

**A Diet High in Saturated Fat Leads to Obesity in  
Female Rats, but Does Not Affect Total, Synaptic, or  
Cell Surface Expression of NMDA Receptor Subunits  
In Hippocampus**

by  
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### Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## Abstract

Obesity is an accumulation of adipose tissue to the point of negative health outcomes; the association of obesity with type 2 diabetes and cardiovascular disease is particularly profound. Notably, obesity has begun to be associated with impairments in cognitive function; however, the underlying cellular mechanisms are poorly understood. Behavioural studies have demonstrated a link between a high fat diet and impaired hippocampal function, and our aim was to characterize protein level changes in the hippocampus of an obese female rat model.

Female rats were fed either a control diet (CD; 10% kcal from fat), or a high-fat diet (HFD; 45% kcal from fat) for 16 weeks. Body weight, food consumption, fasting blood glucose levels, and glucose tolerance were monitored. Upon sacrifice brain, liver, adrenal glands, spleens and fat pads were harvested and analyzed. Plasma leptin and insulin levels were also measured. The distribution of NMDA receptor subunits was examined by using either cell-surface biotinylation, or differential filtration-centrifugation followed by immunoblotting.

The feeding protocol induced an obese phenotype in female rats characterized by larger fat pads, spleens and adrenal glands, as well as greater problems handling a glucose load. However, cellular, surface and synaptic expression of NMDA receptor subunits (GluN1, GluN2A & GluN2B) were not significantly altered, which suggests that changes downstream of the receptor may be responsible for the effects of HFD on cognitive behaviour.

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## List of Abbreviations

ACSF: Artificial Cerebral Spinal Fluid

AMPA:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid

AMPAR:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor

ASP: Acylation Stimulating Protein

AUC: Area Under The Curve

BDNF: Brain-Derived Neurotrophic Factor

BMI: Body Mass Index

CD: Control Diet

CNS: Central Nervous System

CVD: Cardiovascular Disease

FBG: Fasting Blood Glucose

GABA: Gamma Aminobutyric Acid

HFD: High Fat Diet

LTD: Long Term Depression

LTP: Long Term Potentiation

MCP- 1: Monocyte Chemoattractant Protein- 1

NCAM: Neural Cell Adhesion Molecule

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate Receptor

OGTT: Oral Glucose Tolerance Test

PAI- 1: Plasminogen Activator Inhibitor- 1

PND: Post Natal Day

PSD-95: Postsynaptic Density Protein 95

SDS-Page: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

T2D: Type 2 Diabetes

WHO: World Health Organization



## **Introduction:**

### 1.0 Obesity and Public Health

Obesity is the excess accumulation of fat to the point of negative health outcomes (Haslam & James, 2006), and is a rising global epidemic that needs to be addressed. Body mass index, or BMI, is a common method of monitoring body composition. In 2008, the World Health Organization (WHO) provided global estimates that 35% of adults aged 20+ were overweight (BMI  $\geq 25$  kg/m<sup>2</sup>) and 12% were obese (BMI  $\geq 30$  kg/m<sup>2</sup>). Global obesity prevalence has dramatically increased since the 1980s when only 5% of men and 8% of women were affected (WHO, 2008). In Canada, approximately 18% of adults were obese in 2011 (Statistics Canada, 2011).

The WHO concluded that women are more likely to be obese than men in all regions of the world, however, the gender gap is most profound in lower income countries. In the WHO regions of Europe, Eastern Mediterranean and the Americas, over 50% of women are overweight, of which, roughly half are obese (23% in Europe, 24% in the Eastern Mediterranean, 29% in the Americas; WHO, 2008). Interestingly, compared to the global pattern, the trend of obesity is reversed in Canada; 60.1 % of Canadian men are classified as overweight, or obese, compared to 44.2% of women (Statistics Canada, 2011).

Obesity is one of the leading contributors to preventable death globally (WHO, 2008); at least 2.8 million people die each year as a result of being overweight, or obese, and mortality increases with increasing BMI (WHO, 2008). Obesity often leads to the development of type 2 diabetes mellitus (T2D), hypertension, cardiovascular disease (CVD) and certain forms of cancer (Das, 2010). Notably, the association between obesity and T2D is highly significant, and ~90%

of people diagnosed with T2D are overweight, or obese (Albu & Pi-Sunyer, 1998). As well, a study of more than 300000 adults over 7 years found a 9% increase in risk of an ischaemic event for every additional unit of BMI (James et al., 2004). In addition to being associated with mortality, excess weight can significantly impact quality of life through other conditions, such as sleep apnea, osteoarthritis, intracranial hypertension, gastroesophageal reflux, infertility and urinary stress incontinence (Das, 2011). Although obesity cannot directly cause death, the link between obesity and the two most common causes of death (CVD and cancer), along with their rapidly increasing prevalence, highlight the importance of allocating attention and resources to this issue.

### 1.1 Causes of Obesity

Accumulation of adipose (fat) tissue results from an energy imbalance wherein the consumption of energy exceeds expenditure. Obesity can arise as a consequence of genetic factors affecting metabolism, and by lifestyle factors, such as diet and exercise. Genetic variability in weight gain between individuals can be attributable to resting basal metabolic rate, thermic response to food, nutrient partitioning, and energy expenditure associated with physical activity (Das UN, 2010). Energy-rich diets and limited physical activity are considered to be the primary factors contributing to the excess accumulation of fat. In particular, excess consumption of saturated fats and refined carbohydrates, the principal components of the “Western Diet”, are believed to significantly impact excess weight gain, and contribute to the rising prevalence of obesity (Hu et al., 2001). A typical human obesogenic diet contains 35% of calories from fat (Miller et al., 1990). In addition, according to the WHO, physical activity levels are key determinants of energy expenditure, and are fundamental to energy balance and weight control (WHO, 2008). Other potential factors affecting obesity development include: exposure to certain

microorganisms, advanced maternal age, insufficient sleep, endocrine disruptors, pharmaceutical iatrogenesis, reduction in variability of ambient temperatures, and intrauterine and intergenerational effects (McAllister et al., 2009). However, increased consumption and decreased expenditure of calories are believed to be the primary, or at least most intuitive and frequently studied, contributors to the current global obesity epidemic.

## 2.0 Metabolic Characteristics of Obesity

Obesity is a main component of the metabolic syndrome, which also includes hyperglycemia, insulin resistance, dyslipidemia (high triglyceride levels and low levels of high density lipoprotein) and hypertension (Abete et al., 2010). As well, hyperleptinemia is highly prevalent in obese individuals (Considine et al., 1996). The described changes are influenced by multiple organ systems, and nearly all are related to energy homeostasis.

Energy homeostasis is highly influenced by the central nervous system (CNS), gastrointestinal tract, and adipose tissue. The regulation of energy homeostasis can be subdivided into short and long term signaling pathways. Short term signaling pathways involve a variety of signals, mainly from the GI tract and adipose tissue to the CNS, and regulate eating behaviour, such as meal sizes and frequency. Adipose tissue is able to secrete a variety of hormones (termed adipokines) which are affected by changes in fat mass, including: adiponectin, apelin, angiotensin II, acylation stimulating protein (ASP), leptin, plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1), omentin, resistin, visfatin, proinflammatory cytokines and glucocorticoids (Caylak, 2012). While the relationship between the signaling molecules involved in obesity is very complex, the two primary long-term adiposity signals thought to mediate energy balance are the hormones leptin and insulin.

### 2.1 Leptin

Leptin is a hormone secreted predominantly by white adipose tissue, and plasma levels of leptin are proportional to body fat content (Harvey & Ashford, 2003). Leptin levels positively correlate with body fat levels more than any other factor in humans (Considine et al., 1996), and

decrease with physical activity (Koutsari et al., 2003). Notably, leptin is able to enter the brain by a saturable transport mechanism across the blood brain barrier (Banks et al., 1996).

Appetite regulation is believed to be the primary role of leptin via its action on the hypothalamus, although the presence of leptin receptors in other brain areas and bodily tissues suggests additional roles, such as being a permissive factor for puberty, a signal of metabolic status, and, most interestingly, a modulator between maternal and fetal metabolism (Margetic et al., 2002). Direct manipulation of leptin in the brain is able to affect food intake and energy homeostasis (Baskin et al., 1999; Campfield et al., 1995; Cheung et al., 1997; Weigle et al., 1995).

Mutation in the *ob* (leptin) and *db* (leptin receptor; Lee et al., 1996; Wang et al., 1998) genes gives rise to phenotypically obese mice with classical metabolic signs of T2D, including hyperglycemia and hyperinsulemia (Zhang et al., 1994). Leptin administration to *ob/ob* (obese with T2D) mice corrects some of the metabolic defects and aids in weight loss by influencing the endocrine hypothalamus (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). In addition to the endocrine hypothalamus, leptin receptors are found in other brain areas, including the hippocampus (Hakansson et al., 1998), a brain area responsible for memory formation and storage (Ergorul and Eichenbaum, 2004; Section 4.0). Leptin has been shown to affect hippocampal function and alter expression of NMDA receptors in neonatal rats (more information in section 2.4) (Walker et al., 2007).

## 2.2 Insulin

Insulin is a peptide hormone, produced by the beta cells of the pancreas, that primarily functions to increase tissue uptake of glucose from the blood stream. In addition, insulin was the

first hormone to be identified in the control of food intake by the CNS (Woods et al, 1979), and direct manipulation of insulin in the CNS is able to affect food intake due to the presence of insulin receptors in key areas of energy control (Baskin et al., 1988; Sipols et al., 1995).

Virtually all vertebrate tissues, including the brain, contain the tetrameric insulin receptor (IR) (Havrankova et al., 1978; Zhao et al., 1999). Insulin secretion increases with greater weight, in order to maintain the same level of glucose homeostasis in the blood stream, eventually causing tissues to become more resistant to its effects (Stockhorst et al., 2004). Failure of insulin to maintain glucose homeostasis because of tissue resistance to the hormone is the basic causal factor of T2D. Both human and animal data show that the consumption of high fat diet can lead to peripheral insulin resistance and negatively affect cognitive performance (Greenwood & Winocur, 2005; Kalmijn et al., 2004). Mielke et al. (2005) have demonstrated that nutritionally induced insulin resistance affects the neural insulin signaling pathway, and impairs synaptic plasticity in the hippocampus, which links insulin resistance and memory impairment.

### 3.0 Obesity and Brain Function

#### 3.1 Human Studies

Human epidemiological data suggest that obesity in young and middle adulthood significantly increases the risk of dementia in old age (Wilson and Bennet, 2003), and is linked to poor cognitive performance across the lifespan (Elias et al., 2003; Jeong et al., 2005; Smith et al., 2011; Sorensen et al., 1983). For example, in a large prospective study, Elias et al. (2003) found that obesity and hypertension positively correlated with poor memory performance in men, but not women; therefore, obesity may have sex specific effects. In addition, general intelligence scores were lower in obese men, independent of their socio-economic status (Sorensen et al., 1983).

#### 3.2 Animal Studies

##### a. Mice

High fat diet (HFD)-induced obesity caused a significant impairment in glucose regulation and impaired procedural learning and consolidation processes in mice (tested via a bar-pressing task) (Mielke et al., 2006). In contrast, Messier et al. (2007) found that feeding mice a HFD did not affect procedural learning (also tested via a bar pressing task). The difference in results between the studies could be due to the variable lengths of dietary protocols used (12 months vs 3 months, respectively; see Table 2). There are additional inconsistencies with regards to the effects of HFD consumption on spatial memory. While Mielke et al., (2006) did not see a significant effect of the diet on hippocampal-dependent spatial memory (Morris water maze), Farr et al., (2008) reported that diet induced obesity, and hypertriglyceridemia, affected performance of mice on several hippocampal-dependent memory tasks (Morris water maze, bar

pressing task, and active avoidance T-test). The length of the diet was not reported by Farr et al., as they used a 30% difference in body weight (between mice in HFD vs control conditions) as a threshold of obesity onset, which complicates comparison between the studies. In addition, Valladolid-Acebes et al., (2011) also confirmed spatial learning impairment in HFD-fed mice (four-arm baited version of the eight-arm radial maze test) after 8 weeks of diet consumption.

The effects of high fat diet consumption on cognition might be reversible. A HFD protocol (45% kcal from fat) induced significant weight gain, increased plasma glucose levels, and impaired performance on a hippocampal-dependent object recognition memory test (Gault et al., 2010; Porter et al., 2011). However, switching the animals to a normal chow for 28 days, or prolonged activation of the glucose dependent insulinotropic polypeptide receptors (Porter et al., 2011), as well as exendin-4 therapy (an enzyme-resistant glucagon-like peptide-1 mimetic; Gault et al., 2010), improved the cognitive deficits induced by the HFD to varying degrees.

All of the previously mentioned studies were performed on male mice, and might not be generalizable to female animals. Indeed, Hwang et al. (2010) showed that 9-12 months of HFD consumption affected male and female mice to different degrees. Male mice were more vulnerable to HFD-induced weight gain and metabolic alterations (hyperglycemia, hypercholesterolemia, hyperinsulemia, and hyperleptinemia), which are typical obesity-related changes seen in obese humans with metabolic syndrome. Synaptic plasticity (e.g., Long Term Potentiation (LTP) and Long Term Depression (LTD)), but not basal transmission, were also adversely affected by the HFD intervention in male, but not female, mice. HFD-fed male mice also performed significantly worse on learning and memory tasks, which was not attributable to locomotor deficits. Collectively, the mouse studies show that diet-induced obesity is able to alter



certain memory processes, and that the effects could be sex specific; however, differences in dietary protocols across studies complicates the ability to compare the results.

#### b. Rats

A link between HFD-driven obesity and poor cognitive function has been well established in rats. For example, HFD consumption significantly impairs hippocampal-dependent spatial working memory tests (*Radial Arm Maze Task*, Murray et al., 2009; Kanoski & Davidson, 2010; Winocur & Greenwood, 2005; *8-Arm Water Radial Maze Task*, Granholm et al., 2008; *Morris Water Maze Task*, Jurdak et al., 2008; Molteni et al., 2002; Pintana et al., 2012; Stranahan et al., 2008). Notably, some of the studies supplemented their diets with sucrose, or glucose (Jurdak et al., 2008; Kanoski & Davidson, 2012; Molteni et al., 2002; Stranahan et al., 2008). In contrast, McNeilly et al., (2011) failed to see a diet induced difference on water maze performance after 8 weeks of HFD feeding, but did report a significantly worse performance of the HFD group on operant based delayed matching to position task (operant learning). Winocur & Greenwood, (2005) also observed HFD-induced impairment on an operant test of delayed alteration performance with variable intervals between trials (thalamic and hippocampal dependent short and long term memory test), and a series of complex blind-alley maze tasks (a measure of general intelligence).

