

Role of acute exercise-induced brain-derived neurotrophic factor on beta-site amyloid precursor protein cleaving enzyme 1 and amyloid precursor protein processing in the brain

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## **Abstract**

Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is the rate limiting enzyme in the pathway responsible for beta-amyloid production, a pathological feature of Alzheimer's Disease (AD). Exercise has been shown to reduce BACE1 activity, although the mechanisms responsible are unknown. Exercise has also been shown to increase brain-derived neurotrophic factor (BDNF) content and signalling, however whether this neurotrophic factor mediates the effects of exercise on BACE1 regulation requires further investigation. C57BL/6J male mice were placed on a low (LFD) or high fat diet (HFD) for 10-weeks. Following the intervention, the mice either remained sedentary or underwent an acute bout of treadmill running. Mice were euthanized and the prefrontal cortex and hippocampus were collected for analysis. The remaining sedentary mice (n=24) were used for an explant experiment where the tissue was directly treated with BDNF. The HFD reduced BDNF content in the hippocampus, however, an acute bout of exercise was able to significantly increase BDNF content in the prefrontal cortex. We further demonstrated that direct treatment with BDNF results in reductions in BACE1 activity in the prefrontal cortex. This novel finding demonstrates that BDNF can reduce BACE1 activity, independent of an exercise stimulus. Moreover, this finding shows for the first time, that there is a direct link between BDNF signalling and BACE1 regulation in this region of the brain. This highlights the viability of using exercise and BDNF to modulate BACE1 activity as a potential therapeutic intervention, without the negative consequences of drug-induced inhibitions.

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## **List of Abbreviations**

AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
APP	Amyloid precursor protein
A $\beta$	Amyloid beta
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
BDNF	Brain-derived neurotrophic factor
CaMK2	Calmodulin-dependent protein kinase 2
CREB	cAMP response element binding
CTF	Carboxyterminal fragment
ERK	Extracellular signal-regulated kinase
eWAT	Epididymal white adipose tissue
GSK	Glycogen synthase kinase
GTT	Glucose tolerance test
HE	High fat/Exercise
HFD	High fat diet
HS	High fat/Sedentary
IDE	Insulin degrading enzyme
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue
JNK	c-Jun NH <sub>2</sub> -terminal kinase
LE	Low fat/Exercise
LFD	Low fat diet

LS	Low fat/Sedentary
MAPKs	Mitogen-activated protein kinases
NFTs	Neurofibrillary tau tangles
PDKs	3-phosphoinositide-dependant kinases
PGC1- $\alpha$	Peroxisome proliferator-activated receptor-gamma coactivator 1- $\alpha$
PI3K	Phosphatidylinositol-3-kinase
PLC- $\gamma$	PhospholipaseC $\gamma$
sAPP $\alpha$	Soluble amyloid precursor protein alpha
sAPP $\beta$	Soluble amyloid precursor protein $\beta$
T2DM	Type 2-diabetes mellitus
TBST	Tris buffered saline/0.1% Tween 20
TNF $\alpha$	Tumour necrosis factor alpha
TrkB	Tyrosine receptor kinase B

## **Chapter 1: Introduction**

Alzheimer's disease (AD) is a progressive neurodegenerative disorder putatively linked to aging and characterized by neuroinflammation and perturbations in brain energy metabolism (1-4). It is considered to be the most common form of dementia in elderly people, accounting for approximately 60-80% of all cases (5). Disease progression results in irreparable damage to neurons, specifically associated with the cerebral cortex and the hippocampus. The neuronal loss seen in AD can impact the individual's memory, thinking and reasoning, as well as change personality and behaviour (6). As of 2010, it was estimated that at least 35 million individuals worldwide had dementia, and the prevalence is expected to double every 20 years, meaning by the year 2050, it is expected that 115.4 million people will be living with some form of dementia (7). Despite this alarming increase in prevalence, there has yet to be an effective treatment/prevention for AD, which is why elucidating therapeutic strategies to prevent or slow the progression of the disease is critical.

There are two major distinctions of AD, sporadic and familial. Less than 5% of the population accounts for cases of familial AD, which is typically a result of genetic mutations, specifically of the amyloid precursor protein gene, Presenilin-1, and Presenilin-2 (8, 9). Conversely, sporadic AD accounts for the vast majority of cases (10). While there can be a genetic component to sporadic AD (ApoE4 allele), it can be heavily influenced by lifestyle choices, such as diet and exercise (11-14). The exact etiology of sporadic AD is poorly understood, but most efforts to elucidate it focus on two histopathological hallmarks: 1) neurofibrillary tau tangles (NFTs) and 2) amyloid beta (A $\beta$ ) plaques (15). These A $\beta$  plaques and NFT have been established as key players in the

neuronal death that occurs in AD (16), and play a fundamental roles in the mechanisms of early progression of AD (17, 18).

Previous work has highlighted obesity, type 2-diabetes (T2DM), and brain hypometabolism as major risk factors for the progression of sporadic AD (11, 19-21). The exact mechanisms behind adiposity contributing to AD have yet to be elucidated, however, it is thought that chronic low-grade peripheral inflammation can activate microglia, resulting in neuroinflammation (22, 23). Pro-inflammatory cytokines, such as interleukin 1 $\beta$  and tumour necrosis factor alpha (TNF $\alpha$ ) are released by immune/inflammatory cells due to physiological stressors and can activate the mitogen-activated protein kinases (MAPKs) in the brain (24, 25). In an AD brain, perturbed activation of the MAPKs are thought to contribute to the pathology of the disease by increasing neuronal apoptosis (26-28) and enzymatic activity of proteins responsible for the accumulation of A $\beta$  peptides (29).

In addition to obesity related inflammation, systemic and central insulin resistance are contributing factors to neurodegenerative progression (11, 12, 30-33), in fact, many researchers have begun calling AD, Type 3 diabetes (11, 12, 32, 33). In the brain, insulin signalling is responsible for various roles, including regulating synaptogenesis, synaptic remodeling, expression of neurotransmitters (e.g., acetylcholine and norepinephrine), and may act to increase brain glucose metabolism by increasing glucose uptake via insulin-stimulated glucose transporter translocation (34-37). Brain hypometabolism has also been linked to AD progression (21). Glucose uptake into the brain requires a transporter in order to pass the blood brain barrier, this transporter is primarily GLUT1 (38) and down-regulation of GLUT1 in the brain is thought to contribute to the hypometabolism

commonly seen in AD (39). Hypometabolism in the brain can be characterized as reductions in glucose transport and mitochondrial function and is thought to manifest in patients with AD well before clinical symptoms are evident (40). All of these outcomes can have significant implications on cognition.

Recently, exercise has been looked at as a potential preventative and therapeutic strategy, as it has been shown to reduce AD pathology-related markers. Furthermore, exercise has the capacity to increase neurotrophic growth factors, such as brain-derived neurotrophic factor (BDNF), that are responsible for synaptic plasticity, neurite growth and neuronal survival (41, 42). BDNF has also been shown to reduce the production of and have a protective effect against the neuronal toxicity induced by pathological AD markers (43). However, it is currently unknown if there is a direct link between BDNF signalling and AD markers.

## **Chapter 2: Literature Review**

### **2.1 Alzheimer's Disease**

#### *2.1.1 Histopathological Hallmarks of Alzheimer's Disease*

NFTs and A $\beta$  plaques are the pathological hallmarks of AD. NFTs are intracellular, insoluble, twisted fibers comprised mainly of hyper-phosphorylated tau proteins (44). Tau is produced by the splicing of a gene called “microtubule associated protein tau” and plays a role in vesicle transport by promoting assembly and stabilization of microtubules (45-49). Tau protein activity is regulated by phosphorylation and dephosphorylation by various kinases and phosphatases. However, hyper-phosphorylated tau proteins can cause tau to aggregate into paired helical filaments, which subsequently form NFTs (50-58). In the NFT state, the tau proteins are incapable of stabilizing cellular microtubules resulting in microtubule and cytoskeleton destabilization. The number of NFTs correlates to the progression and symptoms of the disease, however not to the neuronal loss (59). As well, NFTs are observed later in the progression of AD whereas increases in A $\beta$  is detectable during early stages of the disease. Thus, in the effort to determine the mechanisms responsible for the early progression and prevention of AD, many researchers predominantly focused on A $\beta$  as the more important histopathological hallmark.

#### *2.1.2 Amyloidogenic Cascade*

A $\beta$  originates from a large type 1 transmembrane protein, known as the amyloid precursor protein (APP) (60, 61). Competing pathways metabolize the precursor protein to begin the amyloidogenic process; these pathways are known as the non-amyloidogenic

$\alpha$ -secretase pathway and the amyloidogenic  $\beta$ -secretase pathway (Figure 1) (17, 62, 63). Under non-pathological conditions,  $\alpha$ -secretase cleaves APP at the Leu<sup>17</sup> site, resulting in a soluble ectodomain, soluble APP $\alpha$  (sAPP $\alpha$ ) fragment and a membrane-bound carboxyterminal fragment (CTF) 83 (64). Subsequently, CTF83 is cleaved by  $\gamma$ -secretase, which results in the intracellular release of amyloid precursor protein intracellular domain (AICD) and extracellular release of p3 (65). The cleavage of APP by  $\alpha$ -secretase cleavage occurs in the A $\beta$  sequence and therefore prevents A $\beta$  formation. However, under pathological conditions, APP is cleaved by beta-site amyloid precursor protein cleaving enzyme 1 (BACE1 or  $\beta$ -secretase) at either Asp<sup>1</sup> or Glu<sup>11</sup> site. BACE1 cleavage results in the release of a soluble ectodomain, soluble APP $\beta$  (sAPP $\beta$ ) fragment, and a membrane-bound CTF89/99 fragment is formed, subsequently  $\gamma$ -secretase imprecisely cleaves the CTF89/99 fragment and typically releases either a A $\beta$ 40 or A $\beta$ 42 peptide (66).

Due to the imprecise nature of  $\gamma$ -secretase, the A $\beta$  produced in this pathway can vary in length between 38 to 43 amino acids however; A $\beta$ 40 is the predominant A $\beta$  species. Conversely, the longer length A $\beta$  species (A $\beta$ 42) are more hydrophobic and more prone to aggregate (67). A pathological issue arises when the A $\beta$  peptides begin to aggregate causing A $\beta$  oligomerization. A $\beta$  oligomers appear to be a potent neurotoxin, resulting in neuronal apoptosis and senile plaque buildup (68, 69). BACE1 is the rate-limiting enzyme in the amyloidogenic pathway and therefore plays a large role in A $\beta$  production. Therefore, understanding how BACE1 activity and content can be regulated could prove to be vital for future therapeutic interventions.

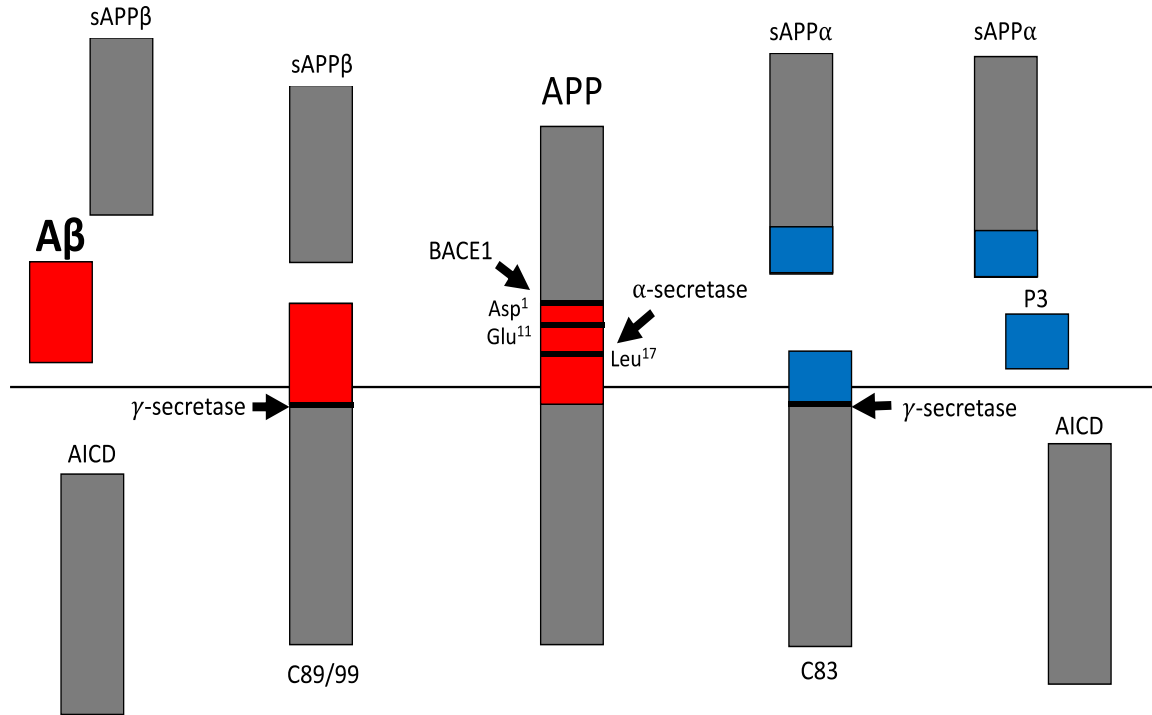


Figure 1: Amyloidogenic Cascade, APP processing and products. The non-amyloidogenic pathway (on the right) is cleaved by  $\alpha$ -secretase and  $\gamma$ -secretase to generate sAPP $\alpha$ , C38, P3 and AICD. While the amyloidogenic pathway (on the left) is cleaved by BACE1 ( $\beta$ -secretase) and  $\gamma$ -secretase to generate sAPP $\beta$ , C89/99, AICD and the extracellular release of an A $\beta$  peptide. (Adopted from Grant Hayward)

### 2.1.3 Production and Removal of A $\beta$

A $\beta$  production on its own does not appear to be harmful; individuals will have intracellular and extracellular A $\beta$  present at any given time, however, an imbalance between production and removal leads to the accumulation of A $\beta$  and subsequent aggregation (70). The imbalance of production and removal of A $\beta$  is one of the major factors driving aggregation, aside from the variable  $\gamma$ -secretase cleavage site. BACE1 has a primary role in regulating A $\beta$  production (71-74) as it is the rate-limiting enzyme in the amyloidogenic cascade. In terms of A $\beta$  removal, insulin degrading enzyme (IDE) and neprilysin appear to be the major players for the degradation of A $\beta$  monomers and oligomers, resulting in steady-state levels of A $\beta$  found in healthy brains (75, 76). IDE is



specifically responsible for the degradation of A $\beta$  monomers while neprilysin has been shown to degrade both monomers and oligomers. Research has shown that deficiencies in IDE can cause ~50% reduction in A $\beta$  degradation (75) while neprilysin deficiencies cause cerebral accumulation of A $\beta$  (76). Studies have shown that in AD, there is increased expression of BACE1 in both human and animal brains (71-73, 77), as well as decreased levels of both IDE and neprilysin (75, 76, 78, 79). Although it is unclear which enzyme(s) have the greatest influence, it is evident that the changes in protein expression and activity of each enzyme contribute to pathologically increased A $\beta$  aggregation.

