glucose tolerance (~90% increase of AUC compared to WT CON). We are unclear why this occurs, but this result has been replicated by our collaborators. Moreover it has been shown that APDE9 mice crossed with heterozygous leptin receptor deficient (db/+) mice developed glucose intolerance and insulin resistance, but had no change in fasting blood glucose, which support a link of AD models and obesity (Jiménez-Palomares et al., 2012).

Previous studies have shown that the diabetes phenotype may accelerate memory impairment in other AD models (Ho et al., 2004, Takeda et al., 2010). AD transgenic mice (APP23) once crossed with *ob/ob* mice showed elevated fasting glucose and insulin levels and glucose intolerance and an exacerbated spatial memory deficit in the MWM compared to *ob/ob* mice (Takeda et al., 2010). Moreover, APP^{+}-*ob/ob* mouse brains from this study showed increased vascular Aβ deposition and inflammation compared to WT, but no change compared to APP23 mice, tentatively suggesting that AD amyloid pathology may affect the pathogenesis of diabetes (Takeda et al., 2010). Another AD model using Tg2576 transgenic mice fed a 60 kcal% HFD also showed an increase of body weight, glucose intolerance, and a >2 fold

increase of Aβ hippocampal generation relative to transgenic mice fed a control diet (Ho et al., 2004).

Studies have also demonstrated commonalities between mechanisms triggered by Aβ plaque and mechanisms involved in peripheral insulin resistance in diabetes via defective brain insulin signalling (Bomfim et al., 2012, Craft, 2012, Ma et al., 2009, Takeda et al., 2011). One such mechanism through which HFD exposure in AD mice can significantly promote AD-type amyloidosis in the brain is through the impairment of insulin receptor signalling, resulting in elevation of γ-secretase, a protease complex which can cleave APP (Ho et al., 2004). APDE9 mice fed a 45 kcal% HFD for 20 weeks showed a working memory deficit, assessed by NORT, which was independent to brain Aβ levels (Petrov et al., 2015). Significant decreases of insulin signalling and mitochondrial complex proteins suggests that mitochondrial dysfunction may also be a contributing factor to cognitive impairment in AD models (Petrov et al., 2015). In cell culture experiments, researchers have also shown that insulin may promote intracellular Aβ plaques accumulation through the acceleration of APP/Aβ trafficking to the plasma membrane (Gasparini et al., 2001).

The presented results demonstrate that WD consumption for 8 weeks significantly increases body weight and fat content in both WT and APDE9 transgenic mice. Furthermore WD exposure impairs spatial memory, as assessed by the Y-maze, in APDE9 mice suggesting a link between the APDE9 phenotype and WD exposure to accelerate spatial memory deficits. Impaired glucose tolerance in APDE9 WD mice signify impaired insulin receptor signalling and may play a role in memory deficit.

Chapter 7 - General discussion

Obesity is a common problem worldwide, and is becoming more prevalent as countries become more affluent and industrialised. Consumption of a HFD, complemented with an increasing sedentary lifestyle, can dramatically increase this risk. Persistent and prolonged consumption of HFDs can instigate significant health impairments with dramatic increases in the risk of cardiovascular disease, type 2 diabetes and cancer. Recent epidemiological studies suggest an association with obesity and cognitive impairments. However, there is conflicting findings in both human clinical studies and animal studies on the presence and the exact nature of cognitive deficits seen in the obese phenotype. The studies undertaken in this thesis were designed to investigate the capacity of the obesity phenotype to impair specific types of cognitive behaviour in a rodent model. This was performed by examining behavioural outcomes in different animal models, being the WD and neonatal models of obesity. We also investigated whether cognitive impairments observed in the WD model of obesity is potentially mediated through the modification of higher order brain pathways. Moreover, we examined the interaction between AD and obesity to potentiate spatial memory impairments in APDE9 transgenic mice fed a WD.

7.1. Summary of findings

7.1.1. WD consumption effects on memory paradigms.

In the first series of studies, we investigated whether WD consumption could sensitise food related cues and overwhelm internal physiological feeding control.