While the water maze and radial arm tasks primarily measure learning ability, Davidson et al., (2012) used a hippocampal-dependent serial feature-negative discrimination problem, and showed that a high fat/carbohydrate diet was able to adversely affect retention, and retrieval, of memories learned prior to dietary intervention. The authors also tested blood-brain barrier (BBB) permeability by injection of sodium fluorescein (dye) into the right femoral artery; subsequent

analysis revealed more dye in the hippocampus of the obese group, suggesting a diet-induced increase in BBB permeability around the hippocampus.

Human epidemiological data show a positive correlation between obesity, cognitive impairment, and increased risk of neurodegenerative disorders. Experimental data support the ability of HFD-induced obesity to affect cognition in mice and rats; in particular, HFD-induced obesity seems to impair hippocampal-dependent memory. While the mechanistic link between obesity and brain function is not completely understood, some authors propose metabolic changes (such as brain insulin resistance) to be a key link (Mielke et al., 2005; McNeilly et al., 2011).

## 4.0 The Hippocampus

The hippocampus belongs to the limbic system, and is responsible for memory formation and storage, as well as spatial navigation. Two basic cellular mechanisms believed to be involved in memory that have been extensively studied in the hippocampus are LTP and LTD (Bear & Abraham, 1996). LTP is characterized by an increase in strength of basal synaptic transmission (Bliss & Collingridge, 1993). Although, LTD (an activity-dependent reduction in the efficacy of neuronal strength) is often thought of as a “forgetting” phenomenon, Willshaw & Dayan (1990) argue that LTD-like modifications add flexibility and information storage capacity to the memory system.

LTP maintenance (beyond ~1 h) requires novel protein synthesis and changes at the post-synaptic density (Nicoll & Malenka, 1995). In addition, LTP induction and maintenance requires the participation of glutamate receptors, specifically the NMDA and AMPA subtypes (to be discussed in the next section; Bliss & Collingridge, 1993; MacDonald et al., 2006; Perez-Otano & Ehlers, 2005; Williams et al, 2007). NMDA receptor activation is also required for the induction of LTD, as NMDA receptor antagonists can block the phenomenon (Mulkey & Malenka, 1992).

### 4.1 The Glutamate Receptors (GluRs)

There are three types of glutamate receptors (NMDA, AMPA and kainate) that can be subdivided into ionotropic (ion channels), or metabotropic subtypes. Upon activation, the glutamate receptors can either permit cation transfer across the cell membrane (ionotropic cation channels), or initiate intracellular signaling cascades (metabotropic G-protein coupled receptors).

All of the ionotropic glutamate receptors are permeable to sodium ( $\text{Na}^{2+}$ ) and potassium ions ( $\text{K}^+$ ), while a fraction of them are also permeable to calcium ions ( $\text{Ca}^{2+}$ ).

Glutamatergic excitatory neurotransmission can be broken down into 3 basic elements: the release of glutamate from pre-synaptic vesicles, its passage across the synaptic cleft, and activation of glutamate receptors on the post-synaptic membrane. The strength of the post-synaptic response can be influenced by the amount of glutamate released into the synaptic cleft, the efficiency of glutamate clearance mechanisms, the quantity and inherent structural characteristics of glutamate receptors on the post-synaptic membrane, as well as post translational modifications of the post-synaptic receptors (e.g., phosphorylation status). Basal synaptic transmission is primarily mediated by AMPA receptors, while high frequency stimulation recruits NMDA receptors (Bartlett et al., 2007; Pavlov & Mielke, 2012).

In addition to their role in synaptic plasticity, GluRs participate in multiple physiological processes, including the formation of neuronal networks during development (Constantine-Paton, 1990; Komuro & Rakic, 1993), and the pattern of ongoing synaptic communication (Traynelis et al., 2010). Due to the abundance of glutamate receptors in the CNS, the GluRs have been implicated in neurodevelopmental disorders (e.g., schizophrenia) (Gilmour et al, 2012), mood disorders (e.g., depression) (Machado-Vieira et al, 2009), chronic neurodegeneration (e.g., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis) (Ahmed et al., 2011; Bogaert et al., 2010; Hu et al., 2012), pain transmission (Bleakman et al., 2006), and brain injury (e.g., cerebral ischemia) (Pavlov & Mielke, 2012).

## 4.2 *N*-methyl-D-aspartate Receptors (NMDARs)

The NMDARs are thought to be heteromeric complexes formed from a combination of 4 subunits: GluN1, GluN2 (A-D subtypes), and GluN3 (A-B subtypes). The GluN1 subunit is an obligatory subunit for all functional receptors (Forrest et al., 1994), while the supplementary GluN2 and GluN3 subunits contribute to various functional properties, including channel gating and ion conductance (Traynelis et al., 2010). Despite a great degree of developmental and anatomical heterogeneity, the majority of NMDARs are believed to consist of two GluN1 with two GluN2 subunits, or, in some cases, a combination of GluN2 and GluN3 subunits (Traynelis et al., 2010). In the hippocampus, synaptic NMDARs are believed to contain primarily GluN2A subunits, while NMDARs containing GluN2B subunits are found extra-synaptically (Petralia, 2012).

Each NMDA receptor subunit shares a similar general architecture: a large extracellular region that consists of the amino-terminal and ligand binding domains, a pore-forming transmembrane region, and an intracellular region containing the carboxy-terminal domain (Frank, 2011; Traynelis et al., 2010). Activation of the NMDA receptor requires binding of the ligand glutamate to the extracellular segment of the GluN2 and GluN3 subunits, binding of the co-agonist glycine to the extracellular segment of the GluN1 subunit, and membrane depolarisation. Depolarisation of the membrane expels magnesium ions, which block the channel pore of NMDA receptors in a non-active state. Due to the unique activation requirements (i.e., requiring both membrane depolarisation and agonist stimulation), the NMDA receptors are often called coincidence detectors. Upon activation, NMDA receptors become permeable to sodium, potassium and calcium ions. Notably, the unique ability to transfer calcium ions involves NMDA

receptors in many important physiological and pathological states (Groc et al., 2009; Szydlowska & Tymianski, 2010; Traynelis et al., 2010).

As with other cell surface receptors, NMDARs are assembled in the endoplasmic reticulum and transported through the Golgi network towards the cell membrane. Within the cell membrane, distribution of NMDARs is not limited to the synaptic cleft, but is also observed in the peri-synaptic and extra-synaptic regions of the membrane (Groc et al., 2009). The NMDARs are not permanently restricted to one location, but can be trafficked between various organelles and the plasma membrane (Dunah & Standaert, 2001; Grosshans et al., 2002; Lavezzari et al., 2004; Rao & Craig, 1997). The receptors also have a great degree of mobility between synaptic and extra-synaptic sites (Goebel-Goody et al., 2009; Groc et al., 2004), and ~65% of synaptic NMDARs are considered mobile (Tovar & Westbrook, 2002). Trafficking of NMDARs can be triggered by biological phenomena (such as LTP; Grosshans et al., 2002; Rao & Craig, 1997), or in response to pathological phenomenon (such as cerebral ischaemia; Pavlov & Mielke, 2012). Trafficking is able to alter the number of functional receptors in places where they can be accessed by ligands, and presents an additional level of regulation of synaptic activity (Collingridge et al., 2004). Trafficking of NMDARs can occur by endo/exocytosis utilizing clathrin-coated pits, or through lateral diffusion along the plasma membrane (Groc et al., 2009; Lavezzari et al., 2004; Prybylowski et al., 2005; Tovar and Westbrook, 2002), and mobility dynamics of NMDARs are highly dependent on their association with other synaptic proteins (e.g., PSD-95, SAP 102; Groc et al., 2009; Prybylowski et al., 2005; Roche et al., 2001). Subunit composition of NMDARs appears to affect the trafficking rates as well (Groc et al., 2009; Roche et al., 2001), however, most current knowledge of NMDA receptor trafficking was obtained from embryonic cultured neurons, and may not accurately reflect mobility dynamics in adult brain.

### 4.3 Obesity Effects on NMDARs.

Eight weeks of HFD (45% kcal from fat) consumption significantly increased body weight and plasma leptin concentrations in C57BL/6J mice (Valladolid-Acebes et al., 2011). As well, the authors report altered density of glutamate carriers and transporters, glutamate kinetics, expression of NMDAR subunits and basal synaptic transmission and plasticity in the hippocampus (Valladolid-Acebes et al., 2011). The GluN2B subunits were significantly downregulated in the HFD fed animals (Cohen's estimate of effect size: 1.7), while no changes in GluN2A, GluN1 and GluA2 subunits were found. Importantly, the study had insufficient power (18.7%; SD=52.91, Mean 1=100, Mean 2=90; based on the data from the GluN2A figure) to detect significant results with respect to GluN2A subunit expression.

As discussed in section 2.1, circulating leptin levels are increased as a result of obesity, and since leptin receptors are found in many brain areas, there is potential for leptin mediated brain alterations in obese individuals. Indeed, neonatal exposure to leptin was shown to affect hippocampal function and alter expression of NMDA receptor subunits in rats (Walker et al., 2007). Two weeks of prenatal leptin administration caused a dramatic impairment in LTP on post natal day 10 (PND10), increased GluN1 expression, and decreased GluN2B expression. Whether, the observed effects of leptin on NMDA receptor expression are limited to the examined developmental period is unclear, since the study did not examine adult brain. Additional evidence suggests that leptin impairs LTP only in the early developmental period (PND 5-18) while enhancing LTP in adult rats (Moult & Harvey, 2011). The authors also indicate that LTP suppression was mediated by the GluN2B containing NMDA receptors, while enhancement of LTP was mediated by the GluN2A containing NMDARs; however, the subunit

expression was not measured directly. In a comprehensive review, Moulton and Harvey (2010) argue that leptin is able to alter NMDA receptor trafficking.

Leptin is able to facilitate NMDAR-dependent synaptic plasticity by converting short-term potentiation into LTP. Administration of leptin enhances currents evoked by maximal, as well as sub-maximal, concentrations of NMDA, which suggests an increase in the density of NMDA receptors at the cell surface (Harvey et al., 2005). The exact mechanism through which leptin is able to alter NMDA receptor trafficking is unknown, but the authors suggest that intracellular cascades are a necessary intermediate.

Circulating levels of insulin are also increased in obese patients (see section 2.2), and insulin was shown to facilitate NMDA receptor exocytosis to the cell membrane (Skeberdis et al., 2000), as well as AMPA receptor clathrin-dependent endocytosis (Man et al., 2000). Insulin can also regulate trafficking of gamma-aminobutyric acid (GABA) receptors and impact synaptic functions associated with learning (Chapouthier and Venault, 2002; McGaugh, 2002; Mielke et al., 2005; Paulsen and Moser, 1998).

Acute administration of leptin enhances NMDAR-dependent plasticity, yet hippocampal function in HFD-induced animals is impaired, which suggests that chronic elevation of leptin and peripheral leptin resistance (common in obese individuals) can affect NMDARs differently. If both leptin and insulin are able to regulate NMDA receptor trafficking, expression and distribution of NMDARS in obese individuals could be affected by a synergistic action of both hormones.



## 5.0 Rationale for the study:

Obesity is a globally significant health concern. Human epidemiological and experimental animal data provide evidence for the ability of obesity, and associated metabolic changes, to affect brain function. In particular, a region of the brain responsible for memory (hippocampus) is affected. Within the hippocampus, basic cellular mechanisms of memory (LTP and LTD) are susceptible to dietary influence, including the consumption of high fat diet leading to obesity. More specifically, a link between obesity and glutamate receptors, which are involved in memory, is suggested. High circulating levels of leptin and insulin, as well as tissue resistance to these hormones, is a likely mechanism that alters expression and trafficking of NMDA receptors. Most animal studies have assessed the effects of dietary modification on males, while epidemiological data suggests that obesity is more prevalent in women.

The proposed study will assess the effects of obesity on total expression, as well as distribution of NMDA receptors (cell surface and synaptic) in the hippocampus of female rats. We hypothesize that, in addition to a total reduction of GluN2B subunit expression, synaptic and surface expression of all NMDA receptor subunits will be reduced (see Table 3 for details). Reduction of NMDA receptor expression could provide structural evidence for a link between obesity and impaired hippocampal function.

## 6.0 Methods

The study consisted of three phases: high-fat feeding to induce an obese phenotype, tissue collection, and biochemical analyses (Figure 2). Notably, two rounds of the study were conducted, and were identical except for slight modifications to the synaptoneurosome technique (Section 6.2.3). All animal care procedures were approved by the University of Waterloo Animal Care Committee.

### 6.1 Phase 1: Diet Assignment

Each week for 5 weeks, 6 female, non-sibling Sprague-Dawley rats at post-natal day (PND) 21, were received and housed in polypropylene cages with woodchip bedding and stainless steel wire lids. Animals were provided a black plastic tube for enrichment, and fed a standard Harlan Teklad rodent diet (#8640) ad libitum with free access to water. Rats were kept on a 12 hour dark/light cycle. At PND56, animals in the weekly groups were weighed and randomly separated into two cages (N = 3/cage). Importantly, animals were screened for the presence of outliers (a body weight more than 2 standard deviations from the mean); no outliers were found.

After PND 56, rats were placed on either a HFD (20% protein, 35% carbohydrate, 45% fat; Research Diets D12451), or CD (20% protein, 70% carbohydrate, 10% fat; Research Diets D12450B) ad libitum (Table 1) for 16 weeks. During the feeding phase, the hoppers were topped up to 500 g every Monday. Food consumption and body weights were collected twice weekly (Monday & Friday).

### 6.1.1 Oral Glucose Tolerance Test (OGTT)

An OGTT is a functional measure of glucose utilization commonly used in clinical practice to diagnose diabetes. An OGTT was performed every 4 weeks for all groups throughout the study. On weeks when group 1 and 5 OGTTs would overlap, group 1 was tested 1 day earlier. Animals were fasted for 12 h before every OGTT, and baseline glucose levels determined using a standard glucose meter with blood gathered via a tail poke. Each rat was then administered (via gavage) a 50% glucose solution (2 g/kg) freshly prepared before the experiment, and blood glucose measurements were taken 30 min, 60 min, 90 min, 120 min and 180 min later. The data were plotted on a graph and the area under the curve (initial glucose spike and subsequent return to baseline) was compared between groups.