## 2.2 Beta-site Amyloid Precursor Protein Cleaving Enzyme 1

BACE1 is an aspartic protease, a part of the pepsin family that is endogenous to humans (80) and is also a type 1 transmembrane protein, a feature considered unique among pepsin proteins. Due to its structural attributes, BACE1 is hypothesized to have specialized functions distinct from other aspartic proteases. Being the rate-limiting enzyme in the amyloidogenic pathway and playing a large role in A $\beta$  production, aberrant BACE1 activity is considered to be a major contributor in AD progression (17, 18, 71-73). As such, BACE1 inhibition has been highly explored as a therapeutic target. To date, there have been several studies that have utilized BACE1 knockout mice (81-84), as well as several clinical trials at various phases aimed at inhibiting BACE1. These studies attempt to eliminate or sequester BACE1 enzymatic activity and reduce A $\beta$  production (83, 85). However, all attempts, as of yet, have either been discontinued (86-88) or exhibited undesired side effects or phenotypes. Specifically, BACE1 knockout mice display reduced body weight, a reduction in nerve myelination, deficits in muscle spindle

formation and most concerning, increased mortality (89). As reviewed by Vassar et al. (83) BACE1 knockout mice have been shown to have: increased axon targeting errors (82), reduced myelination (90), memory impairments (91), alterations in neurogenesis (92), neurochemical abnormalities (93), endophenotypes of schizophrenia (94) and seizures (84), further corroborating a potential role of BACE1 in synaptic function. This indicates that BACE1 or the products of BACE1 cleavage (sAPP $\beta$ , CTF $\beta$ , A $\beta$ ) play a pivotal role in health and not only in AD pathology, as non-pathological functions of these proteins have yet to be elucidated. For instance, despite being associated with AD, A $\beta$  is also thought to have normal physiological functions. Through overexpression of APP, Kamenetz et al. (95) demonstrated that the production of A $\beta$  can depress synaptic activity. To demonstrate this, Kamenetz et al. stimulated neuronal activity in isolated brain slices and found that this was accompanied by a significant increase in A $\beta$  production, suggesting the presence of a negative feedback loop where A $\beta$  plays a role in modulating synaptic activity. To further test the direct effects of A $\beta$  on synaptic transmission, they transfected hippocampal slice neurons with Sinus virus harboring cDNA encoding CTF $\beta$  (containing the A $\beta$  sequence). Increased expression of CTF $\beta$  was enough to depress synaptic transmission, however, this depression was diminished when the slices were treated with a  $\gamma$ -secretase inhibitor (L-685,458), providing further evidence of the role of A $\beta$  and synaptic activity. This is crucial because without synaptic depression, activity can become excessive and induce excitotoxicity (96). Therefore, A $\beta$  provides critical synaptic feedback inhibition, which is important to consider when looking at BACE1 as a therapeutic target, A $\beta$  production cannot be eliminated entirely without severe consequences. Furthermore, BACE1 content is highly concentrated at the

presynaptic terminals of neurons (81, 97) supporting a role in synaptic function. An important consideration when discussing knockout models of BACE1, is that BACE1 is absent throughout the lifespan and therefore it is difficult to tease out the role of BACE1 in developmental stages vs stages later in life. This would be an important distinction to make, especially when considering reducing BACE1 content or activity as a therapy for AD, which typically occurs in the later stages of life. However, a recent study looked at a conditional BACE1 knockout model in mice by using a tamoxifen-dependent Cre/lox genetic recombination system to slice exon 2 of BACE1 (98). In one group of mice, they administered tamoxifen at 3 months of age, which activated the BACE1 knockout, successfully allowing them to grow until full adulthood with intact BACE1 content. The findings from this study showed there were fewer undesirable phenotypes, specifically hypomyelination and no apparent memory/learning impairments in the adult mice. Unfortunately, the conditional BACE1 knockout mice still displayed uncharacteristically short infrapyramidal bundles - a tract of mossy fiber axons in the hippocampus, a serious concern that leads to impaired hippocampal function and loss of synaptic plasticity. This further highlights the need to consider the important physiological functions of BACE1, aside from A $\beta$  production, as future therapeutic interventions should modulate BACE1 enzymatic activity rather than eliminate it all together, in order to be effective.

### *2.2.1 Obesity and BACE1*

Inactivity and obesity have been identified as significant risk factors for the progression of AD (13, 20, 99-101) but the exact mechanisms underlying how increased adiposity contributes to the progression of the disease remains largely unknown.

Putatively, chronic low-grade peripheral inflammation induced by obesity, causes a release of pro-inflammatory cytokines (TNF- $\alpha$ ) from adipose tissue into the circulatory system, which can pass through the blood brain barrier and activate microglia. Activation of microglia results in microglial proliferation, further releasing inflammatory factors, such as, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and activating MAPKs. The release of these inflammatory factors and activation of these kinases can perpetuate neuroinflammation and lead to brain insulin resistance (22, 23). Neuroinflammation and brain insulin resistance are thought to guide APP processing towards the amyloidogenic pathway and increase the expression and activity of BACE1 (29). In fact, studying the effects of an obesogenic diet on BACE1 content and activity, as it specifically relates to A $\beta$  accumulation, has been a focal point in this area of research. In humans, there appears to be a relationship between obesity and circulating plasma A $\beta$  (102-104), along with this, evidence from several longitudinal studies support the hypothesis that higher levels of plasma A $\beta$  can be used to predict the risk of developing AD and cognitive impairments (103, 105, 106). Further, increased circulating A $\beta$  can be detected decades before clinical symptoms of AD appear. For example, a recent study, by Luciano et al. examined biomarkers for AD in normal weight (n=172), overweight (n=135), and obese (n=129) adolescents ranging from 12-18 years of age and evaluated insulin resistance via the Homeostasis Model Assessment of IR index (102). Luciano et al. found that obese adolescence with insulin resistance had significantly higher levels of circulating A $\beta$  when compared to overweight and normal weight peers, suggesting that obesity and insulin resistance may be playing a role in the production of A $\beta$  in humans, even at a younger age. However, longitudinal work in this

area is needed before coming to a definite conclusion with regards to its clinical relevance.

Many researchers have also shown that a high fat diet (HFD) will increase both protein and mRNA expression of BACE1 in rodent models (107-109). In corroboration with this, rodent models of T2DM (diet-induced, streptozotocin, and genetically induced) have increased BACE1 protein content in the brain, accompanied by increased APP processing (109, 110). In contrast, other studies have demonstrated increased APP processing, measured by CTF $\alpha/\beta$  content, with no changes in BACE1 protein content following a HFD, suggesting that activity of BACE1 is altered rather than its expression (99). In accordance with this hypothesis, recent work by our lab fed C57BL/6 male mice a HFD for 7 weeks and found significant increases in BACE1 activity with no changes in protein content or mRNA expression (111). These results were also accompanied by increases in markers of neuroinflammation and aberrant phosphorylation of proteins in the insulin signalling cascade, both of which are thought to phosphorylate APP at the Thr668 site, resulting in increased APP processing (112, 113).

Furthermore, a HFD has been shown to exacerbate AD-related pathology in transgenic rodent models of AD (114-118). Theriault et al. (114) used APP<sub>swe</sub>/PS1 mice, a well-studied familial AD-causing mutation that leads to enhanced A $\beta$  production, and placed them on a HFD for a 4-month period. The results of this study showed that a HFD accelerated cognitive decline, induced A $\beta$ 40 accumulation and increased oxidative stress at the blood brain barrier when compared to a normal chow-fed control. In agreement with these findings, Vandal et al. (116) used 3xTG-AD mice, a more aggressive transgenic model of AD due to additional point mutations, and placed them on either a

HFD (60% fat) or a control diet for a 9-month intervention. As expected, the HFD group exhibited an increase in body weight and reductions in insulin sensitivity; however, the HFD further deteriorated memory function, determined by a Barnes maze test, and exacerbated A $\beta$  accumulation compared to the 3xTG-AD mice on a control diet.

Alternatively, Puig et al. (118) used C57BL6/J mice to determine if diet-induced obesity would alter APP processing in the absence of a genetic mutations. 6-week old C57BL6/J mice were placed on either a 21.2% by weight HFD diet or 5.5% by weight control diet for 22 weeks. Feeding non-transgenic mice a HFD for 22 weeks resulted in a concomitant increase in APP protein content, as well as, increases in proinflammatory markers (TNF- $\alpha$ ) in the brain. Together these studies provide evidence that an obesogenic diet pushes APP processing towards the amyloidogenic pathway not only in models of familial AD but also in a model of diet-induced obesity, which can be representative of sporadic AD. In accordance with this hypothesis, Thirumangalakudi et al. (107) placed 4-month old C57BL6/J mice on either a HFD (21%) or normal chow (5%) for a 2-month period. With no significant differences in weight gain over the 2-month period, the HFD mice had increased neuroinflammation, characterized by glial activation and cytokine expression (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ), impaired working memory, measured using a water radial arm maze, and increased APP processing. Specific markers of APP processing and the amyloidogenic pathway included increased BACE1 mRNA expression and total protein content, CTF $\alpha/\beta$ , and A $\beta$  content. This is particularly interesting as this study highlights that a HFD can drive APP processing, but also that it is likely driven by increased BACE1 expression and protein content, further elucidating the relationship between sporadic AD pathogenesis and an obesogenic diet. Collectively, these studies indicate that

an obesogenic diet has the capacity to increase APP protein content, BACE1 protein content/activity, neuroinflammation and impair insulin signalling, all of which can alter APP processing and ultimately lead to exacerbated A $\beta$  accumulation and impaired cognition (111).

### *2.2.2 Exercise and BACE1*

Evidence indicates that regular exercise can combat obesity and related diseases and interestingly, can also slow down the progression of cognitive impairments initiated by neurodegenerative disorders (119-122). Recent epidemiological studies by our group and others have highlighted a positive correlation between physical inactivity and AD mortality rates (13, 122-124). Exercise training has also been shown to improve cognitive function and memory in both humans and rodent models (125-127). For example, Berchtold et al. (127) placed 2 month old C57BL/6J mice in a cage either with or without a running wheel for 3 weeks and examined cognitive performance using a radial arm water maze. Mice that had access to the running wheel showed significantly shorter latency times, fewer errors, and better retention when compared to the sedentary group. This study provides a line of evidence that mice are able to respond to exercise training with improvements to cognition. Moreover, several lines of evidence indicate that long-term exercise has neuroprotective properties against A $\beta$  accumulation and prevents AD-like pathologies (99, 127-133). Adlard et al. (133) used TgCRND8 mice, a transgenic model of AD that overexpresses APP, and found that a long-term exercise protocol preserved cognitive capacity. This was measured by using a Morris water maze and was indicated by significant reductions in their escape latencies when compared to sedentary

littermates (133). Collectively, these studies suggest that exercise can be utilized to promote overall brain health, improve memory and cognition, and provide a neuroprotective effect against neurodegenerative disease. Given the clear relationship between exercise and cognition, researchers have begun to investigate the “if and how” exercise alters the pathogenesis of AD.