Instrumental conditioning paradigm was performed in WD and CON animals. WD was shown not to affect instrumental conditioning acquisition using a random interval 30 schedule. However, when tested in a progressive ratio instrumental conditioning task there was an observed reduced lever press response compared with control diet counterparts. Instrumental conditioning extinction was then tested under food deprivation. WD exposed

rats were not sensitive to motivational changes due to variations in states of food deprivation compared to control counterparts. Considering Pavlovian conditioning, there was no difference between WD and CON animals using a variable 90 s interval.

Considering our previous usage of the WD model, these results were disappointing. However no one to date has shown a robust Pavlovian or instrumental conditioning deficit after manipulation of fat in the diet. One point to note is that we did not find consistent metabolic markers with the WD model of obesity in our two cohorts of animals considering they were of the same strain, had the same food source and were exposed to the same laboratory conditionings. In cohort one, WD animals were significantly heavier but no differences were observed in epididymal adipose tissue weight, while the opposite was found in cohort two. This suggests that the WD model of obesity may have variable effects amongst individuals. Conceivably the observed cognitive impairments reported in the literature may be associated with total adiposity rather than HFD consumption (see Section 7.2.1).

Further to this, a third cohort (Chapter 3) demonstrated significant weight gain after WD consumption from week 8. We employed this cohort to investigate spatial memory in the DWSh task. WD did not affect acquisition or recall of the DWSh task indicated by (1) no difference in time required to criterion in the training phase nor (2) the number of within- and across-phase errors during the test phase between CON and WD rats.

7.1.2. WD consumption alters brain neurochemistry.

Considering the variable cognitive effects we have observed, we next turned to examining what brain changes occur as a result of WD consumption. Neuronal activation was determined in basal conditions and following environmental stimulation using Fos immunohistochemistry. The number of basal Fos neurons was unaffected by WD consumption in the PFC, striatum and HPC. However following environmental stimulation,

WD exposed rats had a selective increased number of immunoreactive Fos neurons in the striatum.

The striatum is a convergence point for many different types of inputs all around the brain that include but are not limited to serotoninergic inputs (Pan et al., 2010b, Vertes, 1991) and dopaminergic (Nicola et al., 2000, Smith et al., 1994). Findings from Chapter 4 showed that WD consumption induced changes to the serotoninergic and dopaminergic systems. Western blot measurements showed a selective increase of 5-HT_{2C} receptor and 5-HTT but not 5-HT_{2A} receptor expression in the striatum. This increase was independent to any observed spatial memory deficit evaluated by the spontaneous alternation test in the Y-maze. The selective increase of expression may be due to negative feedback from reduced 5-HT concentration in the striatum however, total 5-HT levels would need to be measured to test this hypothesis.

DA levels measured in the striatum were reduced in WD rats relative to control counterparts. DA turnover was also markedly reduced in the HPC of WD rats. Previous studies have reported that D2 receptor expression is also reduced in response to HFD feeding animals (Huang et al., 2005, Johnson and Kenny, 2010) and obese humans (Davis and Fox, 2008). It is known that D2 receptor plays an inhibitory role in dopaminergic transmission in the mesolimbic dopaminergic system (Nestler, 1994) and D2 knockout mice have demonstrated sensitivity to the locomotor and enhanced motivation for food reward in response to cocaine (Bello et al., 2011). Therefore, D2 receptor might be crucial in motivational behavioural responses food rewards. It could be hypothesized that WD consumption alters D2 receptor expression which can lead to the neuroadaptive response to decrease DA level in the striatum and DA turnover in the HPC.

7.1.3. Neonatal overfeeding can impair spatial memory acquisition in the DWSh task but not the Y-maze.

Results from previous chapters indicate that WD consumption in adulthood can induce central neuronal and neurochemical changes, but not robust cognitive impairments. As such we turned our focus to a longer-term obesity model. The neonatal overfeeding model of obesity involves pups suckled in small litters, allowing for increased weight gain from P7 to adulthood. The early life nutritional environment can influence body weight and has important repercussions for metabolism and weight regulation as an adult. Our data from Chapter 5 indicated that although the early life over feeding model of obesity can cause significant weight gain in early life which persists until adulthood. Behavioural effects were variable. No impairment of spatial memory was observed in the Y-maze. However in the DWSh task, neonatally overfed rats made more spatial working memory errors than control litter counterparts indicating that adult neonatally overfed rats had an impairment of spatial memory acquisition. Work performed in conjunction with our collaborators, but outside the scope of this dissertation, indicate that central inflammation may be involved with impairments of spatial memory acquisition observed in neontally overfed rats (De Luca et al., 2016). Neonatally overfed rats also exhibited microgliosis in the HPC after 14 days overfeeding and this persisted into adulthood whilst also associated with poor performance in DWSh task and NORT relative to controls (De Luca et al., 2016).