### 6.2 Phase 2: Tissue Collection and Sample Preparation

At the end of the feeding phase, two of the three rats in each cage were sacrificed; while the remaining animals were bred, and used for a subsequent study examining the gestational effects of maternal HFD. Animals were fasted for 12 hours, and sacrificed via decapitation after anesthesia with carbon dioxide. Each brain was quickly removed and placed in chilled ( $<4^{\circ}\text{C}$ ) oxygenated (95%  $\text{O}_2$ : 5%  $\text{CO}_2$ ) artificial cerebrospinal fluid (ACSF; containing 127.0 mM NaCl, 2.0 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 26.0 mM  $\text{NaHCO}_3$ , 2.0 mM  $\text{MgSO}_4$ , 2.0 mM  $\text{CaCl}_2$ , 10.0 mM glucose; pH 7.4; 300-320 mOsm). Hippocampi were extracted and either homogenized, or cut with a McIlwain tissue chopper into 350  $\mu\text{m}$  thick slices. In some experiments, immediately after sectioning, four slices were manually homogenized in cold Krebs buffer (Section 6.2.3b), and used to prepare synaptoneurosomes (Section 6.2.3a); in other cases, the entire right hippocampus was used for synaptoneurosome preparation. The remaining slices were incubated at an interface,

and allowed to recover for 60-90 min in a chamber with warm ACSF (35°C) and flowing carbogen (95% O<sub>2</sub>: 5% CO<sub>2</sub>).

### 6.2.1 Biometrics

Body weight, body length (nose to tail base), retroperitoneal fat pad weight, adrenal gland weight, spleen weight, liver weight and liver volume were measured. Two vials of trunk blood were collected for analysis of leptin and insulin levels. Trunk blood was allowed to clot for 30 min at room temperature, spun at 2000 x g for 15 min, and the supernatant (serum) was removed and stored at -80°C. The quantification of hormone levels in serum was performed with either an insulin ELISA kit (Millipore), or leptin ELISA kit (Millipore), according to the manufacturer's recommended protocols. Analyses were performed using a plate reader (SPECTRAMax Plus; Molecular Devices).

### 6.2.2 Biotinylation.

Immediately prior to each experiment, the biotinylation solution was prepared by diluting biotin (Thermo Scientific) in ACSF (1.0 mg/mL). Two cell culture inserts (used to hold the slices in the maintenance chamber with ACSF) were removed and placed in a 6-well culture plate. Each insert was incubated with either biotinylation solution, or ACSF (1 mL below insert and 0.5 mL above) using gentle agitation for 45 min at 4°C. After incubation, inserts were placed over ice, and then washed twice with a 50 mM Tris-ACSF quenching solution. Slices were then gently removed from inserts (3 slices/condition), homogenized in 750 µL of non-ionizing lysis buffer [10 mM Tris, 25 mM EDTA, 100 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) IGEPAL CA-630], supplemented with a protease inhibitor cocktail. Following manual homogenization over ice, lysates were centrifuged at 1000 x g for 10 min at 4°C, and the protein concentration of

supernatants was determined with a BioRad DC protein assay kit according to the manufacturer's recommended protocol. Washed neutravidin beads (40  $\mu$ L; Thermo Scientific) were then added to a volume of lysate containing 50  $\mu$ g of protein, and samples incubated for 4 h at 4°C on a roto-shaker. Proteins not isolated through neutravidin–biotin interaction were removed by three successive washes wherein mixtures were spun at 9300 x g, supernatants removed, and 500  $\mu$ L of non-ionizing lysis buffer was used to resuspend the pellets. After the final wash, 50  $\mu$ L of Laemmli buffer [0.0625 M Tris (pH 6.8), 5% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) beta-mercaptoethanol, and 0.001% (w/v) bromphenol blue] was added to each vial, vortexed, placed in a heating block at 95°C (5 min), vortexed again, and pulsed. The supernatants (~35  $\mu$ L) were then resolved with SDS-PAGE.

### 6.2.3 Synaptoneurosome Isolation

#### a. Whole Hippocampus Method

After decapitation and brain removal (described in Section 6.2), the right hippocampus was manually homogenized in 1.5 mL of modified Krebs buffer [118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 24.9 mM NaHCO<sub>3</sub>, 10 mM glucose, pH ~7.4], supplemented with a protease inhibitor cocktail; the homogenate was left on ice for 10 min to allow for gravity sedimentation (to clear nucleic acids), after which ~200  $\mu$ L of the supernatant was saved for SDS-PAGE (designated as the total sample). Each sample was then loaded into a 3 cc syringe attached to a 25 mm diameter syringe filter holder (Millipore). The diluted filtrate was gently forced through three layers of nylon mesh (100  $\mu$ m pore; Millipore) pre-wetted with ~0.2 mL of Krebs buffer, and collected in a 1.5 mL Eppendorf tube. Filtered samples were then loaded into a 1 cc syringe and forced through a pre-wetted 5  $\mu$ m

nitrocellulose filter (Millipore) held in a 13 mm filter holder (Millipore). The homogenate was kept ice cold at all times to minimize proteolysis. Filtered samples were then spun at 1000 x g for 15 min (4°C). The supernatant was removed, and the pellet (synaptoneurosome) was re-suspended in 250 µL of Krebs buffer for analysis with SDS-PAGE. The first cohort of animals was examined with this method of synaptoneurosome preparation (N = 5/condition).

#### b. Hippocampal Slice Method

For the second cohort of animals the method was modified to work with only 4 slices (slices were collected along the length of the hippocampus); this modification allowed a doubling of the sample size without altering other procedures in the study. No difference in protein expression was found between the two methods of synaptoneurosome preparation (Figure 14).

Slices were manually homogenized in 350 µL of Krebs buffer; ~65 µL of the homogenate was saved for SDS-PAGE (total). Each sample was then loaded into a 1 cc syringe attached to a 13 mm diameter syringe filter holder (Millipore). The diluted filtrate was gently forced through three layers of nylon mesh (100 µm pore, Millipore) pre-wetted with ~0.2 mL of Krebs buffer, and collected in a 1.5 mL Eppendorf tube. Filtered samples were then loaded into a 1 cc syringe and forced through a pre-wetted 5 µm nitrocellulose filter (Millipore) in a 13 mm filter holder (Millipore). The homogenate was kept ice cold at all times to minimize proteolysis. Filtered samples were then spun at 1000 x g for 15 min (4°C). The supernatant was removed, and the pellet (synaptoneurosome) was re-suspended in 65 µL of Krebs buffer for analysis with SDS-PAGE.

## 6.2.4 Immunoblotting

### a. Synaptoneurosome samples

Synaptoneurosome samples were loaded in duplicate (10 µg/lane), resolved via SDS-PAGE, and transferred to PVDF membranes (Millipore). For identification of molecular weight in subsequent analysis, 5 µL of protein standard ladder (Precision Plus Protein Western Standards, BioRad) was loaded into each gel. All membranes were blocked for 1 h with 5% (w/v) skim milk powder prepared in TBST [200 mM Tris, 140 mM NaCl, 1% (v/v) Tween-20, pH 7.6]. Immunoblotting for membranes with synaptoneurosome samples was performed by overnight incubation (at 4°C) with an anti-NR1 antibody [1:1000, mouse monoclonal, Millipore 05-432, prepared in blocking buffer] followed by incubation with an anti-mouse secondary antibody (1:5000, Santa Cruz sc-2005, prepared in blocking solution) for 1 h at room temperature. After examination, membranes were placed into stripping buffer (ReBlot, Millipore) for 2 x 15 min incubations at room temperature with constant agitation, and blocked by 2 x 5 min washes with blocking solution before being reprobbed. The same procedure was then repeated with antibodies directed at GluN2A [1:1000, rabbit polyclonal, Millipore 07-632, in blocking buffer; 1:5000 anti-rabbit secondary antibody, Santa-Cruz sc-2004, in blocking buffer], GluN2B [1:1000, rabbit polyclonal, Millipore 06-600, in blocking buffer; same anti-rabbit secondary antibody], PSD-95 [1:2000, mouse polyclonal, Millipore MABN68, in blocking buffer; same anti-mouse secondary antibody] and anti-Actin antibody [1:5000, rabbit polyclonal, Sigma A2066, in blocking buffer; same anti-rabbit secondary antibody].

All synaptoneurosome blots were scanned with Chemi Genius2 Bio Imaging System and the relative density of each band of interest, from within the linear range of exposures, was

measured, background-subtracted, and normalized to actin. Synaptic fractions were taken in relation to total levels within each dietary condition on each blot.

#### b. Biotinylation samples

Biotinylation samples were vortexed and 30  $\mu$ L of surface fraction was loaded per well (15  $\mu$ g of total homogenate was loaded per well for total), resolved via SDS-PAGE, and transferred to PVDF membranes (Millipore). All membranes were blocked for 1 h with 5% (w/v) BSA prepared in TBST. Immunoblotting for membranes with biotinylation samples was performed by overnight incubation (4°C) with an anti-NMDAR1 antibody [1:1000, rabbit polyclonal, Millipore AB9864, prepared in blocking buffer] followed by incubation with an anti-rabbit secondary antibody [1:1000 prepared in 5% (w/v) Milk/ 0.05% TBST, Santa Cruz sc-2004] for 1 h at room temperature. Membranes were placed into stripping buffer (ReBlot) for 2 x 15 min incubation at room temperature with constant agitation, and blocked by 2 x 5 min incubation in 5% (w/v) Milk/ 0.05% TBST before being reprobed.

The same procedure was then repeated for GluN2A [1:1000, rabbit polyclonal, Millipore 07-632, in blocking buffer; 1:5000 anti-rabbit secondary antibody, Santa-Cruz sc-2004, in blocking buffer], GluN2B [1:1000, rabbit polyclonal, Millipore 06-600, in blocking buffer; same anti-rabbit secondary antibody], PSD-95 [1:2000, rabbit polyclonal, Millipore MABN68, in blocking buffer; same anti-rabbit secondary antibody] and anti-NCAM antibody [1:5000, rabbit polyclonal, Santa-Cruz sc-10735, in blocking buffer; same anti-rabbit secondary antibody].

All biotinylation blots were scanned with Chemi Genius2 Bio Imaging System and the relative density of each band of interest, from within the linear range of exposures, was



measured, background-subtracted, and normalized to NCAM. Surface fraction densities were taken in relation to total levels within each dietary condition on each blot.

### 6.3 Statistical Analysis

Analysis was done using the two-tailed Student-Neuman t-test with the significance threshold set at  $p < 0.05$ . Post-hoc power analyses were performed as outlined by Bernard, 2011. Western blots with whole homogenates were analyzed the following way: an absolute relative density for each band was determined, the background subtracted value was then normalized to the background subtracted actin value for the same band (or NCAM for biotinylation blots). An average of duplicates was then calculated to determine the final CD and HFD total expression levels for each blot, and averages for CD blots were statistically compared to those of HFD.

Western blots with synaptic fractions were analyzed the following way: an absolute relative density for each band was determined, and the background subtracted value was then normalized to the background subtracted actin value for the same band. An average of the duplicate values for each condition was then calculated, which was then used to construct a ratio of synaptic to total for each treatment condition (CD Syn/Total; HFD Syn/Total). The averages for CD Syn/Total ratios were then statistically compared to those of HFD Syn/Total. In order to show that no difference exists between the slice and whole hippocampus synaptoneurosome preparation methods, GluN1 expression was statistically compared between the 5 gels from round 1 of the study and the 10 gels from round 2 of the study. No difference in synaptic expression of GluN1 was found between the 2 methods of synaptoneurosome preparation (Whole:  $2.75 \pm 0.6$ ; Slice:  $3.52 \pm 0.5$ ; values are presented as mean  $\pm$  SEM; see Figure 14).

Western blots with surface fractions were analyzed the following way: an absolute relative density for each band was determined, the background subtracted value was then normalized to the background subtracted NCAM value for the same band. An average of duplicates was then calculated to determine the final CD and HFD surface and total expression levels for each blot, and a ratio of surface to total was constructed for each treatment condition (CD Surf/Total; HFD Surf/Total). The averages for CD Surf/Total ratios were then statistically compared to those of HFD Surf/Total.

## 7.0 Results:

*High fat diet consumption for 16 weeks results in greater adiposity without significantly altering body weight.*

Despite eating significantly less food (weeks 2-15;  $p < 0.05$ ; Figure 3A), the HFD group consumed more calories throughout the treatment (significantly more on weeks 1, 8, 11, 14;  $p < 0.05$ ; Figure 3B). However, after 4 months, female Sprague-Dawley rats fed the HFD were not significantly heavier than those fed the CD (Figure 4; Table 4). Notably, animals on the HFD had significantly heavier retroperitoneal fat pads (HFD  $1.8 \pm 0.13$  g vs. CD  $1.3 \pm 0.06$  g;  $p < 0.01$ ; Figure 5A). Liver weight and volume were not significantly affected by diet (Figure 5C & D), but the HFD group did have significantly heavier adrenal glands (CD  $0.060 \pm 0.002$  g vs HFD  $0.067 \pm 0.002$  g;  $p < 0.05$ ; Figure 5B). The HFD fed rats also had significantly heavier spleens (CD  $0.57 \pm 0.02$  g vs HFD  $0.63 \pm 0.02$  g,  $p < 0.05$ ; Figure 5E).

*HFD impairs glucose tolerance without affecting fasting blood glucose levels.*

As early as 4 weeks after diet assignment, animals on the HFD displayed significantly higher levels of blood glucose after a glucose bolus, which is indicative of a reduced ability to clear the glucose from the blood stream (HFD AUC 24% > CD;  $p < 0.05$ ; Figure 6A). The HFD group continued to experience significantly greater difficulty clearing glucose from their blood at each point tested until the time of sacrifice (**8 wks**: HFD AUC 25% > CD,  $p < 0.01$ ; **12 wks**: HFD AUC 29% > CD,  $p < 0.01$ ; **16 wks**: HFD AUC 16% > CD,  $p < 0.05$ ). Notably, fasting blood glucose was not significantly different between groups at any of the time points (Figure 6B).

*Compared to the CD, the consumption of HFD does not alter insulin or leptin levels.*

Plasma insulin levels collected at the time of sacrifice revealed no difference between dietary groups (CD:  $0.49 \pm 0.03$  ng/mL; HFD:  $0.54 \pm 0.09$  ng/mL; N = 10; Figure 7). Compared to the CD rats, the HFD animals had higher levels of leptin, but the results did not reach statistical significance (CD:  $2.17 \pm 0.61$  ng/mL; HFD:  $3.69 \pm 0.85$  ng/mL; N = 5; Figure 8).