From the same study, Adlard et al. (133) also examined the interaction between exercise and the amyloidogenic pathway. Five months of voluntary exercise resulted in reduced extracellular A $\beta$ 40 and A $\beta$ 42 accumulation in both the frontal cortex and hippocampus regions of the brain. Interestingly, these changes occurred in the absence of changes in mRNA expression and protein content of neprilysin or IDE, suggesting that chronic exercise is altering APP processing and not degradation (133), however they did not directly investigate changes in BACE1 expression, content, or activity. Similarly, Maesako et al. (99) found that APP transgenic mice (APP<sub>Swe/Ind</sub>) on a HFD displayed memory deficits and had increased A $\beta$ 40 deposition in the hippocampal region of the brain when compared to APP<sub>Swe/Ind</sub> normal chow controls. This effect was prevented, however, if the mice were housed in a larger cage with access to a running wheel while on the HFD. Furthermore, having access to a running wheel resulted in a down-regulation of BACE1 activity, highlighting the importance and effectiveness of exercise in relation to reducing AD pathogenesis (99). To further examine the effects of exercise training on BACE1 expression, Zhang et al. (129) used APP/PS1 transgenic mice and implemented a long-term exercise protocol. At the age of 5 months, the mice were separated into 4 groups; control sedentary, control exercise, APP/PS1 sedentary, and APP/PS1 exercise. The exercise groups then performed treadmill running for 30 minutes a day, 6 days a



week for a 5-month period. After 5-months of undergoing the exercise training protocol, the APP/PS1 mice exhibited significantly reduced A $\beta$  deposition in the hippocampus, increased neuronal density and reduced APP processing, indicated by reductions in APP and BACE1 expression compared to the APP/PS1 sedentary group. Alternatively, exercise is also able to attenuate A $\beta$  accumulation and APP processing in a non-transgenic model. Selvi et al. (134) used 40 Wistar albino rats, housing them in either standard conditions or environmentally enriched conditions (access to running wheel). The rats were then further divided into either standard chow, HFD or high sucrose diet. After 4 weeks of intervention, the obesogenic diets resulted in increased levels of A $\beta$ 40 and A $\beta$ 42 in the hippocampus, however this was mitigated in the groups with the environmentally enriched cages. Collectively, these studies indicate that long-term exercise provides a neuroprotective effect against A $\beta$  accumulation and the associated cognitive impairments, regardless if it was induced by genetic mutations of the APP or induced by an obesogenic diet. This highlights the validity of using exercise training as a therapeutic or preventative intervention to combat AD, especially due to it being relatively safe, inexpensive and easily implementable in a variety of populations. Moreover, this emphasizes the ability for exercise to modulate the amyloidogenic pathway without the negative side effects of drug-induced inhibition. How exercise directly elicits these changes in the brain remains to be determined.

It is clear that long-term exercise training has a protective effect against a HFD and the progression of neurodegenerative diseases; however, with chronic exercise, comes a constellation of physiological changes that may indirectly affect brain health and signalling pathways. For example, exercise training reduces adiposity and improves

whole body glucose homeostasis/insulin response (135, 136). In order to elucidate the direct effects of exercise on the brain and the corresponding signalling pathways, it needs to be in the absence of changes in other tissues that could be acting as a confounding factor. A recent study from our lab examined whether an acute bout of exercise could ameliorate BACE1 activity and/or content after 7 weeks of a high fat feeding, similarly to that seen with long-term exercise training (111). The findings revealed that a single bout of exercise was able to reduce BACE1 content and activity in obese, insulin resistant mice. These reductions were presumably independent of alterations in adiposity and acute improvements in skeletal muscle insulin sensitivity and glucose uptake, providing evidence that exercise has a direct effect on APP processing. This study highlights direct effect of exercise on the brain, however, the exact physiological mechanisms behind exercise-induced reductions of BACE1 have yet to be explored. It is essential to elucidate these signalling pathways in order to efficiently prescribe exercise programs and develop pharmaceutical targets to slow AD progression and eventually prevent it entirely.

### 2.3 Brain-derived Neurotrophic Factor (BDNF)

Many studies suggest that the synthesis and release of neurotrophins and the binding to their respective receptors are necessary for mediating the effects of exercise on cognition and neuronal health (137-139). Neurotrophins play an essential role in several aspects of neuronal function, with applications ranging from basic neuronal development to neurodegenerative disorders (140). In mammalian brains, there are 4 main neurotrophins that have been identified; nerve growth factor, BDNF, neurotrophin-3 and neurotrophin-4, all of which share similarities in structure and amino acid sequence

(141). These neurotrophins are generated from precursors, pro-neurotrophins, which are 31-35 kDa proteins that contain hydrophobic signal peptides and pro-regions containing sequences of basic amino acids within their N-termini (142). Pro-neurotrophins are proteolytically cleaved by the mammalian proprotein convertases to produce 12-13 kDa mature neurotrophins (140, 142). Of particular interest is BDNF as it plays a specific role in the proliferation, differentiation and growth of neurons, not only during development as originally thought, but also in mature neurons and is responsible for synaptic plasticity and neuronal survival and function (41, 42). Normal cognitive function can be associated with proficient neuronal plasticity, which can be defined as “the capacity of neurons or glial cells to improve or to depress the synaptic efficacy through biochemical or morphological changes which evolve in a dynamic fashion” (143). BDNF is highly expressed in brain regions that exhibit a high degree of plasticity (i.e., prefrontal cortex and hippocampus) (144). However, with AD, there is a noticeable decrease in synaptic plasticity, cognitive function, and BDNF content. As such, the role of neurotrophins, particularly BDNF, in the potential therapeutic benefits with regards to neurodegenerative diseases, has been an area of interest.

### *2.3.1 BDNF Signalling*

BDNF is initially synthesized in the endoplasmic reticulum as a precursor protein, where it is then packaged into dense core vesicles in neurons (145). It is almost exclusively localized to presynaptic dense core vesicles (146) further corroborating the requirement of BDNF release in synaptic plasticity events. However, BDNF can also be secreted from astrocytes (147), microglia (148) and post-synaptic dendrites (145, 146).

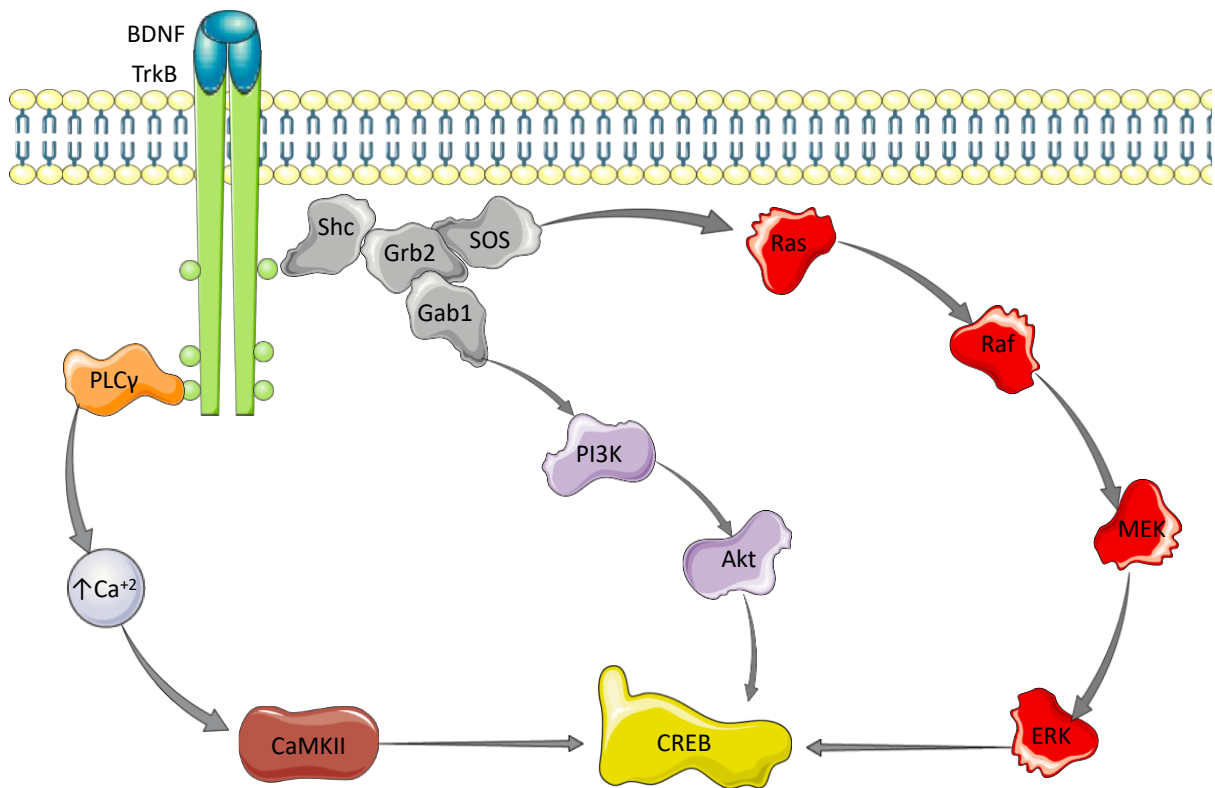
BDNF is secreted in response to neuronal activity and derived from both pre- and postsynaptic sites (149). Once released, it binds to its receptor, tyrosine receptor kinase B (TrkB; also known as tropomyosin receptor kinase B), which results in receptor dimerization and kinase activation at the Tyr512 site and Tyr 816 site (41, 150). Kinase autophosphorylation of TrkB results in the activation of several downstream intracellular pathways. There are three significant pathways, including: 1) phospholipaseC $\gamma$  (PLC-  $\gamma$ ); 2) Ras/MAPK; and 3) phosphatidylinositol-3-kinase (PI3K)/Akt, all of which phosphorylate cAMP response element binding (CREB) (41, 140, 151, 152). CREB is a transcription factor that regulates genes that are important for neuronal survival, such as, c-fos, Nr4a2 and has also been shown to increase the expression of BDNF (153). CREB has been implicated in regulating the synaptic efficacy required for memory storage, as well as mediating long-lasting changes in synaptic plasticity (154). In AD, CREB phosphorylation, transcription and synaptic plasticity are all decreased, implicating the importance of CREB activation and signalling; specifically in regards to maintaining overall brain health and potentially therapeutic targets (154).

Phosphorylation of TrkB Tyr 816 recruits PLC-  $\gamma$  and subsequently increases intracellular calcium levels. Increased calcium has the potential to activate many different pathways, including calcium-calmodulin dependent kinase pathway, which has been shown to phosphorylate CREB (41). TrkB Tyr 515 can activate the Shc/Grb2/SOS complex, a group of adaptor proteins responsible for the recruitment of various proteins including Ras and PI3K. The activation of Ras via Shc/Grb2/SOS complex regulates neuronal differentiation and promotes neuronal survival through the MAPK/ERK pathway (41), which in turn, also leads to direct phosphorylation of CREB. Ras via the

Shc/Grb2/SOS complex is also able to activate PI3K (155, 156). PI3K can be activated by insulin receptor substrate-1, which can also be activated by BDNF (157). PI3K recruits several proteins to the membrane, including Akt and 3-phosphoinositide-dependant kinases (PDKs). At the membrane, Akt is activated at the threonine 308 site by PDK, which in turn phosphorylates various important proteins responsible for cell survival including; Bcl-2/Bcl-x-associated death promoter, I $\kappa$ B, the forkhead transcription factor and caspase 9 (41, 140, 158, 159). Akt is also upstream from CREB and thought to play a role its activation as well. Furthermore, Akt phosphorylates glycogen synthase kinase (GSK) 3- $\beta$  at the Ser 9 site, which inhibits the protein (160). Elevated levels of active GSK 3- $\beta$  have been shown to increase neuronal apoptosis, phosphorylate Tau protein and APP (161, 162). Thus, activation of Akt through PI3K promotes cellular survival in a multifaceted manner. However, in AD and in an obesogenic model, Akt activation has been shown to be perturbed and dysregulated (162, 163).

BDNF signalling occurs on synapses quickly with the onset of stimulation and results in sustained TrkB phosphorylation (164). Rapid synaptic effects are thought to be a result of the PLC-  $\gamma$  pathway, whereas longer lasting transcriptional effects are putatively a product of the PI3K and MAPKs pathways (165). These cascades have been linked to the neuroprotective effects of BDNF, likely through the phosphorylation of CREB, along with mediating cellular survival, proliferation and differentiation (166). However, a link between these downstream targets of BDNF and the amyloidogenic

pathway has yet to be elucidated.