7.1.4. Effect of WD consumption and APDE9 phenotype to impair spatial memory.

By studying APDE9 transgenic mice we investigated the potential effect of WD consumption to accelerate AD behavioural pathogenesis. Findings from Chapter 6 show that both WT and APDE9 mice displayed a similar increase of weight and total fat percentage compared to control diet counterparts. Glucose intolerance was observed only in APDE9 WD mice and

not in WT WD mice or control diet equivalents. When we consider memory, a spatial memory deficit was only discernible in APDE9 mice fed a WD. This indicates again that WD consumption alone does not affect spatial memory.

Speculatively and beyond the scope of this dissertation, defective brain insulin signalling has been seen in both AD and peripheral insulin resistance observed in diabetes which may be the fundamental mechanism underlying link between WD consumption and APDE9 phenotype to impair spatial memory (Bomfim et al., 2012, Craft, 2012, Ma et al., 2009, Takeda et al., 2011).

7.2. Conclusions to specific aims

7.2.1. Are cognitive impairments associated with HFD consumption or weight gain?

The aims of these animal models are to extrapolate to the human condition (HFD, obesity, cognitive impairment). It is clear from the previous literature there is much variability in experimental procedures and findings (**Tables 1.2 and 1.3**). Moreover in our laboratory, after developing multiple cohorts of rats fed the same composition of diet and fed for the same time period, variable amounts of weight gain, epididymal adipose tissue weight and cognitive performance was observed (Kosari et al., 2012, Jenkins et al., 2016, Ali et al., 2016, Nguyen et al., 2017), Chapters 2,3, and 4).

The question exists whether HFD consumption itself or subsequent weight gain can influence cognitive status. The majority of rodent studies report body weight increases after HFD consumption and impairments in spatial learning and memory, motivation, Pavlovian conditioning, working memory and behavioural flexibility (**Table 1.2**). Thus, it is possible that increased adiposity and body weight *per se* might mediate the neurological perturbations,

as excess adipose tissue is highly metabolically active and is involved in several physiological and biochemical events such as inflammation, angiogenesis, hypertension, and vascular homeostasis.

Nevertheless there have been some studies suggesting that cognitive impairments can be independent to any change of body weight after HFD consumption (Arvanitidis et al., 2009, Beilharz et al., 2016, Bocarsly et al., 2015, Boukouvalas et al., 2008, Francis et al., 2013, Hardy and Selkoe, 2002, Hargrave et al., 2016, Kanoski and Davidson, 2010, Kosari et al., 2012, Underwood and Thompson, 2016a, White et al., 2009a). Indeed in the study from our laboratory, 12 weeks of 60 w/w% HFD feeding impaired spatial memory but actually caused a decrease in overall weight gain (balanced by an increase of calorific content) (Kosari et al., 2012). Moreover, Kanoski and Davidson showed that HFD (40 kcal% fat) fed rats had a spatial working and reference memory impairment in the RAM that emerged as early as 72 h and was persistent till 10 days after HFD commencement without any change in body weight (Kanoski and Davidson, 2010). These data suggest that weight gain through the consumption of WD, or in broader context HFD consumption, is not a prerequisite for cognitive deficits observed in animal models of obesity. Furthermore WD consumption may not directly affect cognitive ability but is mediated through central and/or peripheral changes.

7.2.2. Cognitive impairments and disparities between HFD animal studies.

A challenge with the use of HFDs to induce an obese phenotype in animals is that there are variable parameters including duration of feeding, source of ingredients, and composition of dietary fat leading to varying responses in weight gain, glucose tolerance, insulin resistance, triglycerides, and other metabolic markers (Buettner et al., 2006, Ikemoto et al., 1996, Wang et al., 2002).