*HFD does not significantly alter total expression of NMDA receptor subunits.*

A selection of key NMDA receptor subunits (GluN1, GluN2A & GluN2B) were examined by Western blot. The HFD did not significantly alter cellular expression of NMDAR subunits in female hippocampus (GluN1: CD  $100 \pm 12.5\%$ , HFD  $97.4 \pm 14.3\%$ ; GluN2A: CD  $100 \pm 20.3\%$ , HFD  $96.8 \pm 28.9\%$ ; GluN2B: CD  $100 \pm 12.4\%$ , HFD  $98.5 \pm 18.9\%$ ; Figure 9). Total expression of PSD-95 was also similar between groups (PSD-95: CD  $100 \pm 23.2\%$ , HFD  $83.2 \pm 18.2\%$ ). All values are presented as % CD mean  $\pm$  SEM.

*HFD does not significantly alter synaptic expression of NMDA receptor subunits.*

The HFD did not significantly alter synaptic expression of NMDAR subunits (GluN1, GluN2A & GluN2B) in female hippocampus. Synaptic expression of PSD-95 was also similar between groups (GluN1: CD  $100 \pm 12.5\%$ , HFD  $82.9 \pm 7.8\%$ ; GluN2A: CD  $100 \pm 12\%$ , HFD  $90.5 \pm 9.0\%$ ; GluN2B: CD  $100 \pm 13.8\%$ , HFD  $86.4 \pm 8.6\%$ ; PSD-95: CD  $100 \pm 12.5\%$ , HFD  $90.2 \pm 9.0\%$ ; Figure 10). All values are presented as % CD mean  $\pm$  SEM.

*HFD does not significantly alter cell surface expression of NMDA receptor subunits.*

HFD consumption over 16 weeks did not significantly alter cell surface expression of NMDAR subunits (GluN1, GluN2A & GluN2B) in female hippocampus (GluN1: CD  $100 \pm$

9.4%, HFD  $102.8 \pm 8.2\%$ ; GluN2A: CD  $100 \pm 17.4\%$ , HFD  $96.4 \pm 13.7\%$ ; GluN2B: CD  $100 \pm 19.2\%$ , HFD  $104.1 \pm 16.1\%$ ; Figure 11). All values are presented as % CD mean  $\pm$  SEM.

## 8.0 Discussion:

Obesity is now recognized as one of the major contributors to preventable death in the world (WHO, 2008). In addition to being associated with T2D and CVD, human obesity is associated with impaired cognitive function (section 3.1). While behavioural studies using obese animals have demonstrated a link between obesity and impaired hippocampal function, the mechanisms by which obesity affects cognition are poorly understood. In our study we aimed to create an animal model of dietary-induced obesity, and characterize structural changes in the hippocampus of adult female rats.

The results from this study show that our HFD feeding protocol (4 months, 45% of calories from fat) is sufficient to induce an obese phenotype in adult female rats. However, no effect of the HFD upon total, synaptic, or cell surface expression of hippocampal NMDA receptor subunit expression in the hippocampus was observed.

### *Characterizing an obese phenotype in adult female Sprague-Dawley rats.*

Unlike the BMI threshold in humans, there is currently no clearly defined standard for characterizing obesity in animal models (Hariri & Thibault, 2010). Therefore, to accurately detect the onset of diet-induced obesity with our feeding protocol, several anatomical and metabolic changes were assessed. Authors of previous studies of dietary induced obesity used a combination of factors to characterize obesity including crude biometrics [body weight, Farr et al., 2008; adiposity, Lingvist et al., 2006) and metabolic measures such as: fasting blood glucose, glucose tolerance and hormonal imbalances (Hwang et al., 2010; Messier et al., 2007; Mielke et al., 2006).

Despite being similar in body weight to the CD group, rats in the HFD group had significantly heavier retroperitoneal fat pads, and this effect is consistent with the literature (Lin et al., 2000). Rats have 3 main fat depots: retroperitoneal, mesenteric and inguinal. Retroperitoneal fats were chosen for analysis in our study because this fat depot could be extracted with consistent accuracy, which limited technical variability between surgeries. We assumed that fat deposition would be consistent across all of the fat depots, therefore, retroperitoneal fat pad weight was taken as representative of general adiposity level. Since we did not test differences between fat depots directly, the possibility does exist that our adiposity estimate may not fully reflect depot changes. If we were able to use dual energy x-ray absorptiometry (DEXA; Chen et al., 2012), we could more accurately determine adiposity levels, as well as the proportion of other types of tissues. Since the crude body weights were similar between groups despite greater adiposity in the HFD group, it would be interesting to determine which type of tissue in the CD group contributed to their body weight (instead of fat).

Liver was examined because obesity increases the risk of non-alcoholic fatty liver disease (Fabbrini et al., 2010). Accumulation of fat in the liver impairs hepatic insulin signaling, and results in increased gluconeogenesis that is normally suppressed by insulin (Konstantinos et al., 2009). Ilhami et al., (2007) previously reported an increase in liver volume in female rats after 12 weeks of high fat diet consumption (30% kcal from fat). In addition, male rats fed a high fat diet for 11 weeks were shown to have higher liver weights compared to CD animals (Handjjeva-Darlenska & Boyadjieva, 2009). In our study liver weight and volume were similar between dietary groups, but the lack of significant findings could be a function of sexual dimorphism, as will be discussed later.

The HFD fed animals had significantly larger adrenal glands, and this increase could be related to an increase in corticosterone levels caused by an onset of obesity. Although we did not test corticosterone levels directly, previous work has shown that HFD consumption increases corticosterone levels in rats (Lindqvist et al., 2006); notably, the increase in corticosterone was only significant for male animals. Since obesity is known to induce low-grade systemic inflammation (Lumeng & Saltiel., 2011), we hypothesized that spleen, being the largest lymph organ, could be affected by the HFD treatment. Our results indicate that rats in the HFD group had significantly heavier spleen weights compared to controls. A significant enlargement of spleen has been previously shown in response to 3 months of HFD consumption in female rats (Altunkaynak et al., 2007). Changes in these organ weights could be explained by increased cell mass needed to produce higher levels of corticosterone (in the case of adrenal glands), and inflammatory cytokines (in the case of spleen).

In our study we fed dietary groups defined diets with the only difference between them being the fat to carbohydrate ratio. The primary advantage to using a defined diet was limitation of the variability in macronutrient composition between batches (Buettner et al., 2006), which could have introduced variability into our study. Although a defined diet is not representative of a typical human diet because of its consistency, we decided against using a cafeteria-style diet (wherein animals are fed a selection of human foods containing high levels of fat and/or sugar) due to potential difficulties in reproducibility of our results by others in the future, and in other parts of the world, where certain products would be difficult to obtain. The calories in our HFD were primarily from saturated fat, and consumption of saturated fat has been previously shown to be highly obesogenic (Yaqoob et al., 1995). In our study, despite eating significantly less food in grams, the HFD group consumed more calories weekly (statistically significant at weeks 1, 3, 8,



11 and 14). The excess consumption of calories was likely the primary factor contributing to greater fat mass in the HFD group. Excess energy consumption by rats on a high fat diet has been previously reported (Buettner et al., 2006). Notably, one limitation in our choice of diet was the level of sucrose found in our CD (35% kcal from sucrose) in comparison to the HFD (17% kcal from sucrose; Table 1), given that rats were shown to exhibit a preference for saccharide solutions (Sclafani & Nissenbaum, 1987). The CD diet may have been more palatable than the HFD, due to high sucrose levels, which would have caused the CD fed animals to overeat and reduce the difference in weight gain between dietary groups. Interestingly, we still observed greater adiposity, and impaired glucose handling, in the HFD group. Potentially, our results indicate that consumption of fat is a greater contributor to adiposity than consumption of sugar. Indeed, in a comprehensive review of animal models of obesity, Hariri and Thibault (2010) suggest that diet high in fat with low carbohydrate levels are more obesogenic than those with a reversed ratio of fat to carbohydrates.

Results from the monthly oral glucose tolerance tests indicate that HFD consumption decreased the ability to handle glucose as early as 4 weeks after diet initiation, and this impairment was maintained at all subsequent testing points. A decreased ability to clear glucose from the blood stream is a defining characteristic seen in diabetic patients (Efendic et al., 1980). In diabetes, the decreased ability to clear glucose from the blood stream is primarily attributed to decreased tissue response to circulating insulin, and results in hyperglycemia. Surprisingly, no differences in fasting blood glucose between treatment groups were detected throughout the study period. Although, we also found no difference in plasma insulin levels between dietary conditions, therefore, our fasting blood glucose and plasma insulin results are complementary. Fasting blood glucose and insulin levels were shown to be affected by HFD in previous studies

(Handjieva-Darlenska & Boyadjieva, 2009; McNeilly et al., 2011; Mielke et al., 2006; Stranahan et al., 2008), but differences in characteristics of study subjects (species, gender), and dietary treatment (length, % fat/sugar) could explain the difference in results. For example, Stranahan et al. fed male rats a combined diet of 50% kcal from fat and a 20% sucrose solution for 8 months, while Mielke et al. fed male mice for 1 year with a diet containing 45% kcal from fat. McNeilly et al. used male Wistar rats fed a diet consisting of 45% kcal fat for 12 weeks. While McNeilly's group had a design similar to our study, the gender difference between the 2 studies is very important. As will be discussed later, male animals seem to be more susceptible to the effects of dietary induced obesity compared to females (Hwang et al., 2010). The species difference between studies is also important. Pintana et al. (2012) reported no difference in plasma glucose between dietary groups, but significant increase in plasma insulin in the HFD group, after 12 weeks of dietary regiment. Their group fed a diet higher in fat content compared to ours (60% vs. 45% kcal from fat) to male Wistar rats. Stranahan et al. (2008) reported an increase in fasting blood glucose levels with no differences in plasma insulin after 8 months of HFD supplemented with sucrose feeding in male Sprague-Dawley rats. Inconsistencies in metabolic complications resulting from HFD consumption between studies (glucose levels, plasma insulin) demonstrate that variables such as species, and gender, might be important factors to consider when interpreting the results from various groups.

In addition, in contrast to previous findings in male animals (Cha & Jones, 1998; Handjieva-Darlenska & Boyadjieva, 2009; Hwang et al., 2010; Lin et al., 2000; Valladolid-Acebes et al., 2011), we did not see significant differences in plasma leptin between dietary groups. Lin et al. (2000) have also shown that high fat diet induces leptin resistance characterized by a reduced response to leptin administration. While Hwang et al., (2010) did

report a similar degree of increase in plasma leptin between sexes in response to HFD consumption, our leptin analysis was performed on only 5 animals per dietary group, and the observed variability may have prevented the observation of statistically significant differences. Plasma leptin levels are typically proportional to fat content (Harvey & Ashford, 2003), and since we saw greater levels of adipose tissue we expected to see higher levels of plasma leptin in our HFD group. We conclude that an obese phenotype was reached after 16 weeks of feeding based on significantly greater fat mass in the HFD group, which is the key characteristic of obesity, and their decreased ability to handle glucose.

*The effects of diet-induced obesity on NMDA receptor expression.*

Based on human epidemiological and experimental animal studies, which indicate an effect of dietary induced obesity on cognitive function (particularly impaired memory, Elias et al. 2003; Mielke et al., 2006), we expected to see a reduction in expression of NMDA receptor subunits in the hippocampus. The cellular, synaptic and cell surface expression of selected subunits were examined using a variety of preparative biochemical techniques followed by Western blotting. Hippocampal NMDARs are believed to predominately contain GluN1, GluN2A, and GluN2B subunits (Petrulia, 2012), hence we assessed expression of these subunits. The results indicate that there were no significant differences in the expression of GluN1, GluN2A and GluN2B subunits in any of the examined fractions, or total levels, in response to dietary treatment. While synaptic levels of GluN1, GluN2A and GluN2B were slightly reduced (17%, 9%, and 14%, respectively), the changes were not statistically significant, which could be due to low power (31%, 16%, and 11%, respectively).

The lack of a dietary induced effect on NMDAR subunit expression in this study is contradictory to previous research, but the difference between our study results and previous work (primarily by Valladolid-Acebes et al., 2011 and Mateos et al., 2009) could be attributed to species, gender and other differences in study design. For example, Valladolid-Acebes et al. found that HFD consumption decreased total expression of GluN2B subunits, while having no significant effect on GluN1 and GluN2A subunits; however, they used C57BL/6J male mice fed for 8 weeks (45% calories from fat). Western blotting by Mateos et al. revealed a significant reduction of GluN1 subunit expression and a decrease in phosphorylation of the Y1325 residue on the GluN2A subunit in HFD animals; however, they used C57BL/6J mice and a much longer treatment protocol (9 months, 21% calories from fat; the sex of animals used was not specified).

Notably, the results between the Valladolid-Acebes et al. and Mateos et al. groups, with respect to expression of NMDA receptor subunits, are also contradictory. While Valladolid-Acebes et al. found a reduction in GluN2B total expression, as a result of HFD treatment, they report no difference in GluN1 or GluN2A expression. In contrast, Mateos et al. report a significant reduction in total GluN1 levels (~40%). Since Mateos et al. fed their animals for a total of 9 months, a significantly greater time period may be required to induce changes in expression of GluN1 subunits.

Stranahan et al. (2008) reported that male rats maintained on a high energy diet (50% kcal from fat + 20% sucrose) for 8 months performed worse than control animals on spatial memory tasks, and also had reduced hippocampal dendritic spine density, and reduced LTP in the CA1 region of the hippocampus. LTP in the CA1 region requires participation of the NMDA receptors (Grosshans et al., 2002), which suggests that a reduction in the receptor number could be one of the structural alterations responsible for impaired LTP in the hippocampi of the HFD

animals reported by Stranahan et al. Although, they did not assess expression of NMDA receptors, and they suggest that a reduction of brain-derived neurotrophic factor (BDNF) might be part of the causal chain.

Compared to male animals, females are known to be less susceptible to the effects of dietary induced obesity. For example, Hwang et al. (2010) showed that male mice are more vulnerable to HFD-induced weight gain and metabolic alterations (hyperglycemia, hypercholesterolemia and hyperinsulemia). Potentially, a difference in susceptibility of female rats to obesity related complication can explain the absence of fasting blood glucose, and plasma insulin level differences between dietary groups in our study. Notably, Hwang et al. used mice for their animal model, and the same effect due to sexual dimorphism may not be present in rats.

Hwang et al. also show that male mice were more susceptible than females to learning deficits, and impairment in hippocampal synaptic plasticity (LTP & LTD), as a result of high fat diet induced obesity; this group used a 45% kcal from fat modified diet over 8-11 months of feeding. Also, hippocampal neurogenesis was previously shown to be impaired in male, but not female rats, as a result of high fat diet consumption (Lindqvist et al., 2006). In addition, human epidemiological studies support a similar trend where general intelligence scores are lower in obese men compared to obese women (Sorensen et al., 1983). Collectively, experimental and observational data demonstrate that women are less susceptible to obesity-associated complications, and this phenomenon may explain the lack of differences between dietary groups with respect to fasting blood glucose and NMDAR subunit expression in our study. Importantly, having used female animals is a strength of our study, given that previous studies primarily focused on male animals; as well, in human populations obesity is more prevalent in women (WHO, 2008).