*Figure 2: BDNF/TrkB signalling. BDNF binding to its receptor (TrkB) causes receptor dimerization and kinase activation. Kinase autophosphorylation of TrkB results in the activation of several downstream, intracellular pathways. Of particular interest is the Ras/MAPK pathway (red), PI3K pathway (purple) and the PLC $\gamma$  pathway (orange). All mentioned pathways have been shown to activate CREB, which can further increase BDNF expression, as well as increase gene expression of proteins responsible for neurite growth and survival.*

### 2.3.2 Alzheimer's Disease and BDNF

Several lines of evidence suggest that decreased levels of BDNF could be associated with AD pathogenesis (167, 168). For example, clinical evidence shows that there is a decrease in the expression and protein content of BDNF and TrkB in the post-mortem brains of patients with AD (167, 169-172). Phillips et al. examined hippocampal samples obtained from the national neurological research bank for study, 9 of which were diagnosed with AD and 9 which were absent of AD pathology (169). Using an RNAase protection assay, they determined that BDNF mRNA abundance was reduced in the

hippocampus of patients that were diagnosed with AD when compared to non-AD patients. Providing early evidence that decreased expression of BDNF is involved with the pathogenesis of AD. In further examination of these findings, Holsinger et al. used the parietal cortex from normal, neurologically unimpaired subjects, and those with AD, provided by the Institute for Brain Aging and Dementia tissue repository at the University of California and quantified BDNF mRNA levels (170). Through the use of competitive RT-PCR, BDNF mRNA levels were compared between the normal, neurologically unimpaired tissue, and that of the AD subjects. There was a 3.4-fold reduction of BDNF mRNA in the parietal cortex of the AD subjects. These two studies highlight that BDNF mRNA expression is lower in brain regions commonly affected by AD, signifying that a reduction of BDNF expression may be contributing to AD progression. On top of this, research from Hock et al. demonstrated that BDNF protein content is significantly lower in post-mortem parietal cortex and hippocampus brain regions of patients with AD when compared to non-neurologically impaired control patients (173). However, the major issue with post-mortem sampling is the integrity of protein and/or mRNA, as post-mortem delay can range from 3 to 33 hours making it difficult to be certain that the decreases seen are solely from the disease (144). With this in consideration, several studies have looked at circulating BDNF levels in patients with AD rather than using post-mortem brain samples (174, 175). Two cross-sectional studies examined the serum levels of BDNF in patients with AD and compared them to healthy patients used as a control. Laske et al. found that there was significantly lower levels of circulating BDNF in the serum of AD patients (18.6 ng/ml) when compared to control volunteers (21.3

ng/ml), further highlighting a potential link between BDNF levels and the pathogenesis of AD (174).

Due to the potential issues of studying post-mortem tissue, researchers have examined the relationship between BDNF and AD pathogenesis, both in *in vivo* and *in vitro* models. Peng et al. (176) examined the cortical levels of BDNF mRNA in two different transgenic mouse models of AD (APP<sup>NLh</sup>/PS-1<sup>P264L</sup> and TgCRND8). The mice were euthanized at different time points based on pathological progression of each respective strain. APP<sup>NLh</sup>/PS-1<sup>P264L</sup> mice demonstrate elevated levels of A $\beta$  and plaque deposition at 6 months of age and were euthanized between 15 – 18 months of age, whereas, TgCRND8 mice demonstrate elevated levels of A $\beta$ 42 at 4 weeks and plaque deposition at 3 months and were euthanized at 11.4 months of age. RT-PCR analysis of the frontoparietal cortex revealed that both transgenic strains had significantly lower BDNF mRNA levels when compared to wild-type littermates (176). A $\beta$ 42/A $\beta$ 40 ratio and A $\beta$  oligomers were also measured, where a strong correlation between A $\beta$  oligomers and decreased BDNF mRNA levels was observed. This suggests that the aggregation of A $\beta$  peptides is linked to the lower BDNF expression. This further contributes to the understanding of the vast effects of A $\beta$  oligomer toxicity and highlights the potential link between BDNF and AD. To compliment these findings, Jiao et al. showed that there were significant reductions in serum BDNF levels and total protein content in the cortex of AD transgenic mice (177). Similar results have been shown *in vitro* with a SH-SY5Y cell line, where administration of A $\beta$  oligomers has been shown to downregulate BDNF expression (178). One potential mechanism for the observed BDNF downregulation could be through a A $\beta$ -induced CREB transcriptional downregulation (178, 179).



However, *in vitro* treatment with BDNF appears to have a neuroprotective effect against A $\beta$  toxicity. In cortical neuron cultures, BDNF had a specific and dose-response protective effect against neuronal toxicity induced by A $\beta$ 42 and these results were mitigated through TrkB signalling (180). When treated with K252a, a selective TrkB inhibitor, the protective effect of BDNF was abolished, insinuating that BDNF binding to TrkB elicits a neuroprotective response to A $\beta$  toxicity.

Recent work has shown that a HFD leads to reduced BDNF protein content and impaired BDNF signalling similarly to that seen with AD (181-184). Research done in our lab has shown that feeding mice a HFD reduces BDNF content and hinders its signalling (181). Eight-week-old C57BL/6J mice were fed either a low fat or HFD for a 7-week intervention and the prefrontal cortex of all mice were analyzed. The HFD resulted in lower levels of BDNF protein content as well as decreased phosphorylation of downstream targets including TrkB and CREB. These findings highlighted that high fat feeding-induced obesity leads to decreased BDNF content and its downstream targets. These deficits have recently been associated with the progression of neurodegeneration and AD, providing a potential explanation of the link between obesity and sporadic AD. In agreement with this, Molteni et al. fed 2-month-old female Fisher 344 rats a high fat-high sucrose diet for 2 months and examined changes in the hippocampus. They found a significant reduction in expression and protein content in BDNF, CREB (total and phosphorylated) and other markers of synaptic plasticity (synapsin and GAP-43) (182). As well, the rats demonstrated impairments of spatial learning, shown by increased escape latency times during a Morris water maze. Collectively, these studies suggest that there is a link between decreased levels of BDNF and AD progression, further, a HFD

can result in deficits to BDNF signalling and may provide an explanation as to why obesity increases the risk of developing AD.

### *2.3.3 Exercise and BDNF*

Exercise training increases both circulating BDNF in the periphery and BDNF content in the brain, in both human and rodent models, respectively (139, 153, 181, 185-188). A meta-analysis by Dinoff et al. (189) covered 29 studies that met the inclusion criteria, in which, the studies were examining the effects of exercise training on resting peripheral BDNF levels. The results from these studies indicated that aerobic exercise training was effective in increasing resting peripheral BDNF levels (189). These findings are important as they strengthen the evidence that physical activity can potentially enhance cognition through increased concentrations of neurotrophins. However, to better understand the mechanisms behind the therapeutic effects of BDNF via physical activity, it is vital to examine BDNF levels with a single bout of exercise in the absence of other physiological adaptations associated with exercise training. In humans, acute exercise is a potent stimulus for the increase in circulating BDNF from sites including the brain, liver, muscle and platelets (190). Gold et al. demonstrated that 30 minutes of moderate exercise on a bicycle ergometer can significantly increase serum BDNF production in a male/female population via ELISA (191). This was the first study to highlight that a single bout of exercise has the capacity to increase circulating BDNF levels in humans. In line with these findings, Rasmussen et al. evaluated the contribution of the human brain to plasma BDNF both at rest and in exercise, as well as the brain regions responsible for BDNF release (187). Using human participants, a catheter was used to draw blood from the internal jugular vein (venous) and the radial artery (arterial) at rest, 2

hours, and 4 hours after exercise (rowing). Blood was analyzed for plasma BDNF levels using an ELISA and the cerebral release of BDNF was calculated as venous-arterial difference divided by the venous concentration. The results indicated that BDNF levels increased after 4 hours of exercise (902 pg/ml from 347 pg/ml) but also that the brain contributes significantly to circulating BDNF levels both at rest and during prolonged exercise. Furthermore, the finding shows that almost three quarters of BDNF in the venous circulation originates from the brain, highlighting neuronal tissue as the main contributor; although it is not the sole contributor (187). To determine which brain regions were responsible for the release of BDNF, the investigators had mice perform an acute bout of exercise, 2 hours of treadmill running, and euthanized them either immediately after exercise or after 2, 6, or 24 hours of recovery (187). The cerebellum, hippocampus and cortex were dissected and used for mRNA analysis. BDNF mRNA levels increased with exercise and peaked 2 hours post-exercise in the cortex and hippocampus, but not the cerebellum. This emphasizes brain-regional differences in BDNF expression and release, highlighting the cortex and hippocampus, the areas of the brain involved in memory and cognition, as the main contributors. The differences in exercise modality and intensity and duration pose an issue with translating these findings to humans, nevertheless, both of these studies indicate that BDNF levels are increasing with acute bouts of exercise and furthermore, these increases are derived from the brain (187).

To further explore the effects of exercise on BDNF, a recent study aimed to determine if the relationship between exercise and increased circulating BDNF is intact in individuals with AD (192). A single bout of aerobic exercise on a treadmill was

completed by 21 patients with AD and 18 healthy older adults. Compared to baseline levels, plasma BDNF significantly increased with exercise in both patients with AD and control individuals, confirming that exercise increases circulating BDNF levels in patients with neurodegenerative disorders, as well as healthy individuals. This study highlights the viability of using exercise to induce increases in BDNF content and release with patients with AD. However, the exact mechanisms behind exercise-induced increases in BDNF protein content within the brain remain uncertain. A recent study aimed to elucidate this question and showed that exercising skeletal muscle may increase hippocampal BDNF by organ cross-talk (153). Wrann et al. suggest that muscle contraction leads to the cleavage of fibronectin type 3 domain-containing protein 5 (FNDC5), which is then secreted into circulation as irisin. Irisin is then thought to be able to stimulate BDNF gene expression via peroxisome proliferator-activated receptor- $\gamma$  coactivator 1- $\alpha$  (PGC1- $\alpha$ ) pathway (153, 188). However, this mechanism cannot explain the rapid increase in circulating BDNF following an acute bout of exercise as upregulation of BDNF takes ~3 hours (187) and therefore thought to contribute more so to long-term improvements in cognitive function.

A review by Knaepen et al. highlights that there is a strong positive correlation to exercise intensity and plasma BDNF concentrations in which, higher intensity and graded exercise elicited the greatest response (193). Several lines of evidence also show that other exercise modalities (maximal, submaximal, sprints, endurance) have the capacity to increase circulating BDNF levels in healthy individuals (187, 194-196), which is an important consideration if prescribing exercise as a therapeutic intervention. For example, Ferris et al. demonstrated that with an intensity slightly above the aerobic threshold or at

maximum effort, proportional increases in BDNF occurred (13% and 30%, respectively). In addition to the intensity of the exercise, the duration is also a factor in BDNF response. Saucedo Marquez et al. showed that BDNF concentrations gradually increase over time in both high-intensity interval and continuous intensity exercise models (197).

Studies in humans rely solely on measuring circulating levels of BDNF and make inferences on its ability to pass the blood brain barrier (i.e. bioavailability) as measuring the direct effects of exercise on BDNF content in the human brain remains challenging. However, circulating BDNF is thought to be able to pass the blood brain barrier in a bi-directional manner (198) and is vital to central functions, including the upregulation of central BDNF expression (188). To accommodate for the current inability to directly measure BDNF content in the human brain, several studies aimed to determine the effects of exercise training on mRNA expression and total protein content of BDNF in the brain regions of rodent models (138, 199-202). Similar to increased circulating BDNF in human studies, BDNF mRNA and protein content is up-regulated with chronic exercise (wheel running) in the hippocampus of rodent models (202). Other studies have also examined the effects of acute exercise on BDNF expression and protein content in rodents (181, 202) to corroborate previous findings indicating that acute exercise increases BDNF levels. Soya et al. compared low and moderate intensity treadmill running in a rat model (30 minutes at 15m/min vs 25m/min). This study demonstrated that a single bout of exercise significantly increases BDNF mRNA expression in the hippocampus, with levels peaking at 1.5 hours post-exercise (202). A recent study from in our lab examined if an acute bout of exercise could ameliorate the high-fat feeding induced reductions in brain BDNF content and signalling (181). 8-week old C57BL/6J

mice were fed a LFD (10% kcals from lard) or HFD (60% kcals from lard) ad libitum for 7 weeks, resulting in an obese, glucose intolerant model. Both groups then performed a single bout of treadmill running (2 hours at 15m/min) followed by a 2-hour recovery period. After which the prefrontal cortex and hippocampus were collected for protein content and mRNA analysis. The HFD significantly decreased BDNF content and signalling, indicated by decreases in the phosphorylation status of its receptor TrkB and downstream effector CREB. However, the results demonstrated that a single bout of exercise was successful in reversing high-fat feeding induced reductions in BDNF signalling. These results highlight that BDNF signalling is still intact in an obesogenic model and that a single bout of exercise can induce BDNF signalling, suggesting that exercise may be a simple, non-pharmacological treatment for metabolic diseases where there are decreases in BDNF.

Strong evidence indicates that exercise, in the absence of changes in adiposity, is effective in increasing circulating BDNF and total protein content, making it a simple, inexpensive, non-pharmacological intervention in treating diseases that have impaired synaptic plasticity and cognitive impairments. However, despite the associations between increased BDNF with exercise and decreased BDNF with AD, no studies have examined the mechanistic link between acute exercise-induced BDNF/BDNF treatment and its potential regulation of BACE1 content/activity.

### **Chapter 3: Statement of the problem**

The accumulation of extra-neuronal peptides, known as A $\beta$ , is considered detrimental to neuronal networking and leads to neuronal apoptosis and dysfunction. The etiology behind the accumulation are intricate and poorly understood, however most research focuses on the production of A $\beta$  peptides. BACE1, the rate-limiting protease enzyme in the amyloidogenic process, initiates the processing of amyloid precursor protein leading to the production of A $\beta$ , however, the mechanisms behind the regulation of BACE1 content and activity have yet to be elucidated. Previous work has shown that a single bout of exercise can decrease BACE1 activity and content in high fat fed mice (111). Exercise has also been shown to increase BDNF, a neurotrophic growth factor that is responsible for synaptic plasticity, neurite growth and neuronal survival (41, 42). BDNF has been shown to reduce A $\beta$  production and have a protective effect against the neuronal toxicity induced by A $\beta$  (43). Interestingly, BDNF expression and protein content is lower in brain regions where A $\beta$  production is most prominent, the hippocampus and prefrontal cortex (203, 204). Furthermore, evidence proposes that lower levels of BDNF could be associated with the degradation of neurons seen with the accumulation of A $\beta$  (167, 168). Despite these associations between BDNF and A $\beta$ , a direct link between BDNF and BACE1 has yet to be elucidated.