7.2.2.1. Duration of feeding

The duration of HFD consumption that has been previously discussed in this thesis (see **Table 1.2**) have ranged from days to almost a year. Spatial memory impairments have been described as early as 72 hrs after HFD commencement in the RAM (Kanoski and Davidson, 2010) and 10 days in the spontaneous alternation task (Hargrave et al., 2016). In our laboratory, we could not achieve consistent effects on cognition or metabolic markers after 3 months dietary exposure.

We can also not assume that there is a simultaneous effect on metabolic markers, brain changes and cognition. An interesting series of studies from the Chattipakorn laboratory, investigated this association. Rats were exposed to a HFD and when tested at 4 and 8 weeks after diet commencement there was no memory deficit in the MWM but a reduction of hippocampal long-term potentiation and long-term depression was observed (Pintana et al., 2016, Pratchayasakul et al., 2015). After 12 weeks, once tested again in the MWM these rats did show an impairment of spatial acquisition (Pintana et al., 2016, Pratchayasakul et al., 2015), demonstrating the timing difference in the presentation of molecular marker of memory and actual memory deficit.

7.2.2.2. Fatty acid composition

Fatty acid composition of the diet should also be considered as it may play a role in body weight regulation. It has been suggested that diets high in saturated fats induce the typical HFD phenotype, whereas diets high in polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) may have less adverse and/or beneficial effects on body composition and insulin action (Abete et al., 2011, Shillabeer and Lau, 1994, Silva et al., 2006, Storlien et al., 1991, Storlien et al., 1996a, Storlien et al., 1996b, Takeuchi et al., 1995). Saturated fats are more poorly used for energy and thus remain to be stored as

triglycerides whilst PUFAs and MUFAs are more readily used for energy when initially ingested (Leyton et al., 1987, Storlien et al., 2001). Different types of fatty acids display different metabolic behaviours such as oxidation and deposition rate differences that may contribute to weight change. The general consensus in animal studies is that the oxidation rate of the saturated fats decrease with increasing length of the carbon chains (McDonald et al., 1980). While research suggest that MUFAs and PUFAs have higher oxidation rates than long-chain saturated fats (Marette et al., 1990).

7.2.2.3. Source of dietary fat

The source of fat and fatty-acid composition that comprise HFDs are inherently linked as most animal-derived fats are saturated fats and in contrast plants & fish-derived fats are generally unsaturated fats. The source of the fat component in these diets can vary between animal-derived fats (e.g., lard, fish oil or beef tallow) and plant oils (e.g., corn or safflower oil) (Buettner et al., 2007). Several epidemiological studies have showed the potential adverse effects of saturated fats on increasing the risk of developing cancer (Leosdottir et al., 2005, Staessen et al., 1997), cardiovascular disease (Chen et al., 2011, Tucker et al., 2005) and mortality (Kromhout et al., 2000) whilst purporting the positive effects of fish oil (Gopinath et al., 2011, Konig et al., 2005, León et al., 2008, Yamagishi et al., 2008). Although research suggests that fats derived from animals and plants can produce comparable physiological responses, e.g. weight gain, hyperglycaemia, hyperinsulinemia, and hypertriglyceridemia after several weeks of consumption in animals (Buettner et al., 2007). However, fat derived from fish has been described to engender obesity resistance by decreasing body weight, serum insulin and glucose (Holness et al., 2003, Levy et al., 2004), triglycerides and induce hypertrophy of visceral fat pads (Yaqoob et al., 1995, Buettner et al., 2007). (Catta-Preta et al., 2012). Animal-derived HFD consumption has been reported to

produce impairments in behavioural flexibility measured by the VIDA task with the degree of impairment being highly associated with the level of animal-derived fat (Greenwood and Winocur, 1996, Greenwood and Winocur, 1990, Winocur and Greenwood, 1999). Further experiments are required to accurately compare and provide a qualitative measure whether different sources of fats and proteins can indeed influence cognition.