Offspring born to obese mothers are at an increased risk of developing obesity and metabolic complications in adulthood (Franco et al, 2012), which are known risk factors for cardiovascular disease (section 1.0). Vickers (2011) has also reported that maternal high fat diet consumption increases the risk of offspring obesity, likely by establishing unfavorable metabolic set-points, and epigenetic programming during development. In addition, female obesity may have important trans-generational effects from the perspective of the brain, for offspring born to obese mothers are at an increased risk of cognitive problems, symptoms of attention deficit hyperactivity disorder in adulthood, eating disorders in adolescence, and psychotic disorders in adulthood (Van Lieshout, Taylor & Boyle, 2011). Results reported by Van Lieshout and colleagues indicate a relationship between maternal obesity and fetal brain development, which highlights the importance of studying the effects of obesity in females.

Measuring total, synaptic and cell surface levels of NMDA receptor subunits in the hippocampus was the first step in the assessment of potential structural alterations that could explain the impairment in memory previously observed in obese animals. NMDA receptors play a necessary role in the upstream process of many forms of LTP induction and maintenance by allowing calcium entry into the cell. Although the number of NMDA channels is an important factor underlying LTP, it is only part of the full picture; NMDA receptor stoichiometries, phosphorylation levels, and the association of NMDARs with other intracellular proteins involved in signal transduction can also contribute to NMDAR mediated LTP. For example, Mateos et al., (2009) found a reduction in GluN2A (Y1325) phosphorylation in HFD mice compared to controls. Phosphorylation has been shown to be required for long-term synaptic plasticity in the hippocampus, since inhibition of tyrosine kinases blocks LTP (O'Dell, Kandel & Grant, 1991). An increase in tyrosine phosphorylation was also shown to enhance NMDAR

currents in electrophysiological experiments (Wang & Salter, 1994). If the quality of diet can alter phosphorylation states of NMDA receptors, it would likely alter their function as well, which, in turn, could explain some of the observed behavioural alterations, but this needs to be further tested. In addition, dietary induced obesity could potentially cause memory impairment by affecting other forms of LTP, such as those that are NMDAR-independent (Johnston et al., 1992).

To examine changes in expression of other proteins in the NMDAR complex that may be related to signal transduction, and to assess broad changes in synaptic structure, total and synaptic expression of PSD-95 was assessed. PSD-95 is an important protein in NMDAR complexes at the post synaptic density, which is involved in protein-protein interactions, and is able to affect NMDA receptor trafficking (Kalia LV & Salter MW, 2003; Lavezzari et al, 2003). Also, PSD-95 is considered one of the most abundantly expressed synaptic proteins (Dosemeci et al., 2007). No significant changes in total, or synaptic, PSD-95 expression were detected between treatment conditions in our study. Previous immunohistochemistry by Grillo et al., 2011 revealed redistribution and clustering of PSD-95 in the CA3 region of the hippocampus of genetically diabetic male rats (hypo-IRAS); potentially the expression of PSD-95 is not affected by obesity related complications, but redistribution and clustering causes changes in PSD-95-mediated signal transduction.

AMPA receptor expression could also contribute to cognitive impairment seen in dietary induced obesity. AMPA receptors are required for the initial activation of NMDARs, by allowing membrane depolarization that leads to the expulsion of the magnesium ions from NMDAR channels. Expression of AMPARs was not directly assessed in our study, but previous

studies found no difference of HFD consumption on AMPAR expression (Valladolid-Acebes et al. 2011; Mateos et al. 2009).

### Conclusion and future directions.

High fat diet consumption over 16 weeks lead to obesity in female rats, which was characterized by significant fat accumulation and an impaired ability to utilize glucose. The dietary treatment had no significant effect on total, synaptic, or cell surface expression of NMDA receptor subunits in the hippocampus; PSD-95 expression was also similar between dietary groups.

Future studies should characterize changes in NMDA receptor expression at the synaptic plasma membrane; potentially combining the biotinylation and synaptoneurosome isolation techniques could enrich synaptic cell surface fractions (``zoom in``), but this technique needs to be empirically developed. The receptors embedded in the synaptic membrane are functionally the most relevant for LTP induction since they are the primary targets for synaptically released glutamate during signal transduction. Small changes at the synaptic cell surface fraction could have important functional consequences, but would be hard to detect with the techniques used in the current study due to the noise-to-signal ratio. Since synapses compose a small fraction of the neuronal surface, and NMDA receptors are also located on the plasma membrane outside of synapses, as well as in vesicles inside the cell, detection of changes in cell surface expression only at synapses is particularly challenging.

Phosphorylation levels of NMDAR subunits could be assessed after co-immunoprecipitation and/or immunoblotting against phosphorylated amino acids. In addition, a potential acute stimulus (inducing LTP) could be introduced, and subsequent structural changes



compared between control and experiment groups. LTP maintenance requires protein synthesis to take place (Nicoll & Malenka, 1995), including insertion of novel glutamate receptors into the post-synaptic density (Grosshans et al, 2001). High fat diet induced obesity could potentially interfere with novel protein synthesis, or trafficking of novel proteins to the post-synaptic density. Future studies could utilize a chemical or electrophysiological induction of LTP followed by Western blotting, after allowing sufficient time for protein synthesis and trafficking to take place.

Hwang et al., (2010) demonstrated an important difference in susceptibility to diet-induced obesity between male and female mice. Sexual dimorphism observed by Hwang et al. is systemic in nature, with both metabolic and cognitive effects of the HFD consumption being of different magnitudes between genders. Sexual dimorphism in response to diet should be further investigated in a rat model. Majority of previous studies have focused only on male animals, and their findings might not be universally generalizable.

Overall, the accumulating evidence from human and animal studies leaves little doubt about the ability of diet-induced obesity to impair cognitive function. Particularly worrisome is the increasing prevalence of dementia in countries where high fat and high calorie diets are popular (Kalmijn et al, 1997). Also, high BMI in middle age is associated with higher risk of dementia (Kivipelto 2005, Whitmer et al., 2005). Loss of memory is a hallmark feature of dementia (McKhann et al., 1984); therefore, structural changes, particularly in brain areas involved in memory, could be further examined for potential therapeutic development.

## 9.0 Tables and Figures

Ingredient	Control Diet		High Fat Diet	
	Amount (mg)	Calories (kcal)	Amount (mg)	Calories (kcal)
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Cornstarch	315	1260	72.8	291
Maltodextrin 10	35	140	100	400
Sucrose	350	1400	172.8	691
Cellulose	50	0	50	0
Soybean oil BW200	25	225	25	225
Lard	20	180	177.5	1598
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H <sub>2</sub> O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.05	0	0	0
FD&C Red Dye #40	0	0	0.05	0
Total	1055.05	4057	858.15	4057

Table 1. Composition of experimental diets.

<b>Author/ Year</b>	<b>Species/Age (Weight)/ Gender (M/ F)</b>	<b>Dietary Intervention</b>
<b>Davidson et al., 2012</b>	Sprague–Dawley rats/ 250-275g/ M	40% kcal fat vs 13% control
<b>Farr et al., 2008</b>	CD1 mice/ 12 months/ M	10% kcal fat vs 5% (control); time period N/A
<b>Gault et al., 2010</b>	Swiss TO mice/ 6–8- weeks old/ M	45% kcal fat for 8 months and 21 days
<b>Granholtm et al., 2008</b>	Fisher 344 rats/16 months/M	10% (kcal saturated fat) + 2% (kcal cholesterol) vs 12% (kcal unsaturated fat; control) for 8 weeks
<b>Hwang et al., 2010</b>	C57BL/6J mice/ 3 months /(M/F)	45% kcal fat for ~8-11 months
<b>Jurdak et al., 2008</b>	Long-Evans Rats/ 6 weeks/ M	3 groups: high fat (9.0 kcal/g), high sucrose (1.28 kcal/g) or regular chow (3.6 kcal/g) for 5 weeks
<b>Kanoski &amp; Davidson, 2010</b>	Sprague–Dawley rats/ 60 days/ M	40% kcal fat + glucose (220.5g/kg) vs 13% kcal fat control for up to 90 days
<b>McNeilly et al., 2011</b>	Wistar rats/ 150–175 g/ M	45% kcal fat vs 7.4% for 12 weeks
<b>Messier et al., 2007</b>	C57BL/6J mice/ 7 weeks/ M	60% kcal fat vs 5%, and 2 groups +/- sucrose for 3 months
<b>Mielke et al., 2006</b>	C57BL/6J mice/ 17-20g /M	45% kcal fat vs 5% for 12 months
<b>Molteni et al., 2002</b>	Fisher 344 rats/ 2 months/ F	~39% kcal fat + ~40% kcal sucrose for 2-24 months
<b>Murray et al., 2009</b>	Wistar rats/ ~100g / M	55% kcal fat vs 7.5% for 9 days
<b>Pintana et al., 2012</b>	Wistar rats/ 5 weeks/ M	60% kcal fat vs 20% for 12 weeks
<b>Porter et al., 2011</b>	NIH Swiss mice/ 8 weeks/ M	45% kcal fat for 155 days
<b>Stranahan et al., 2008</b>	Sprague-Dawley rats/ 2 months/ M	~50% kcal fat + 20% sucrose vs chow for 8 months
<b>Valladolid-Acebes et al., 2011</b>	C57BL/6J mice/ 5 weeks/ M	45% kcal fat vs 10% for 8 weeks
<b>Winocur &amp; Greenwood, 2005</b>	rats/ 1 month/ (N/A)	40% kcal fat for 3 months

Table 2. Details on reviewed animal studies involving dietary interventions

Objectives	Assessment Measures (primary)	Hypothesis
1. Establish a model system for the study of the effects of HFD on female rat brain.	body weight, retroperitoneal fat-pad weight, food consumption, fasting blood glucose, glucose metabolism, levels of leptin and insulin, liver weight and volume, spleen weight, and adrenal gland weight.	A 16-week HFD (45% kcal fat) will create an obese phenotype in female rats characterized by increased body weight, increased in selected biometrics and metabolic complications.
2. Using the established model, determine if the 16 week-HFD intervention affects expression of NMDA receptor subunits.	levels of total NMDA receptor subunits (GluN1, GluN2A, GluN2B) assessed via SDS-PAGE and immunoblotting.	The dietary intervention will reduce total expression levels of all NMDA receptor subunits.
3. Using the established animal model, determine if the 16 week HFD intervention affects cellular distribution of NMDA receptor subunits.	levels of NMDA receptor subunits, assessed via SDS-PAGE and immunoblotting, after isolation of cellular compartments (plasma membrane, synaptic terminals).	The dietary intervention will reduce the levels of all NMDA receptor subunits in synaptic terminals, and plasma membrane compartments.

Table 3. Questions and Hypotheses

<b>Measure</b>	<b>CD Mean ± SEM</b>	<b>HFD Mean ± SEM</b>	<b>N/group</b>	<b>p</b>
Final Body Weight (g)	278.43 ± 3.42	285.56 ± 3.93	30	0.18
Fat Pad Weight (g)	1.32 ± 0.07	1.81 ± 0.13	20	0.001
Spleen Weight (g)	0.57 ± 0.02	0.63 ± 0.02	20	0.01
Adrenal Gland Weight (g)	0.06 ± 0.002	0.07 ± 0.002	20	0.03
Liver Weight (g)	6.31 ± 0.13	6.41 ± 0.21	20	0.68
Liver Volume (mL)	5.88 ± 0.16	6.02 ± 0.18	20	0.56
Final OGTT (AUC)	270.55 ± 11.55	314.3 ± 18.11	30	0.05
Final Fasting Blood Glucose (mmol/L)	4.9 ± 0.10	5.1 ± 0.13	30	0.34
Plasma Insulin (ng/mL)	0.49 ± 0.08	0.54 ± 0.09	10	0.65
Plasma Leptin (ng/mL)	2.17 ± 0.61	3.69 ± 0.85	5	0.19
Cellular Expression of GluN1	0.69 ± 0.09	0.68 ± 0.36	15	0.89
Cellular Expression of GluN2A	0.59 ± 0.12	0.57 ± 0.17	15	0.93
Cellular Expression of GluN2B	0.75 ± 0.09	0.74 ± 0.14	15	0.95
Cellular Expression of PSD-95	1.01 ± 0.23	0.84 ± 0.18	12	0.57
Synaptic Expression of GluN1	3.26 ± 0.41	2.70 ± 0.26	15	0.26
Synaptic Expression of GluN2A	2.94 ± 0.35	2.65 ± 0.26	15	0.53
Synaptic Expression of GluN2B	3.35 ± 0.46	2.89 ± 0.29	15	0.41
Synaptic Expression of PSD-95	3.07 ± 0.38	2.77 ± 0.28	12	0.53
Surface Expression of GluN1	0.29 ± 0.03	0.30 ± 0.02	14	0.83
Surface Expression of GluN2A	0.26 ± 0.05	0.25 ± 0.04	14	0.87
Surface Expression of GluN2B	0.09 ± 0.02	0.09 ± 0.01	14	0.87

Table 4. Summary of Results.