#### **3.1 Aim of Thesis**

This thesis aims to provide novel information about the mechanistic link between BDNF and BACE1. It is anticipated that increased BDNF signalling will result in reductions in BACE1 activity. This information will further our understanding of the

regulation of A $\beta$  production and the associated detriments to neuronal tissue. In addition, this study will be the first to determine region specific differences in response to BDNF, as well as, if the response is different following a period of high fat feeding. This study will expand the fundamental knowledge of how the brain responds to physiological stressors, such as, exercise and a high fat diet.

### 3.2 Objectives:

- 1) Characterize the detriments of chronic high fat feeding on cognition, brain metabolism, BDNF signalling, and the amyloidogenic pathway
- 2) Determine the effects of an acute bout of exercise on BDNF content/signalling and the amyloidogenic pathway
- 3) Identify a direct link between BDNF and BACE1 activity.

### 3.3 Hypothesis:

1. It is hypothesized that in a C57BL/6J obesogenic mouse model, a HFD will reduce BDNF content and increase BACE1 activity; however, a single bout of exercise will ameliorate these effects by increasing BDNF content/signalling and reducing BACE1 content.
2. Furthermore, the direct treatment of brain explants with BDNF will reduce BACE1 activity in the absence of exercise and other circulating factors.



## **Chapter 4: Methods**

### *4.1 Materials*

Low-fat (10% kcal fat; cat#D12450B) and high-fat (60% kcal fat; cat#D12492) diets were purchased from Research Diets INC (New Brunswick, NJ). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (Westgrove, PA). Molecular weight marker, reagents, and nitrocellulose membranes for SDS-PAGE were acquired from Bio-Rad (Mississauga, ON) and GE Healthcare Life Science (cat#10600002). Western lightning Plus-ECL (PerkinElmer, 105001EA). Insulin and glucose were purchased from Eli Lilly and BioShop Reagents, respectively. All other sources are listed throughout the text. Antibodies against BDNF (1:500, Santa Cruz cat #sc-65514), CREB (1:500, Cell Signaling cat #9197), pCREB (1:500, Cell Signaling cat#9196), TrkB (1:500, Cell Signaling cat#4603), pTrkB (1:500, abcam cat#ab109684) APP (1:500 Biolegend, cat#SIG039152), pAPP Thr668 (1:500, Cell Signaling cat#6986S), BACE1 (1:500, Cell Signaling cat#5606P), sAPP $\alpha$  (1:500, BioLegend cat#SIG39139, and sAPP $\beta$  (1:500, BioLegend cat# SIG-39138) were obtained and used during this study.

### *4.2 Animals*

Experimental protocols were approved by the Brock University Animal Care Committee (file #17-06-02; Appendix A) and are in compliance with the Canadian Council on Animal Care. Male C57BL/6J mice (18 weeks of age;  $31.48 \pm 2.2$  g,  $n = 72$ ) were ordered from The Jackson Laboratory (Bar Harbor, Maine, USA) and allowed to acclimatize for 3 days in the Brock University Comparative Biosciences Facility. During

acclimatization, mice were fed standard chow (2014 Teklad global 14% protein rodent maintenance diet, Harlan Tekland, Mississauga, ON). All mice were kept on a 12-hour light: 12-hour dark cycle and had *ad libitum* access to food and water through the entirety of the study. Mice were randomized to either: 1) low fat diet and sedentary; 2) low fat diet and acute exercise; 3) high fat diet and sedentary; or 4) high fat diet and acute exercise.

#### *4.3 Experimental Design*

C57BL/6J male mice were randomized into either a high fat fed (n = 36, 60% kcal fat) or control diet group (n = 36, 10% kcal fat). Baseline measures of all mice (N = 72) were conducted immediately after acclimatization and included body mass measurements. The mice remained on their respective diets for a 10-week period, with biweekly body mass measurements. Following the 10-week period, mice underwent glucose and insulin tolerance testing. The two groups were then further randomized into either exercise or non-exercise groups (Low fat/Sedentary (LS, n = 24), Low fat/Exercise (LE, n = 12), High fat/Sedentary (HS, n = 24), High fat/Exercise (HE, n = 12)). The exercise groups performed a single bout of exercise, 2 hours of treadmill running as previously described (111). Following a 2-hour recovery period, the mice performed a cognitive test, euthanized, and the prefrontal cortex and hippocampus were collected.

The sedentary groups were then divided into two experimental groups (n = 12 in each). The first group underwent tissue collection as described for the exercised groups and the second group was used for explant experiments where the tissue was directly treated with BDNF.

# Preclinical Model of Sporadic AD

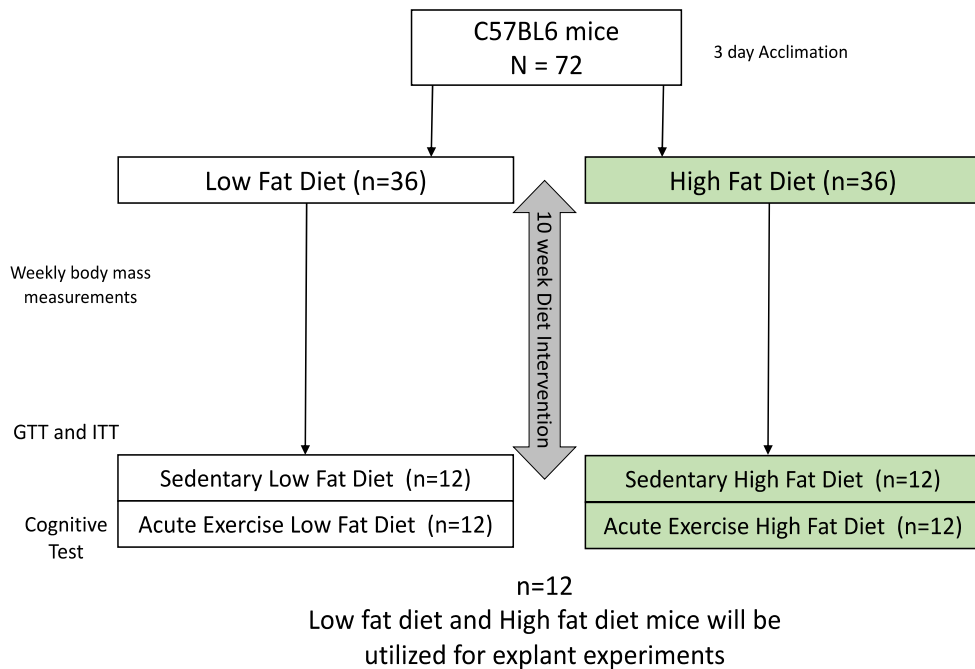


Figure 3: Schematic of experimental design.

## 4.4 Glucose Tolerance Test and Insulin Tolerance Test

Intraperitoneal glucose tolerance tests (GTT) were performed on fasted (6h), non-anesthetized mice during the second last week of feeding (week 9) (Appendix B). Glucose measures were obtained from tail vein blood using an automated glucometer at baseline and at 15, 30, 45, 60, 90, and 120 min following an intraperitoneal injection of glucose (2g/kg body mass).

Intraperitoneal insulin tolerance tests (ITT) were performed on non-anesthetized mice during the second last week of feeding (week 9) following a 2-day recovery period from the glucose tolerance test (Appendix C). Glucose measures were obtained from tail vein blood using an automated glucometer at baseline and at 15, 30, 45, 60, 90, and 120 min following an intraperitoneal injection of insulin (0.75g/kg body mass).

#### *4.5 Acute Exercise Protocol*

To minimize differences between groups and in stress, all mice were acclimated to motorized treadmill running during a 3-day period consisting of 5 minutes of running/day at 8 m/min, 5% grade. This acclimatization period took place during the last week of feeding. 72 hours following the last day of acclimation, mice ran for 120 minutes at 15m/min, with an incline of 5%. The acute bout of exercise started at 8:00AM, which is the beginning of the light cycle. All mice in the exercise treatment group were able to complete the 2 hours of treadmill running without issue and was well tolerated. Following the exercise bout, mice were placed back in their cages to recover for two hours without food. This two hour recovery period was chosen to be consistent with our previous work using this protocol (181)

#### *4.6 Barnes Maze Spatial Memory Test*

All procedures were in accordance with Brock University's SOP (Appendix D) and adapted from Attar's (205) and Rosenfield's studies (206). A Barnes maze (painted white), Virox<sup>®</sup> spray for cleaning the area after each trial, a stopwatch, and a camera attached to the SMART video tracking software (Panlab, SL, Barcelona, Spain) was used during the test. Visual cues were placed around the room and remained in the exact same location and orientation for the duration of testing. Acclimatization was broken down into two phases and was carried out during the final week of feeding. The probe phase (test) was conducted before tissue collection.

During the habituation phase mice were placed under a clear beaker and guided around the maze for 30 seconds. This was followed by the beaker being placed over the escape cage for 3 minutes and the mice gently being nudged in if they did not enter before the time expired. They were then allowed to explore the escape cage for 1 minute. Between each mouse, the maze, beaker, and escape cage were wiped with Virox<sup>®</sup>.

The training phase was performed 24 hours after the habituation phase. Mice were placed under an opaque beaker for 15 seconds. The beaker was then lifted, allowing the mice to explore the maze for 2 minutes. If they found the hole, they were left for 1 minute before being put back into their cage. If they failed to find the escape hole in the 2 minutes, they were guided there in the clear beaker and nudged in after 2 minutes if they still did not enter, after which, the mice were also given 1 minute to explore the escape cage.

The probe phase was performed 48 hours after the training phase and 2 hours after the acute bout of exercise. After 15 seconds under the opaque beaker, the mice were given 3 minutes to find the escape hole. During this phase, data on latency time to correct escape hole and permanence time spent in wrong zone (false holes) were recorded. This test was performed with assistance from Dr. Cheryl McCormick, her lab group, and their equipment.

#### *4.7 Tissue Collection*

Mice were anaesthetized with a weight-adjusted bolus intraperitoneal injection of sodium pentobarbital (5mg/100g body weight). Blood was collected, and mice were sacrificed through exsanguination. Surgical scissors were used to decapitate the mice and

then and a midline incision was made to remove the skin to expose the skull. A small incision was then made at the base of the parietal bone and then cut through to the most anterior part of the skull (i.e., frontal bone). Forceps were then used to peel back/break off both sides of the parietal bone. Once the brain was exposed, closed curved surgical scissors were slid underneath the posterior part of the brain and gently lifted up. The scissors were then used to sever the cranial nerves, emancipating the brain. Once the brain was removed from the skull, a surgical scalpel was used to dissect the brain into the left and right hemispheres. After isolating the brain into its respective hemispheres, the prefrontal cortex and hippocampus was removed. The prefrontal cortex was located at the anterior portion of the brain and identified as the most outer layer of the brain. Curved tweezers were then used to gently peel back the cortex and expose the “glossy” hippocampus. Once successfully removed, the samples were snap frozen in liquid nitrogen and stored at -80°C for Western blotting analysis.

In addition, inguinal and epididymal white adipose tissue (iWAT and eWAT respectively) was collected as a characteristic of body composition. The liver was extracted as well to further characterize the model and for future analyses. All tissues were stored in a -80°C freezer until further analysis.

#### *4.8 Explant Experiment*

The prefrontal cortex and hippocampus from the sedentary groups was dissected into left and right hemispheres and then placed into a tube with pre-oxygenated DMEM media + 0.1% FBS and allowed to incubate for 30 minutes at 37 degrees C as described previously (207). After which, either the left or right prefrontal cortex/hippocampus was

treated with 100ng/ml of BDNF while the other side remained as a control and was treated with the same volume of media and left to incubate for 30 minutes. The tissue was then removed from the media, homogenized and stored at -80 degrees for further analysis. Western Blotting will be used to look specifically at markers of BDNF signalling.

#### *4.9 Western Blotting*

Samples were homogenized (FastPrep®, MP Biomedicals, Santa Ana, CA) in 20 volumes of NP40 Cell Lysis Buffer (Life Technologies; CAT# FNN0021) supplemented with 34  $\mu$ L phenylmethylsulfonyl fluoride and 50  $\mu$ L protease inhibitor cocktail (Sigma; CAT# 7626-5G, CAT# P274-1BIL). The homogenized samples were placed on a shaker in a 4°C fridge for 20 minutes to reduce foam accumulation. Homogenized samples were then centrifuged at 4°C for 15 minutes at 10,000g, after which the supernatant was collected, and protein concentration was determined using a Bicinchoninic acid assay (Sigma-Aldrich - B9643, VWR – BDH9312). The samples were prepared to contain equal concentrations (1 $\mu$ g/ $\mu$ l) of protein in 2x Laemmli buffer and placed in a dry bath at 100°C for 5 minutes. 20 $\mu$ g of protein were loaded and separated on 10% SDS-PAGE gels for 90 minutes at 120V. Proteins were then wet-transferred onto nitrocellulose membrane at 100V for 60 minutes. Membranes were blocked in Tris buffered saline/0.1% Tween 20 (TBST) with 5% non-fat powdered milk for 1 hour at room temperature. The appropriate primary antibody (1:500 ratio) was then applied and left to incubate on a shaker, at 4°C overnight. Following primary incubation, the membrane was washed with TBST 3 x 5 minutes and then incubated with the corresponding secondary antibody conjugated with horseradish

peroxidase (Jackson ImmunoResearch, 1:2000 ratio) for 1 hour at room temperature. Signals were detected using enhanced chemiluminescence and were subsequently quantified by densitometry using a FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA). A representative ponceau stain was measured and analyzed for each membrane to ensure equal loading (<10% variability across the membrane).