7.2.2.4. Source of dietary protein

The protein source in the diets may affect the induction of the obese phenotype and associated cognitive impairment due to the presence of soy. Soy represents the main protein source in almost all natural-ingredient commercially available formulated diets and contains phytoestrogens, a plant compound structurally similar to estradiol that can mimic its effects by binding to estrogen receptors (Lephart et al., 2004, Thigpen et al., 2004). The concentration of phytoestrogens in any diet is directly correlated with the soybean content (Thigpen et al., 1999, Thigpen et al., 2004). There are consistent reports that males outperform females in spatial memory tasks and are suggested to be due to presence of estradiol (Frye and Sturgis, 1995, Luine et al., 1998, Warren and Juraska, 1997). Oral administration of estradiol or phytoestrogens can result in a dose-dependent improvement in the performance of the RAM tests (Lephart et al., 2002, Pan et al., 2000, Pan et al., 2010a). As such, HFDs containing phytoestrogens derived from soy may attenuate or conceal its potential deleterious effects on cognition.

7.3. Future directions

7.3.1. Dopaminergic and serotoninergic systems

Our data suggests that WD consumption impairs dopaminergic system in the striatum and HPC. The specific mechanism, by which WD consumption modifies the dopaminergic system whether it is by DA synthesis, re-uptake or metabolism, is still unknown. Further experiments are required to determine whether WD consumption can induce any changes to DA receptor expression as well as the ability to alter DA synthesis and metabolism. There is a need to examine DA re-uptake with labelled DA to examine whether intracellular levels are reduced.

Another consideration is the serotoninergic system and whether 5-HT_{2C} receptor and 5-HTT expression changes are not localised only in the striatum but in other areas of the brain. HPLC analysis of 5-HT and its primary metabolite, 5-hydroxyindoleacetic acid should also be performed to ascertain to what extent the serotoninergic system is perturbed.

7.3.2. Adipokines

Beyond the scope of this PhD dissertation, the interactions of the adipokines, such as adiponectin and leptin, with cognitive impairment would be an interesting research prospect. As adipocytes produce a number of endocrine hormones that contribute to the regulation fat storage. An increase of visceral fat observed in obesity can reduce lipid storage capacity, leading to increased lipolysis and release of free fatty acids, inflammatory agents, and disturbed adipokine release (Engfeldt and Arner, 1988, McQuaid et al., 2011).

7.3.2.1. Adiponectin

Adiponectin is the most abundant anti-inflammatory adipokine that has the ability to interfere with lipid synthesis & storage, neoglucogenesis and peripheral utilisation of glucose as well as modulating immune responses (Ouchi et al., 2003, Yamauchi et al., 2001). Preclinical studies suggest that adiponectin may induce weight loss by centrally increasing energy expenditure (Qi et al., 2004) and can also protect hippocampal neurons against chemical induced cytotoxicity (Qiu et al., 2011). Furthermore, central administration of adiponectin for 1 week has been reported to increase of neuronal dendritic spines, dendritic arborisation and proliferation of neural progenitor cells in the adult DG (Zhang et al., 2016). In humans, obesity has been associated with lower levels of adiponectin than normal weight counterparts in both children (Stefan et al., 2002) and adults (Arita et al., 1999, Cnop et al., 2003, Jurimae et al., 2009, Matsubara et al., 2002). Whereas studies investigating the link between adiponectin and AD or MCI remains unclear with a study reporting lower serum adiponectin levels associated with worse cognitive performance (Teixeira et al., 2013) whereas others have reported either a non-significant change (Bigalke et al., 2011, van Himbergen et al., 2012) or increase of adiponectin levels (Gu et al., 2010, Une et al., 2011) when compared to healthy counterparts. Authors do suggest the discrepant results may be due to the differences in cognitive status characterisation and sample sizes between studies (Teixeira et al., 2013). There is scarce data concerning adiponectin and its involvement in higher brain function. Only one study has investigated the role of adiponectin and learning behaviour. This study observed that infusions of adiponectin into the DG of the hippocampus in fear-conditioned mice facilitated extinction of contextual fear (Zhang et al., 2017). Adiponectin deficiency did not affect acquisition of contextual fear but impaired extinction learning (Zhang et al., 2017). Deletion of adiponectin receptor protein 2, but not adiponectin receptor protein 1, enhanced fear expression suggesting a link between adiponectin and learning (Zhang et al., 2017).