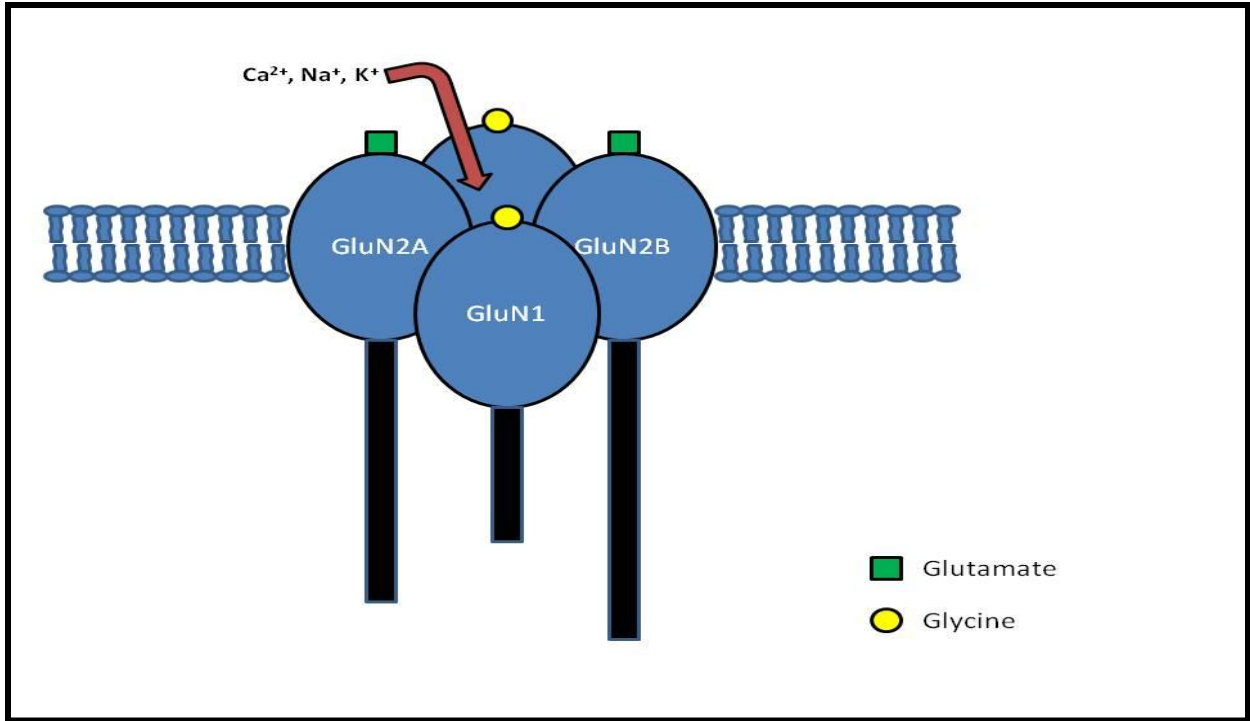


Figure 1. An example of an ionotropic NMDA receptor embedded in the plasma membrane.

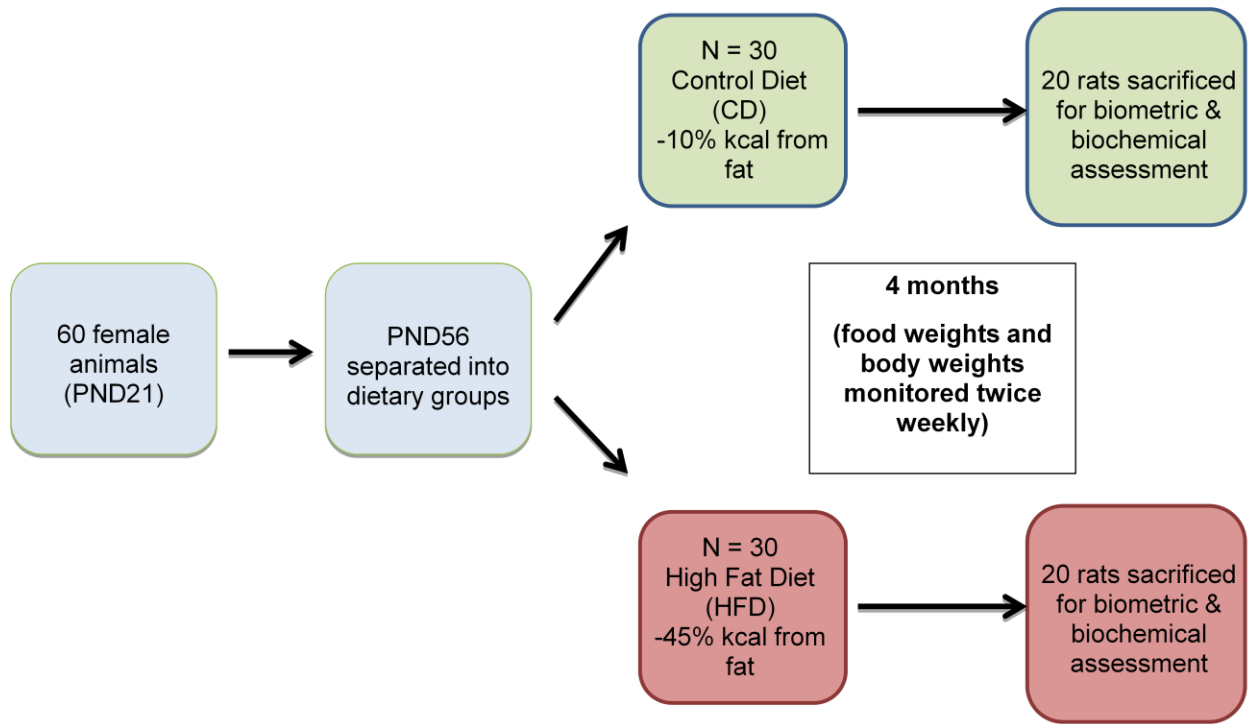


Figure 2a. Experimental Design Flowchart

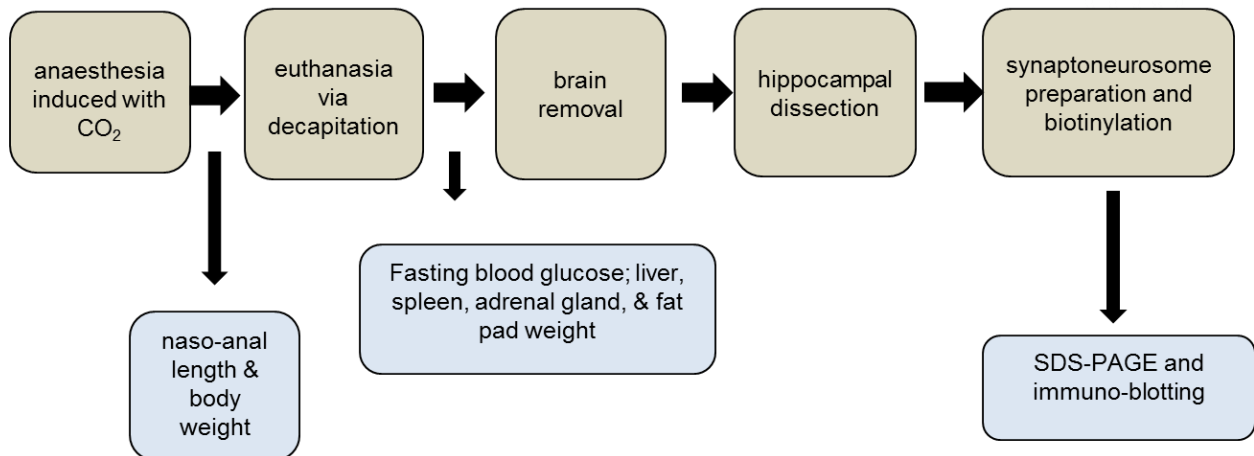
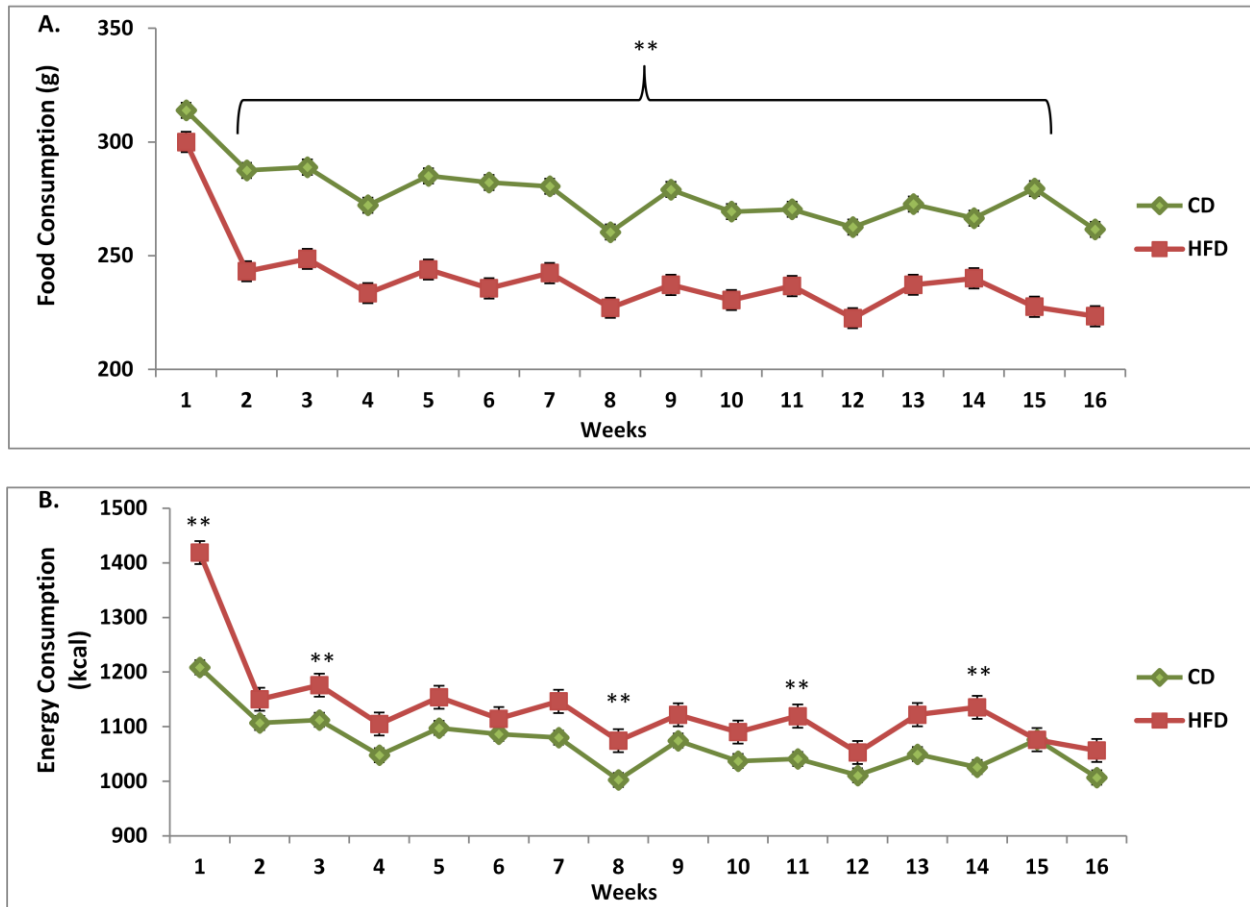


Figure 2b. Experimental Procedures Flowchart



**Figure 3. Eating Profile**

**A)** Food consumption was recorded weekly per cage of 3 animals. The HFD group consumed significantly less food in weeks 2-15 compared to the CD group ( $p < 0.05$ ; Student's t-test). **B)** Energy consumption (determined using: CD = 3.85 kcal/g; HFD = 4.73 kcal/g) was greater in the HFD group compared to the CD group (significant on weeks 1, 3, 8, 11 & 13;  $p < 0.05$ ; Student's t-test). Values are mean  $\pm$  SEM (N = 30/diet).



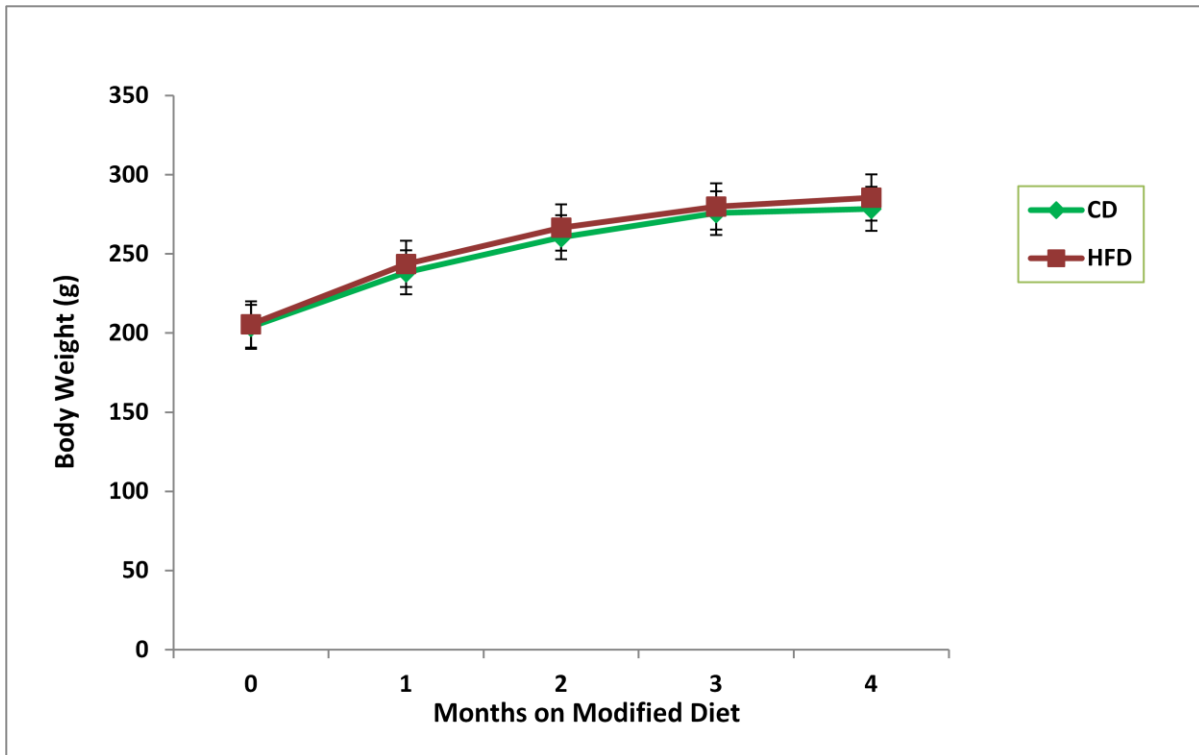
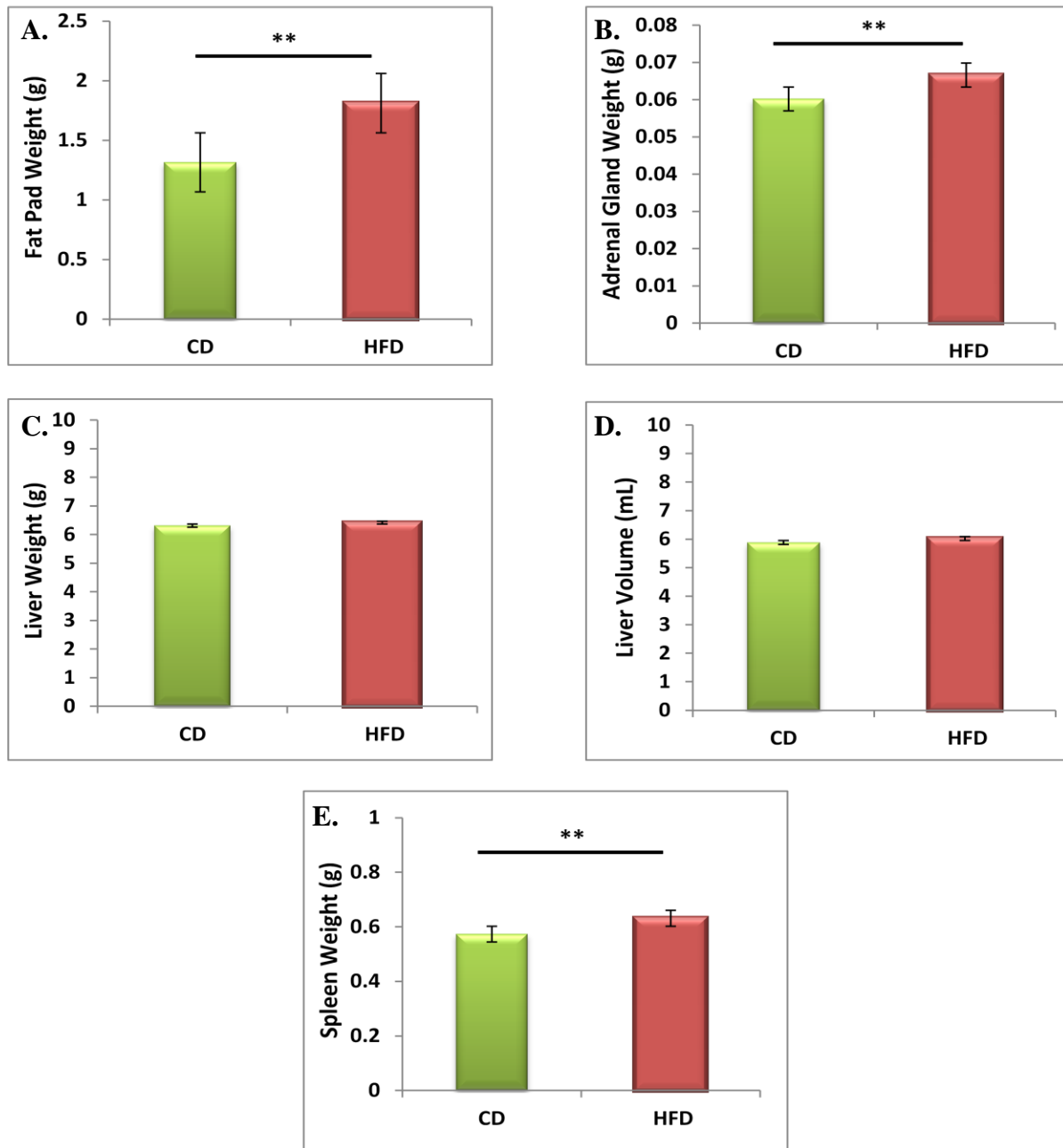