#### *4.10 Real-time PCR*

Prefrontal cortex and hippocampus total RNA was extracted and reversed transcribed into cDNA. Changes in mRNA expression were determined using real-time quantitative PCR as described previously (111). RNA was isolated from the prefrontal cortex and hippocampus following homogenization in Trizol reagent using an RNeasy kit according to the manufacturer's instructions (RNeasy Kit 74106; Qiagen). RNA yield and purity were determined using a Nano-drop system (NanoVue plus; GE healthcare). RNA samples were prepared at 1 µg/µl using RNase free water (total volume of 10 µl). cDNA was synthesized using random primers and dNTP (Invitrogen) at a 1:1 ratio as well as a master mix (5x FSB, DTT, RNase out and SuperScript II Reverse Transcriptase). 7500 Fast Real-Time PCR system (Applied Biosystems) was used to perform the RT PCR. Each sample was loaded in duplicate and contained 10 µl of PCR master mix, 4 µl of RNase free water, 1 µl of gene expression assay and 5 µl of cDNA (diluted with 80 µl of RNase free water). Gene expression assays were purchased for Bdnf (Mm04230607\_s1), Bace1 (Mm00478664\_m1), and Gapdh (Mm9999915\_g1). Gapdh was used as a housekeeping gene. Relative differences in Bdnf and Bace1 mRNA expression were



determined using the  $2^{-\Delta\Delta CT}$  method and normalized to the respective control group (208). The housekeeping gene, Gapdh did not change between groups.

#### *4.11 Beta-secretase Activity*

BACE activity in the prefrontal cortex and hippocampus was determined using a commercially available beta-secretase activity assay kit (Ab65357; Appendix E) as previously described (111, 209). Samples were homogenized (FastPrep®, MP Biomedicals, Santa Ana, CA) and extracted using 20 volumes of ice-cold PBS. Samples were left to incubate on ice for 15 minutes and centrifuged at 10,000 g for 5 minutes at 4°C. The supernatant was collected, and a BCA assay was performed to determine protein concentration. All samples were prepared at 0.75 µg/µL. A total of 50 µL of sample was added to each well in duplicate, followed by 50 µL of 2x reaction buffer and 2 µL of beta-secretase substrate. The plate was left to incubate in the dark at 37°C for 60 minutes and fluorescence was read using spectrometer (SpectraMax M2; Molecular Devices) at excitation and emission wavelengths of 335 and 495 nm, respectively. The assay uses a secretase-specific peptide conjugated to two reporter molecules, EDANS and DABCYL. In the un-cleaved form, the physical proximity of the DABCYL moiety and EDANS quenches the fluorescent signal. Cleavage of the peptide by β-secretase separates EDANS and DABCYL allowing for the release of a fluorescent signal.

#### *4.12 Statistical Analysis*

Body weights, GTT and ITT results were recorded, and a two-way repeated measures ANOVA design was performed. This was followed by calculating area under

the curve and conducting a two-way ANOVA based on exercise by diet. Differences between total and phosphorylated protein content, liver, iWAT, eWAT tissue weights, and Barnes Maze spatial memory test markers were analyzed using a two-way ANOVA (Exercise by Diet). Significant interactions were followed up with Tukey post-hoc analysis. In cases where data were not normally distributed data was logarithmically transformed. Data are expressed as means  $\pm$  SEM with significance set at  $P < 0.05$ .

## Chapter 5: Results

### 5.1 Exercise Study

#### 5.1.1 Diet-induced Changes on Whole-body Measurements

Prior to being assigned groups, there was no significant differences in body weight between the mice ( $p>0.05$ ). Following randomization into either a high fat fed (HFD;  $n = 36$ , 60% kcal fat) or low-fat fed group (LFD;  $n = 36$ , 10% kcal fat), the HFD group had a higher body mass after 1-week of diet intervention compared to LFD group ( $p<0.05$ ). Body mass continued to increase until the end of the 10-week intervention when compared to the low-fat diet group (Figure 4;  $p<0.0001$ ). To further characterize changes in whole-body measurements, inguinal and epididymal white adipose tissue (iWAT and eWAT, respectively) and the liver were collected post-euthanization. The HFD groups demonstrated higher tissue mass with regards to iWAT ( $p<0.05$ ), eWAT ( $p<0.05$ ), and liver ( $p<0.05$ ) when compared to the LFD groups (Table 1).

*Table 1. Body and tissue mass post-10-week intervention*

	<b>Body Mass (g)</b>	<b>Inguinal white adipose tissue (g)</b>	<b>Epididymal white adipose tissue (g)</b>	<b>Liver (g)</b>
<b>LFD</b>	$31.93 \pm 0.47$	$0.41 \pm 0.05$	$0.84 \pm 0.07$	$1.20 \pm 0.04$
<b>HFD</b>	$48.40 \pm 0.54 *$	$2.01 \pm 0.08 *$	$1.73 \pm 0.07 *$	$2.34 \pm 0.08 *$

Values are presented as mean  $\pm$  SEM for  $n=24$ /group. Significance ( $p<0.05$ ) is denoted by \*.

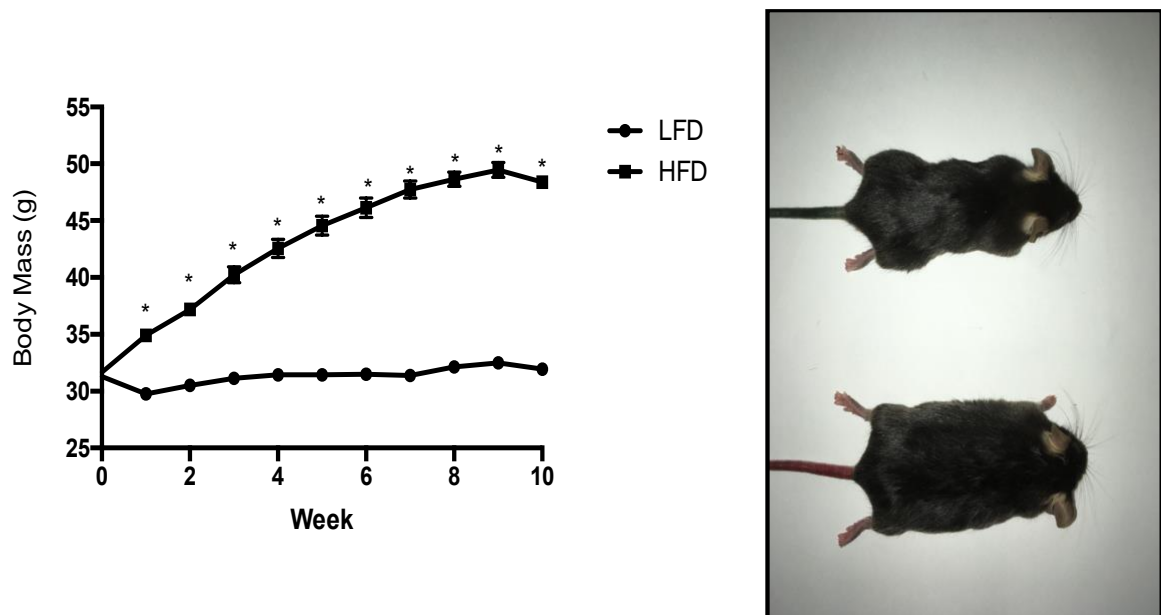


Figure 4: Changes in body mass throughout the duration of the study. LFD; animals were maintained on a low-fat diet while they aged for 10 weeks from 16 weeks of age; HFD; animals were maintained on a high fat/obesogenic diet for 10 weeks from 16 weeks of age. \*; significantly different from LFD at a given time point. ( $p < 0.05$ )  $n = 36/\text{group}$ .

### 5.1.2 Whole Body Glucose Tolerance and Insulin Sensitivity

The HFD group demonstrated a significant reduction in whole body glucose and insulin tolerance, indicated by a higher blood glucose area under the curve in the glucose tolerance test (Figure 5a) and insulin tolerance test (Figure 5c). Mice were then subdivided into exercise and sedentary groups at this time to ensure there were no significant differences in glucose and insulin tolerance between exercise and sedentary groups.

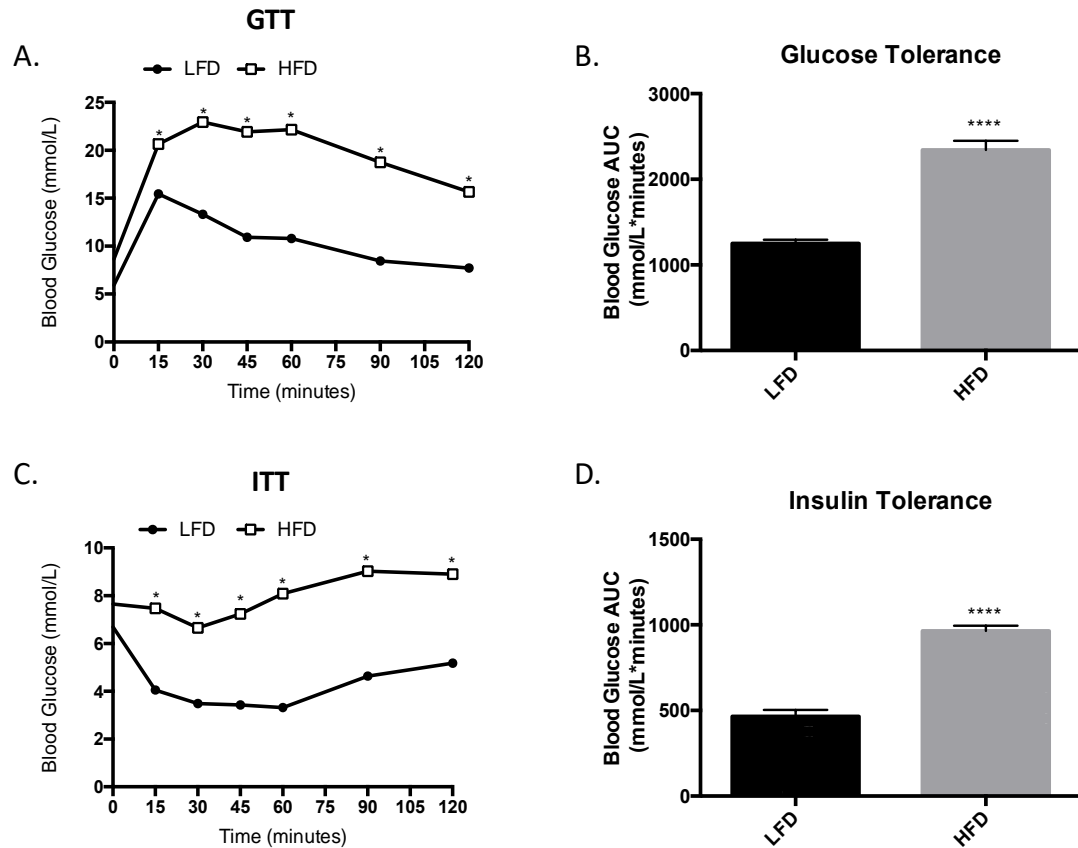


Figure 5: HFD impairs glucose and insulin tolerance. (a) Glucose tolerance test, (b) Glucose tolerance test represented as area under the curve, (c) Insulin tolerance test, (d) insulin tolerance test represented as area under the curve. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD. (\*\*\*\*) =  $p < 0.00001$  compared to LFD.

### 5.1.3 Cognitive Function

To determine the effects of a HFD and exercise on cognition, a Barnes maze test was implemented prior to end-point surgeries but after an acute bout of exercise. The Barnes maze uses visual cues and habituation/training phases to test the spatial memory of the mice. The HFD groups demonstrated greater permanence time in the incorrect zones (Figure 6a;  $p < 0.001$ ), this was measured by the amount of time the mice had spent exploring the wrong zone (away from the escape hole). The HFD groups also had a 28% higher latency time to the correct zone compared to the LFD groups (Figure 6b) however, this did not reach significance. This was measured as the time it took the mice to find the

escape hole. Although not significant, the HFD groups had a lower pass/fail rate than the LFD. The HFD only had a 79% pass rate, in which they successfully found the escape hole, compared to the LFD groups which had an 87% pass rate ( $p>0.05$ ; Figure 6c). However, there were no significant changes in cognitive performance with an acute bout of exercise (data not shown) ( $p>0.05$ ).