7.3.2.2. Leptin

Leptin is an adipokine involved in the regulation energy homeostasis by interfering with hypothalamic signalling that controls long-term caloric intake and fat stores (Elmquist et al., 1998, Friedman, 2010, Farr et al., 2014). Leptin also has been shown to be involved in memory processes with leptin deficient mice having impairments in hippocampal long-term potentiation & long-term depression (Harvey et al., 2006, Li et al., 2002, Shanley et al., 2001), spatial memory in the T-maze (Farr et al., 2006). Whilst central administration of leptin improved performance in the RAM task (Paulus et al., 2005), MWM task (Oomura et al., 2006) and also has been reported to facilitate hippocampal long-term potentiation (Oomura et al., 2006, Wayner et al., 2004). Obese humans and animals have elevated serum leptin and impaired physiological responses to exogenously administered leptin, and are hence considered leptin-resistant (Caro et al., 1996, Considine et al., 1996, Frederich et al., 1995, Halaas et al., 1997). Additionally, serum leptin levels have also been reported to be negatively associated with AD risk (Jane et al., 2014, Johnston et al., 2011, Lieb et al., 2009).

7.3.3. Tumour necrosis factor (TNF)-alpha

Tumour necrosis factor (TNF)-alpha is a cytokine involved in systemic inflammation mainly produced by macrophages but also by a broad variety of cell types such as mast cells and neurons. Its primary role is the regulation of the acute phase reaction of inflammation where it can induce fever, stimulate phagocytosis, and apoptotic cell death. Within the brain TNF-α regulates synaptic transmission, synaptic plasticity and neurogenesis. (Montgomery and Bowers, 2012). Thus it has a broad range of actions which can be either neuroprotective or neurotoxic. Obesity is characterized by low-grade inflammation both in peripheral tissues and in hypothalamic areas critical for energy homeostasis (Das, 2001, Thaler et al., 2012).

Overweight and obese children and adults have elevated serum levels of TNF-α and serum-soluble TNF receptor p55 (sTNF-RI), which are closely associated with cardiovascular risk factors and cardiovascular and non-cardiovascular causes of death (Das, 2001). Whilst an intervention of regular exercise decreased BMI, percentage body fat, serum TNF-α and sTNF-RI (Tsukui et al., 2000, Zahorska-Markiewicz et al., 2000). In mice, differences in cognitive performance have been associated with TNF-α gene polymorphisms and TNF-α knockout mice display cognitive dysfunctions in a variety of tasks involving learning and retention, spatial learning/memory, cognitive flexibility, and learning effectiveness (Baune et al., 2008, Beste et al., 2010). The authors note that the absence of TNF was correlated with poor cognitive functioning and the deletion of both TNF-receptors reduced cognitive functioning (Baune et al., 2008)

Consequently it would be important to inspect potential changes in adiponectin, leptin, circulating inflammatory agents such as TNF-α and adipokine levels in response to WD consumption and examine possible associations to cognitive impairment.

7.4. Overall Conclusion

The results of this PhD thesis have demonstrated that the obese phenotype induced by WD consumption singlehandedly does not affect cognition in the assortment of behavioural tasks employed testing different aspects of conditioning, motivation and spatial memory. Our biochemical data suggest that WD consumption induces central changes, particularly in the striatum. It is still unclear if the central biochemical changes presented in this thesis are associated with impairments of learning and memory reported in the literature. It is conceivable that these central biochemical changes precede impairment of learning and memory. Cognitive impairment arising from HFD consumption may be due to an interaction

of many external factors, such as diet duration and composition, but also internal factors such as consequent changes to neurotransmitter systems, central inflammation and signalling. This thesis also shows that neonatal overfeeding does not impair spatial memory in a one-trial one-test spatial memory task but does diminish acquisition of the multi-trial spatial memory DWSh task. Furthermore, in an AD animal model we demonstrate that WD consumption alone does not affect spatial memory but there could possibly be interplay between the APDE9 mice phenotype and WD consumption having a deleterious effect of spatial memory.

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