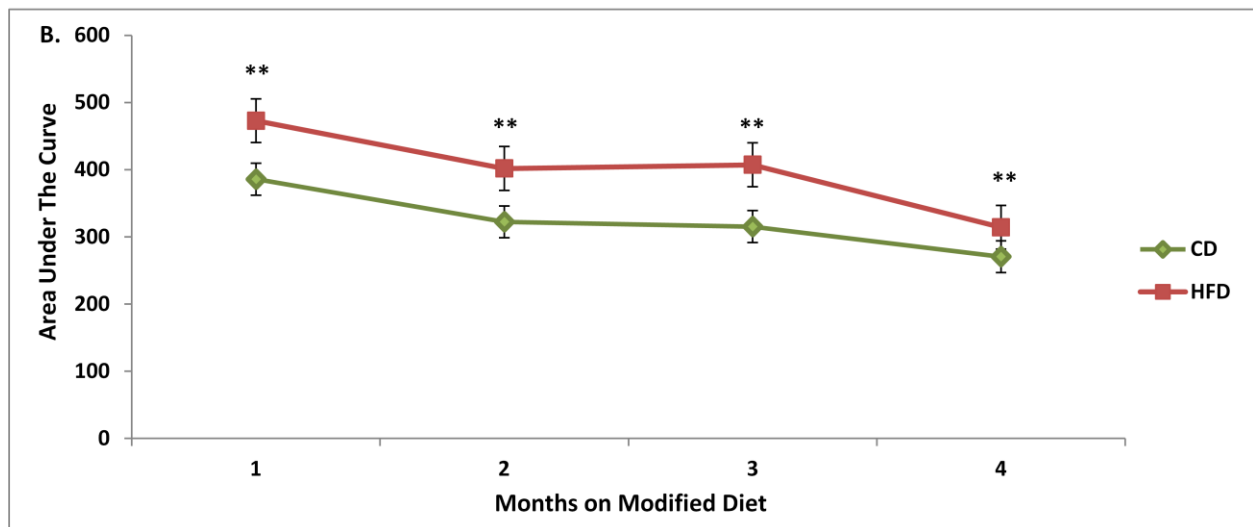
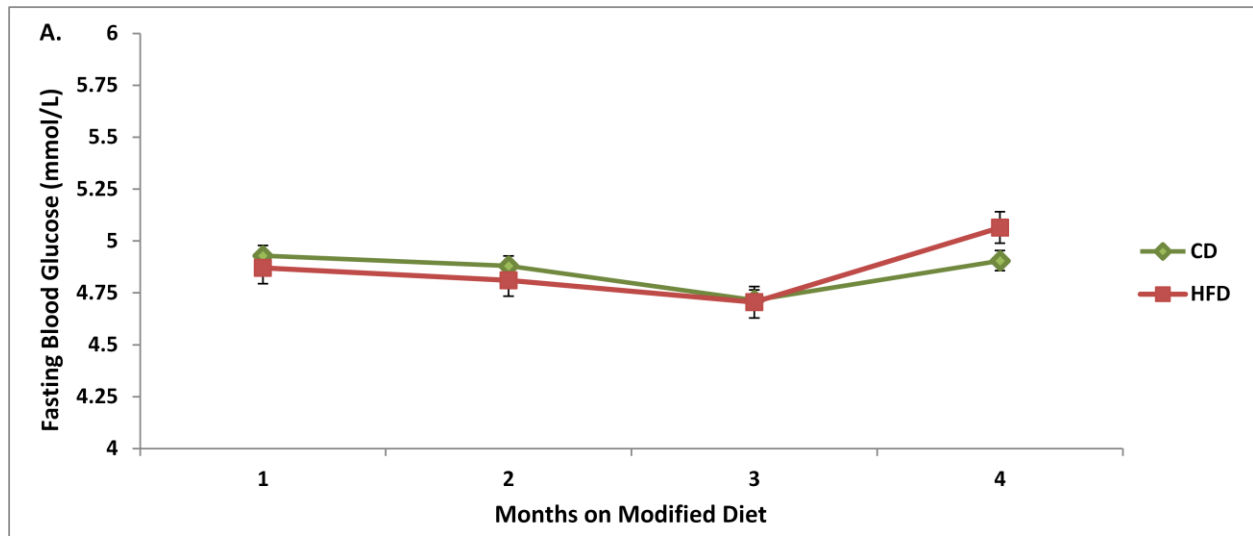
Figure 4. Body Weight Monitoring

Total body weight change in HFD and CD female rats. Body weight was recorded twice a week and is represented as monthly averages per dietary group over the study period (CD: N = 30, HFD N = 30). Values are mean  $\pm$  SEM. No significant difference was present between CD and HFD groups throughout the study.



**Figure 5.** Post Mortem Biometrics

**A)** HFD-fed female rats had significantly greater retroperitoneal fat pad mass compared to CD animals ( $p < 0.001$ ; Student's t-test). **B)** The HFD group had significantly heavier adrenal glands compared to CD animals ( $p < 0.05$ ; Student's t-test). **C)** Liver weight was not significantly altered by treatment. **D)** Liver volume was not significantly altered by treatment. **E)** The HFD group had significantly heavier spleens ( $p < 0.05$ ; Student's t-test). All values are mean  $\pm$  SEM based on  $N = 20$  per group; all data collected at the time of sacrifice.



**Figure 6.** Fasting Blood Glucose and Oral Glucose Tolerance Test Values

**A)** Fasting blood glucose levels were not significantly altered by the difference in diet. **B)** Monthly oral glucose tolerance tests revealed an impaired ability to handle glucose in the HFD group compared to controls ( $p < 0.05$ ; Student's t-test). Data are expressed as arbitrary units from area under the curve (AUC). All values are mean  $\pm$  SEM (total of 30 animals / treatment condition).

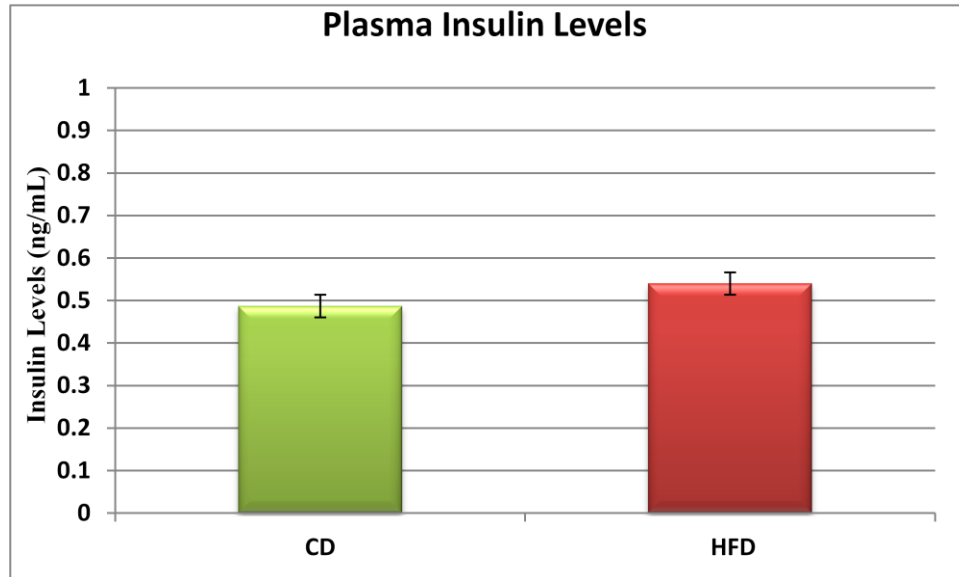


Figure 7. A comparison of plasma insulin levels upon sacrifice. Insulin levels were not significantly different between dietary groups. All values are mean  $\pm$  SEM based on N = 10 per dietary group.