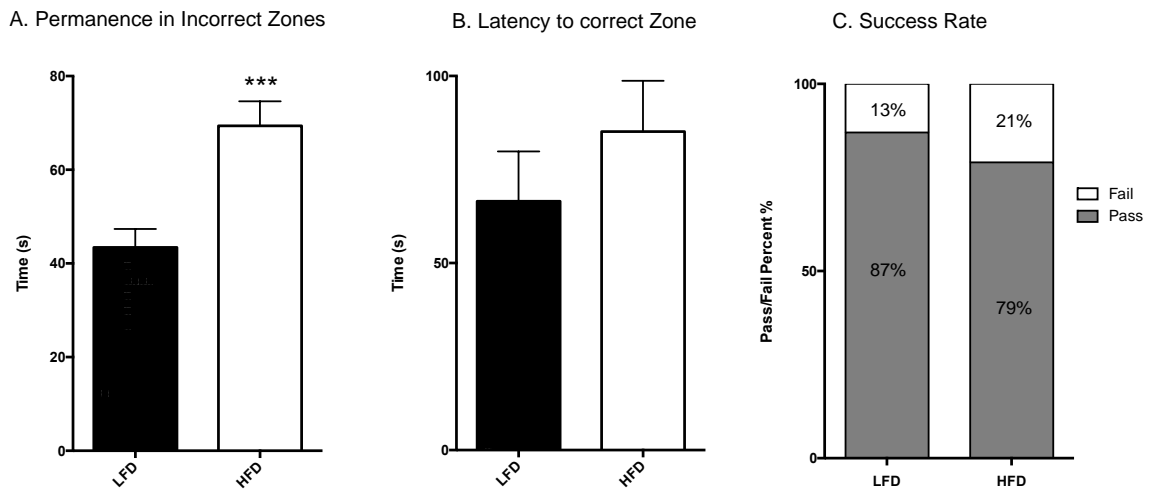
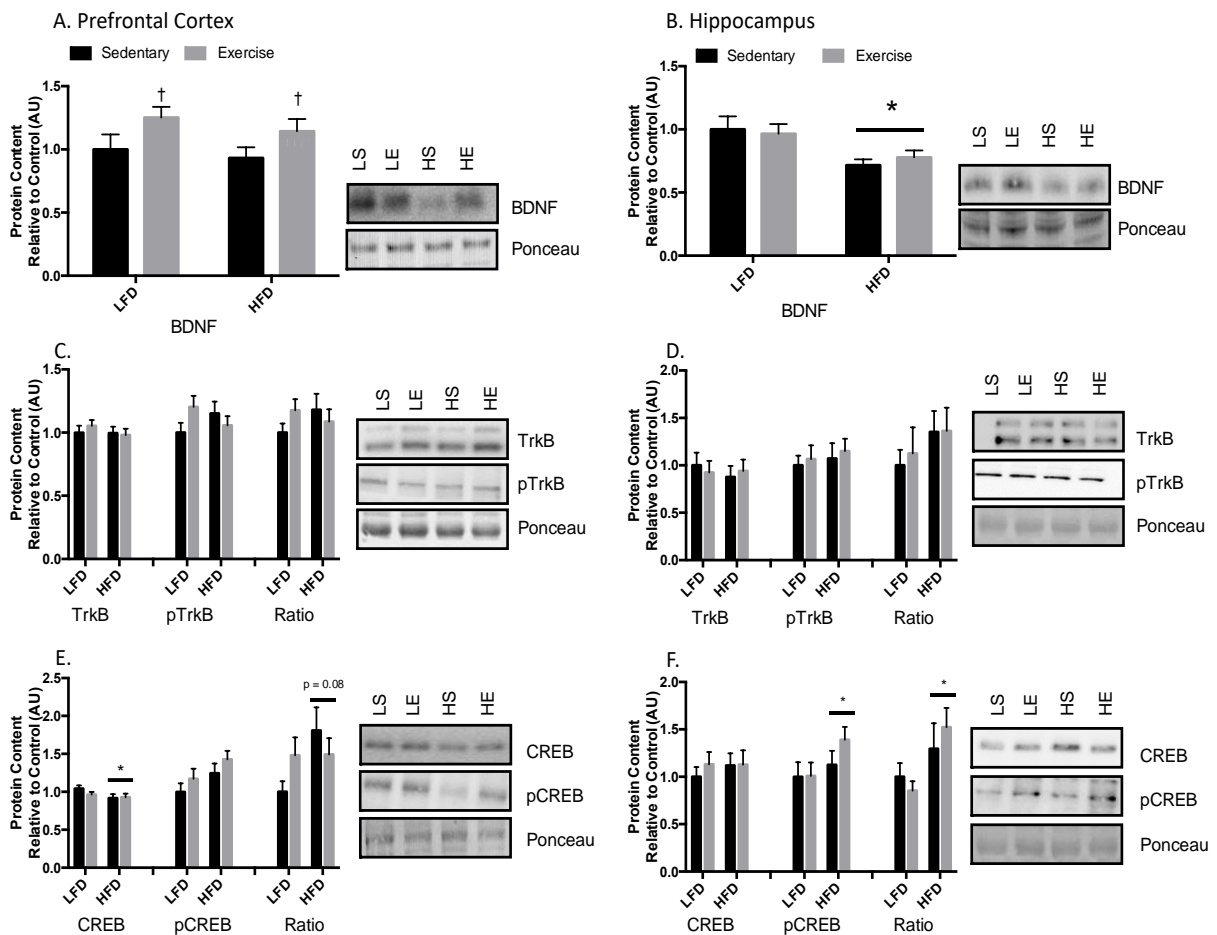


Figure 6: Barnes maze results. a) Permanence in incorrect zones, time the mice spent away from the escape hole. b) Latency to correct zone, time it took the mice to find the escape hole. c) Pass/Fail percentage, indicating whether or not the mice found the escape hole. Sedentary and Exercise groups were pooled together. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD.

### 5.1.3 BDNF Signalling

Western Blotting was used to determine the influence of a HFD and the effects of an acute bout of treadmill running on BDNF and markers of BDNF signalling. In the prefrontal cortex there was no difference in BDNF content between the two dietary interventions, however, findings demonstrate that an acute bout of treadmill running resulted in higher total BDNF protein content in both LFD and HFD groups (Figure 7a;  $p = 0.02$ ). In the hippocampus, the HFD lowered BDNF protein content relative to the LFD groups (Figure 7b;  $p = 0.003$ ), but there were no changes with the acute exercise intervention.

To determine the BDNF signalling response, its receptor TrkB and downstream effector CREB, were measured. No changes were observed in total or phosphorylated TrkB content in either the prefrontal cortex or the hippocampus in response to the HFD or acute exercise (Figure 7c; Figure 7d). In the prefrontal cortex, total CREB content decreased in the HFD groups (Figure 7e;  $p = 0.03$ ), while there were no changes in phosphorylation or in the ratio of phosphorylated to total content ( $p = 0.08$ ). In the hippocampus, there were no changes in CREB total protein content, however with the HFD there were significant increases in CREB phosphorylation and the ratio of phosphorylation to total content (Figure 7f;  $p = 0.03$ ).



**Figure 7: BDNF signalling.** A) An acute bout of exercise significantly increased BDNF total protein content in the prefrontal cortex B) A HFD significantly reduced BDNF protein content in the hippocampus C) There were no detectable changes in TrkB protein content in the prefrontal cortex D) There were no detectable changes in TrkB protein content in the hippocampus E) Total CREB content was reduced with a HFD in the prefrontal cortex F) CREB phosphorylation was higher with a HFD represented as both an absolute value and as a ratio to total content in the hippocampus. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD, (†) =  $p < 0.05$  compared to sedentary.

#### *5.1.4 Amyloidogenic Pathway*

Western blotting was used to measure total protein content of the products of the amyloidogenic pathway, as well as major enzymes involved in the pathway. BACE1 is the rate-limiting enzyme in the amyloidogenic pathway and considered to be a hallmark in AD progression, while IDE is responsible for the degradation of A $\beta$ . In the prefrontal cortex, BACE1 protein content did not change with the HFD or acute exercise intervention, however, there was significantly lower IDE content with the HFD group (Figure 8a;  $p = 0.03$ ). In the hippocampus, BACE1 and IDE protein content remained unchanged (Figure 8b).

There were no significant changes in either full length APP content or phosphorylation in the hippocampus and prefrontal cortex (Figure 8c; Figure 8d). Markers of APP cleavage were also measured in the prefrontal cortex and hippocampus (Figure 8e; Figure 8f). In the prefrontal cortex, there were no changes in CTF, sAPP $\alpha$ , or sAPP $\beta$ . In the hippocampus, there were no changes in CTF and sAPP $\beta$ , however, there was a reduction in sAPP $\alpha$  total protein content with an acute bout of exercise (Figure 8f;  $p = 0.01$ ).



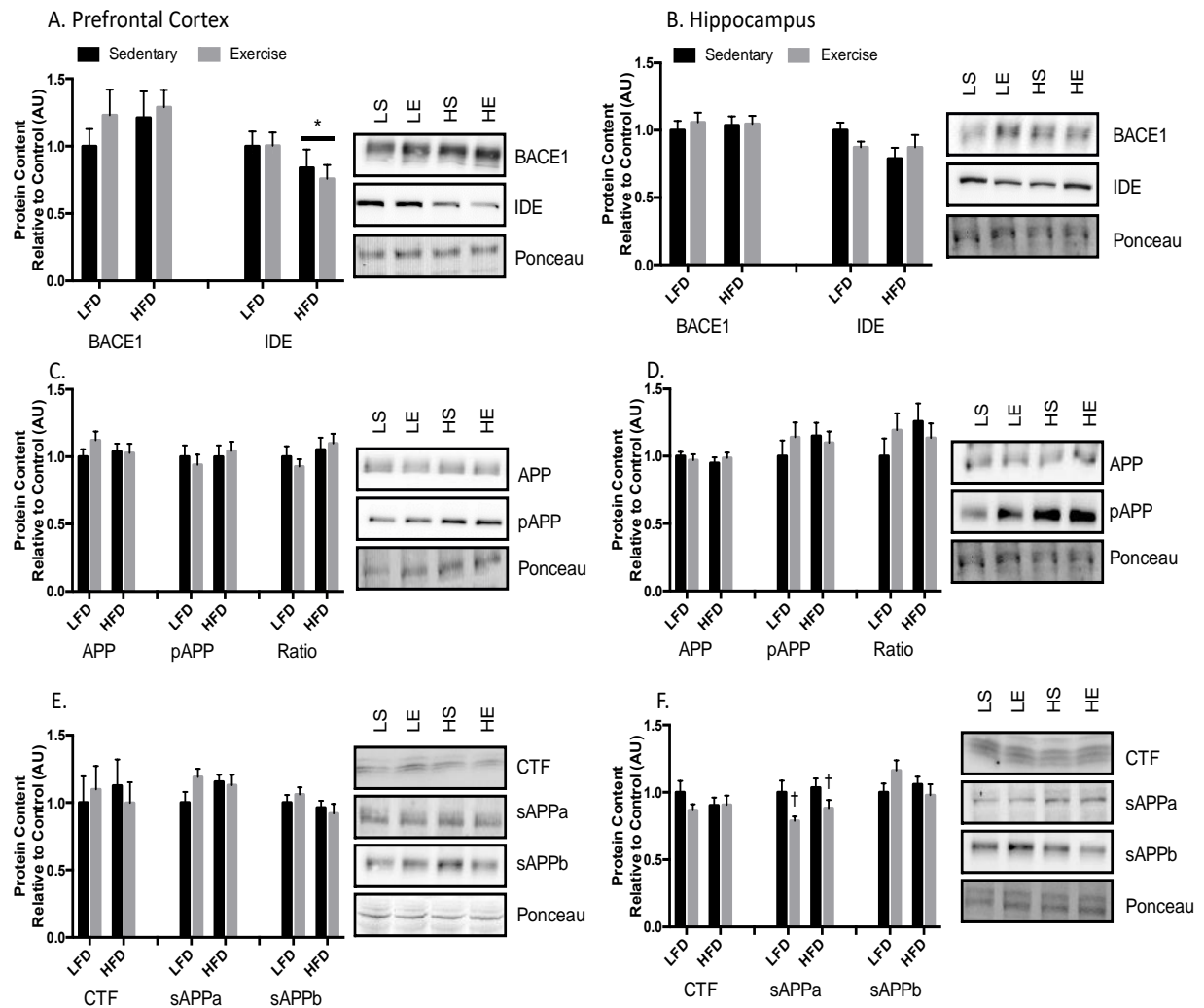


Figure 8: Amyloidogenic pathway. A) There were no detectable changes in BACE1 content but IDE content was significantly reduced with a HFD in the prefrontal cortex B) There were no detectable changes in the hippocampus C) There were no detectable changes in APP protein content in the prefrontal cortex D) There were no detectable changes in APP protein content in the hippocampus E) There were no detectable changes in the protein content of the CTF, sAPPa and sAPPb in the prefrontal cortex F) sAPPa was reduced with exercise in the hippocampus. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD, (†) =  $p < 0.05$  compared to sedentary.

### 5.1.5 Downstream Effectors of BDNF Signalling

To determine the effects of the HFD and exercise-induced increases in BDNF protein content/signalling, major downstream effectors, extracellular signal-regulated kinase (ERK) and calmodulin-dependent protein kinase 2 (CaMK2), were measured. In the prefrontal cortex, there were no changes in total ERK content (Figure 9a) however, in the hippocampus, ERK content was lower in the HFD group (Figure 9b;  $p = 0.045$ ). In

the prefrontal cortex, ERK phosphorylation and the ratio of phosphorylated to total protein was significantly reduced with a HFD, however, this reduction was recovered with a single bout of exercise (Figure 9a;  $p = 0.02$ ). In the hippocampus, there were no changes in phosphorylated ERK (Figure 9b), however, there was a statically significant change in the ratio of phosphorylated to total protein ERK, likely due to the reduction of total ERK ( $p = 0.02$ ). In the prefrontal cortex, there were no changes in total or phosphorylated CaMK2 (Figure 9c) however, in the hippocampus, there was a significantly lower CaMK2 phosphorylation (Figure 9d;  $p = 0.03$ ) with a HFD.