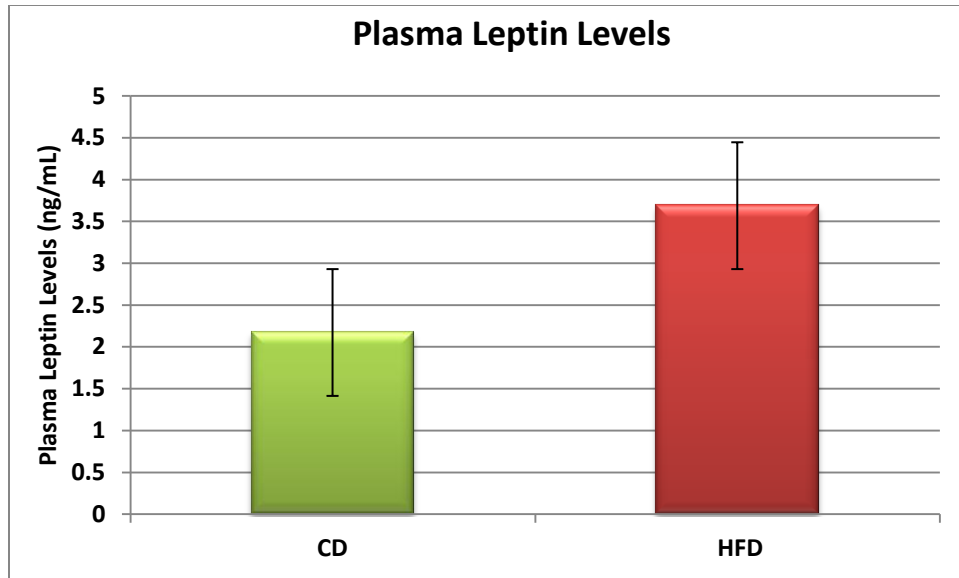
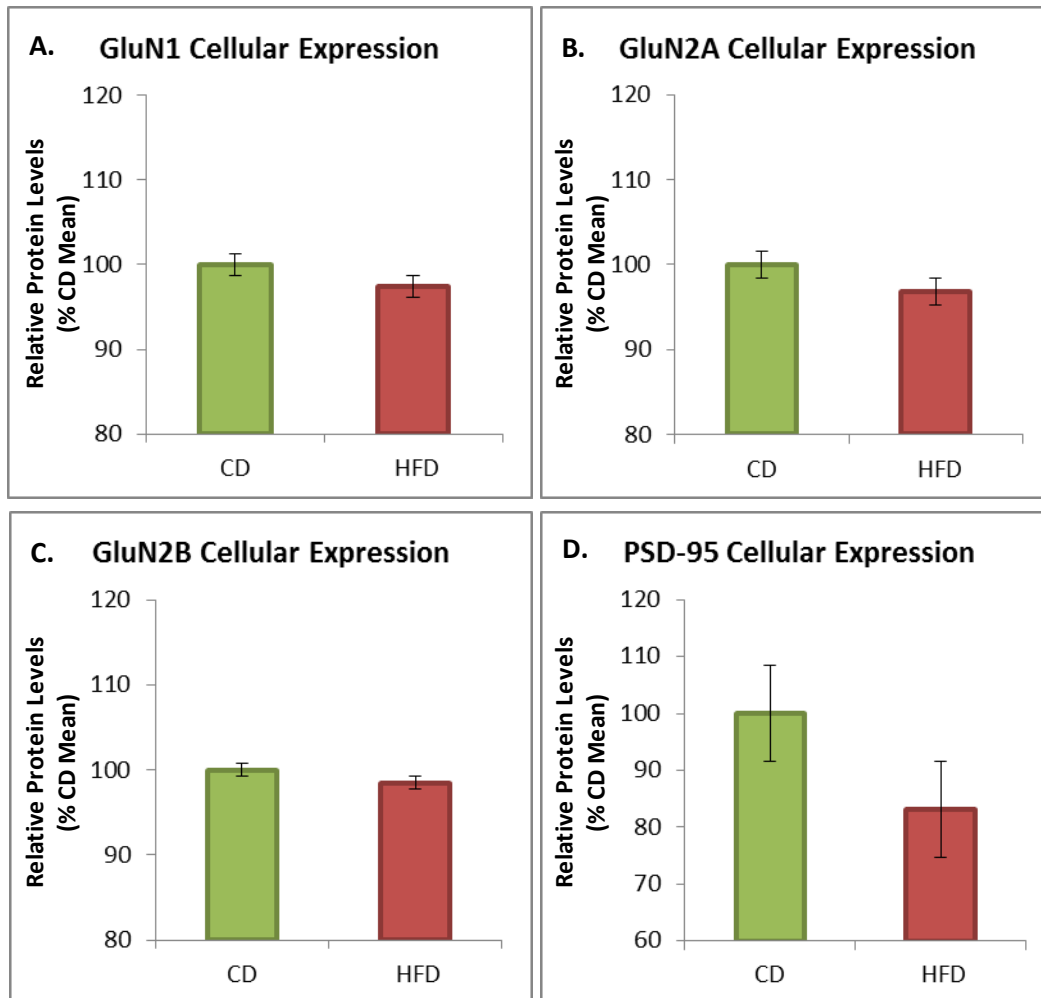
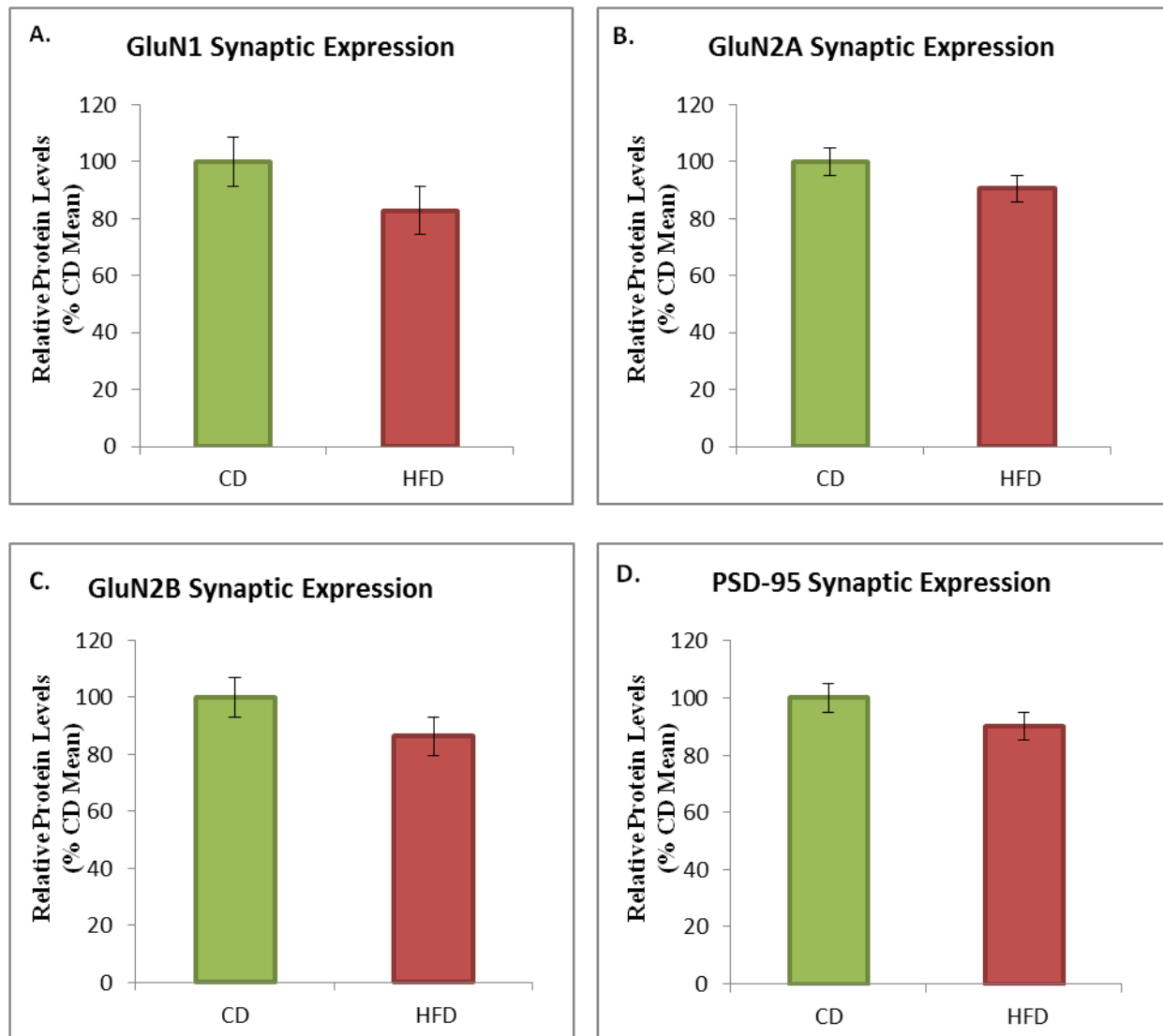


Figure 8. A comparison of plasma leptin levels upon sacrifice. Leptin levels were not significantly different between dietary groups. All values are mean  $\pm$  SEM based on N = 5 per dietary group.



**Figure 9.** Cellular Expression of NMDAR Subunits and PSD-95

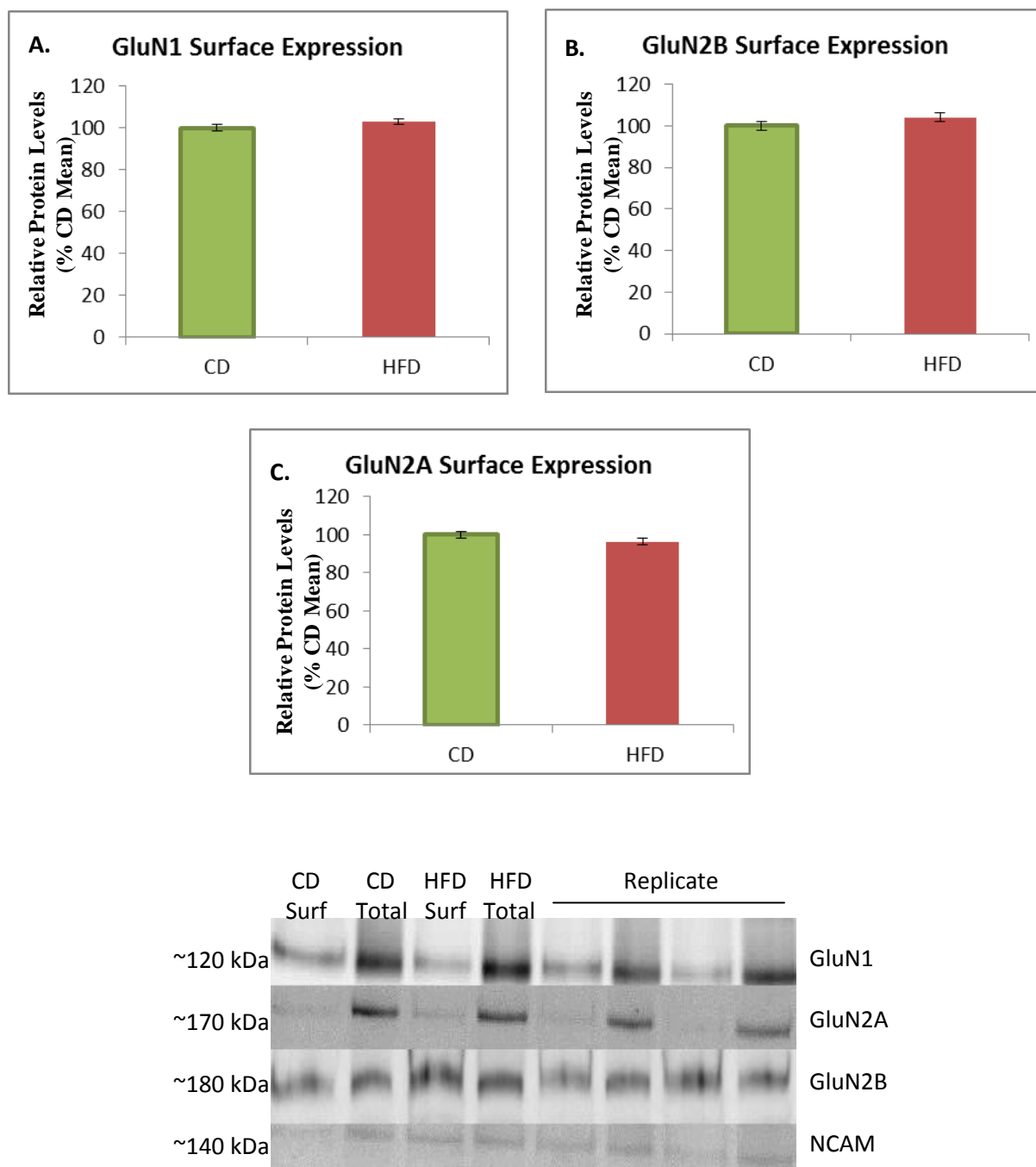
The cellular expression of selected NMDA receptor subunits, and the synaptic protein PSD-95 in female hippocampus after 4 months of dietary treatment. **A)** GluN1, **B)** GluN2A, **C)** GluN2B, and **D)** PSD-95 levels normalized to actin and expressed as % CD mean  $\pm$  SEM (N = 15/treatment condition).



**Figure 10.** Synaptic Expression of NMDAR subunits and PSD-95

No change in synaptic expression of NMDA receptor subunits and PSD-95 was found in female hippocampus after 4 months of dietary treatment. **A)** GluN1, **B)** GluN2A, and **C)** GluN2B levels were normalized to actin, and ratios of synaptic to total lanes were calculated for each respective dietary condition, and expressed as % CD mean  $\pm$  SEM (N = 15/treatment condition). **D)** PSD-95 levels were normalized to actin, ratios of synaptic to total lanes were calculated for each respective dietary condition, and expressed as % CD mean  $\pm$  SEM (N = 11/treatment condition).

\*Representative blot presented in Figure 9.



**Figure 11.** Surface Expression of NMDAR Subunits

Surface expression of NMDA receptor subunits in female hippocampus was not changed after 4 months of dietary treatment. **A)** GluN1, **B)** GluN2A, and **C)** GluN2B levels were normalized to NCAM, and ratios of surface to total lanes were calculated for each respective dietary condition and expressed as % CD mean  $\pm$  SEM (N = 14/treatment condition).



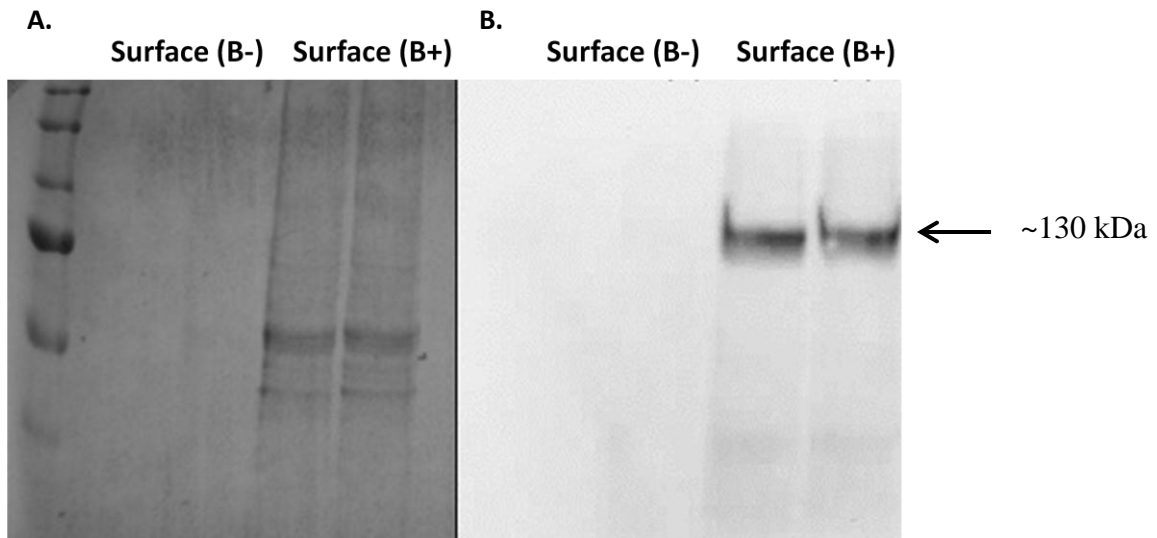


Figure 12. Housekeeping blot with biotinylated samples.

**A)** Ponceau S image showing proteins present only in biotin containing samples **B)** Representative blot showing expression of the GluN1 subunit only in biotin containing samples. Together these data suggest that biotinylation successfully isolated only cell surface proteins. B-, biotin absent; B+, biotin present.

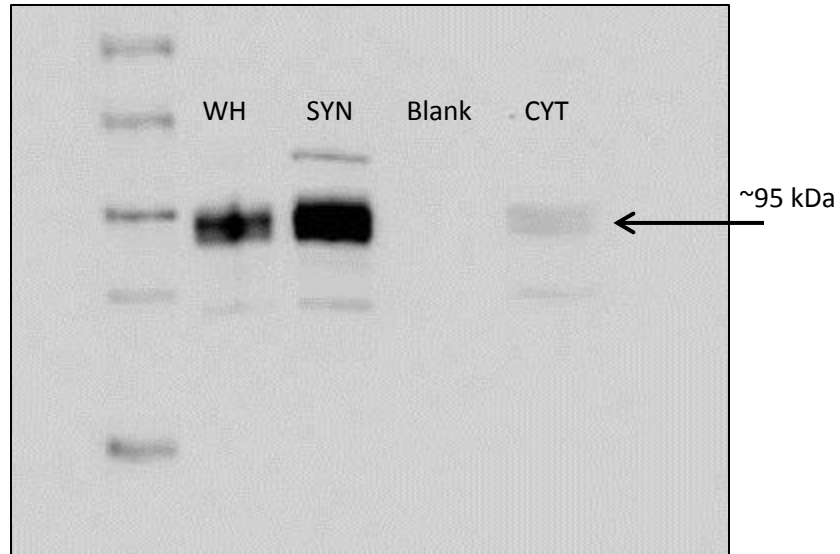


Figure 13. Housekeeping blot with synaptoneurosome samples.

Successful enrichment of synaptic terminals using the synaptoneurosome technique was confirmed by enrichment of PSD-95 the synaptic fraction with very little expression in the cytosolic fraction. WH, whole homogenate; SYN, synaptic fraction; CYT, cytosolic fraction.



Figure 14. Comparison of GluN1 subunit expression between the whole and slice methods of synaptoneurosome preparation from the CD group. GluN1 was normalized to actin, and ratios of synaptic to total lanes were calculated, and expressed as mean  $\pm$  SEM for each type of synaptoneurosome preparation (Whole: N = 5; Slice: N = 10).

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