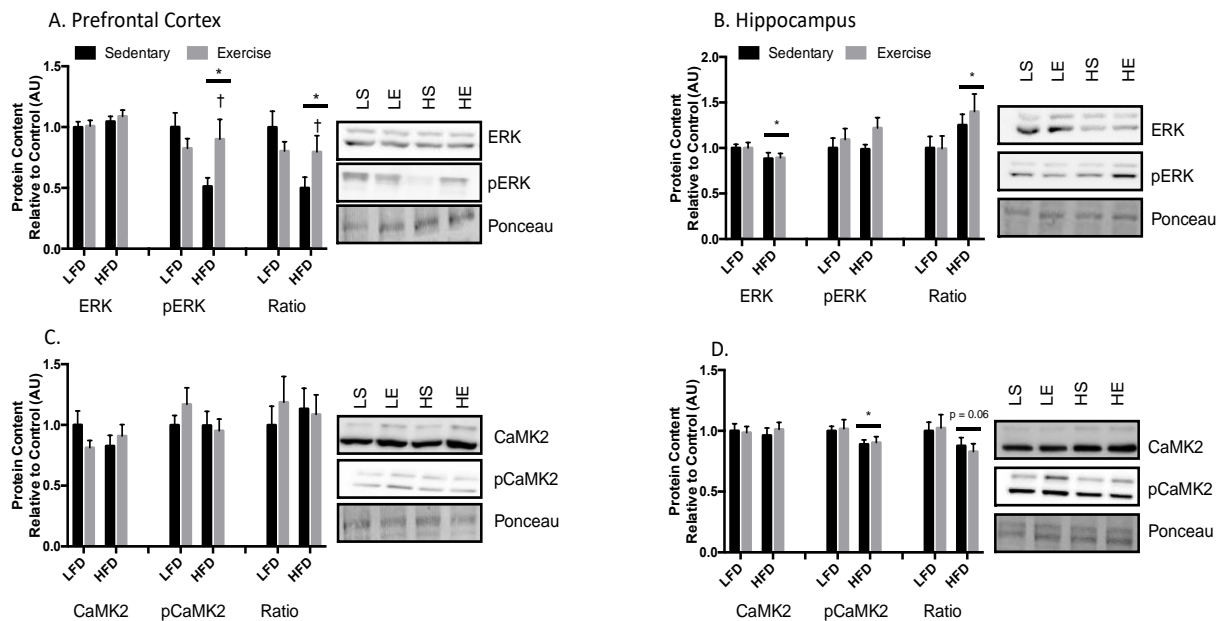


Figure 9: Downstream effectors of BDNF signalling. A) pERK was significantly lower with a HFD, however this was recovered with a single bout of exercise, represented as an absolute value and relative to total content the prefrontal cortex B) total ERK was reduced with a HFD and the ratio of pERK/ERK was significantly higher in the HFD group in hippocampus C) there were no detectable changes in protein content with CaMK2 in the prefrontal cortex D) pCaMK2 was significantly lower with a HFD in the hippocampus Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD, (†) =  $p < 0.05$  compared to sedentary.

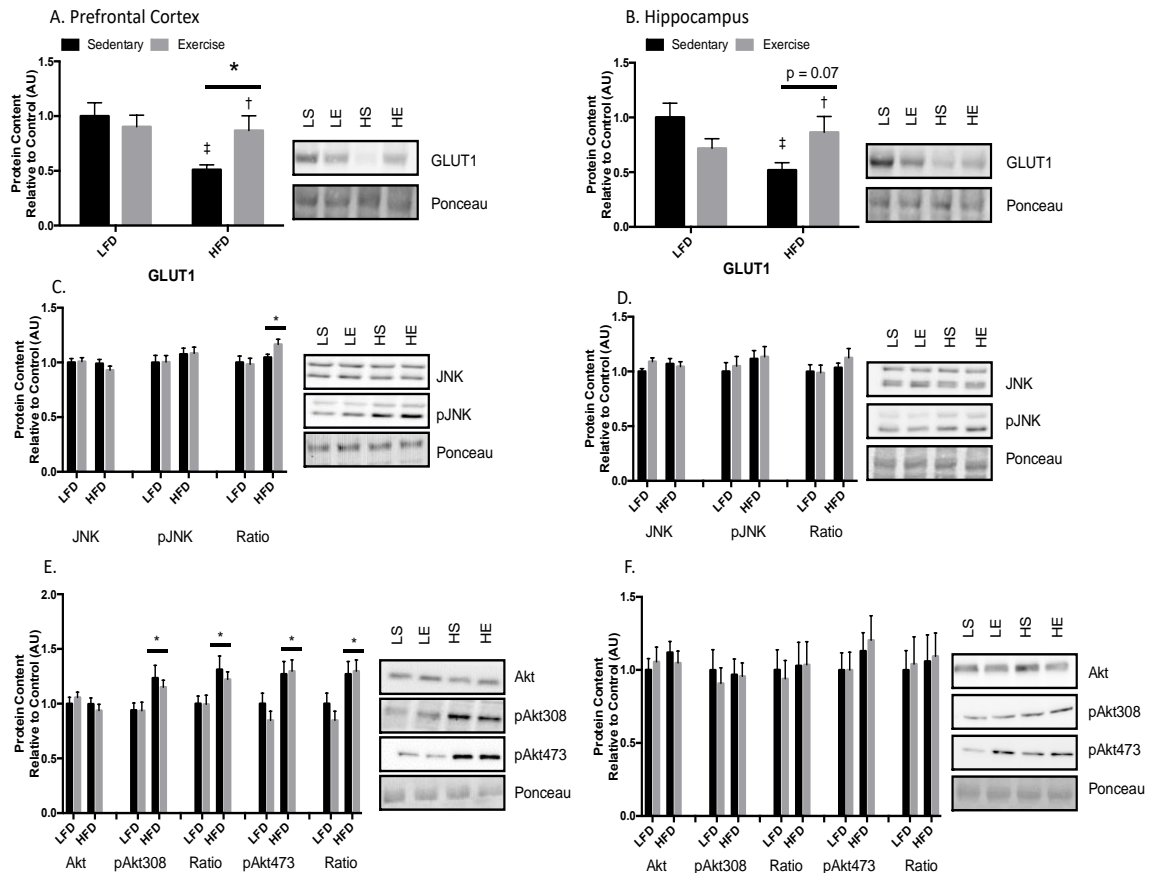
#### *5.1.6 Markers of Hypometabolism*

To further characterize the obesogenic model and the effects of acute exercise, markers of neuroinflammation, insulin signalling, and glucose uptake were measured. Glucose uptake into the brain requires a transporter in order to pass the blood brain barrier, this transporter is primarily GLUT1 (38) and down-regulation of GLUT1 in the brain is thought to contribute to the hypometabolism commonly seen in AD (39). In the prefrontal cortex, GLUT1 total protein content is lower in the HFD group when compared to LFD (Figure 10a;  $p = 0.02$ ) however, this reduction is recovered with a single bout of exercise ( $p = 0.045$ ). In the hippocampus, a HFD significantly reduced GLUT1 content in the sedentary group when compared to the LFD sedentary control (Figure 10b;  $p = 0.002$ ) as well, in the HFD groups, an acute bout of exercise significantly increases GLUT1 content ( $p = 0.015$ ).

The MAPK pathway is thought to be one of the major pathways involved in the progression of AD. MAPKs are serine-threonine kinases that mediate cellular signalling associated with various cellular activities, which include, cell proliferation, differentiation and apoptosis (210, 211). In particular, c-Jun NH<sub>2</sub>-terminal kinase (JNK), is thought to contribute to the pathology of the disease by increased neuronal apoptosis, increased activation of BACE1, and phosphorylation of APP and NFTs (26-28, 212). JNK has been identified as a crucial mediator in insulin resistance and has been shown to directly interfere with insulin signalling (213). In the prefrontal cortex, the ratio of phosphorylated to total protein JNK is significantly higher in the HFD group (Figure 10c;  $p = 0.02$ ), with no changes in total or phosphorylated JNK content or with exercise

intervention. In contrast, there are no changes in phosphorylated or total JNK content the hippocampus in response to the HFD or post-exercise (Figure 10d).

Previous work has highlighted perturbed insulin signalling via Akt phosphorylation in the brain with AD (162, 163, 214). In agreement with this, the prefrontal cortex showed higher phosphorylated Akt at the threonine 308 site ( $p = 0.038$ ) and serine 473 site ( $p = 0.026$ ) with a HFD, while total Akt content did not change (Figure 10e). Contrarily, total and phosphorylated Akt in the hippocampus remained unaltered (Figure 10f).



**Figure 10: Markers of hypometabolism.** A) GLUT1 protein content was significantly reduced with a HFD but recovered with an acute bout of exercise in the prefrontal cortex B) GLUT1 content was significantly reduced with a HFD within the sedentary group and an acute bout of exercise significantly increased GLUT1 content within the HFD groups in the hippocampus C) Ratio of pJNK to total JNK was significantly higher with a HFD in the prefrontal cortex D) There were no detectable changes in total or phosphorylated JNK in the hippocampus E) in the prefrontal cortex, pAKT at the threonine308 and serine473 site were significantly increased with a HFD F) there were no detectable changes in total or phosphorylated Akt in the hippocampus. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD, (†) =  $p < 0.05$  compared to sedentary, (‡) =  $p < 0.05$  compared to LFD sedentary.

### 5.1.7 mRNA Expression

To detect acute changes with the single bout of exercise and the long-term effect of a HFD, mRNA expression of BDNF, BACE1 and IDE were measured via RT-PCR. In the prefrontal cortex and the hippocampus (Figure 11a; Figure 11b) there were no changes in BDNF mRNA with exercise or the HFD. Similarly, there were no changes in BACE1 mRNA expression in both the prefrontal cortex and hippocampus with exercise or HFD (Figure 11c; Figure 11d). In the prefrontal cortex and hippocampus, there were no detectable changes in mRNA expression of IDE (Figure 11e; Figure 11f).

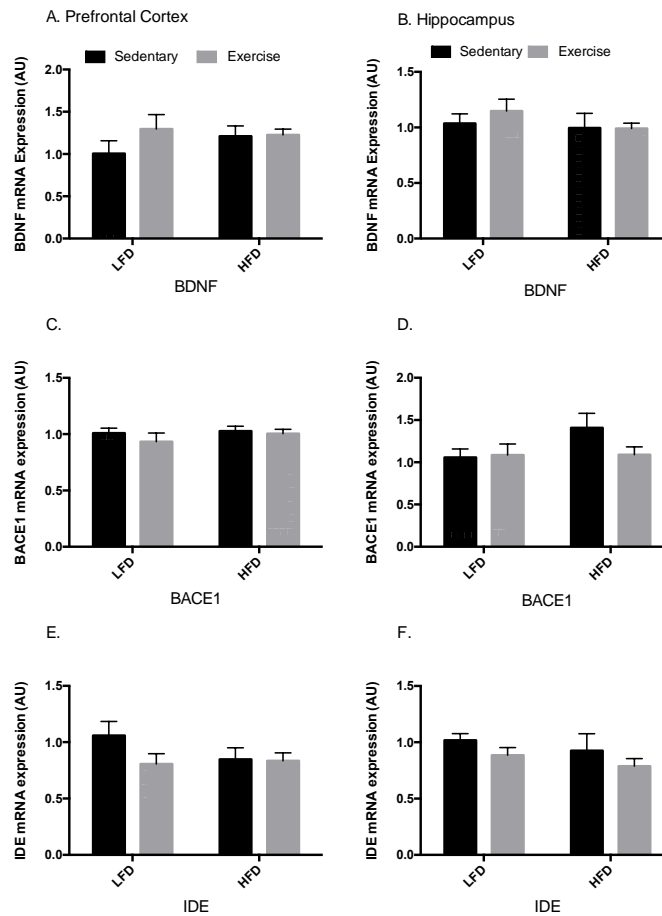


Figure 11: mRNA expression. A) There were no detectable changes in BDNF mRNA expression in the prefrontal cortex B) There were no detectable changes in BDNF mRNA expression in the hippocampus C) There were no detectable changes in BACE1 mRNA expression in the prefrontal cortex D) There were no detectable changes in BACE1 mRNA expression in the hippocampus E) There were no detectable changes in IDE mRNA expression in the prefrontal cortex F) There were no detectable changes in IDE mRNA expression in the hippocampus. Values are represented as mean  $\pm$  SEM.

## 5.2 Explant Study

### 5.2.1 Beta-secretase Activity Assay

To establish if there is a direct link between BDNF signalling and BACE1, a beta-secretase activity assay was utilized on prefrontal cortex and hippocampus samples that were treated with a dose of BDNF post-dissection. In the prefrontal cortex, BDNF treatment resulted in a significant reduction of BACE activity (Figure 12a;  $p = 0.003$ ) however, there were no changes in BACE activity in the hippocampus samples (Figure 12b;  $p = 0.12$ )

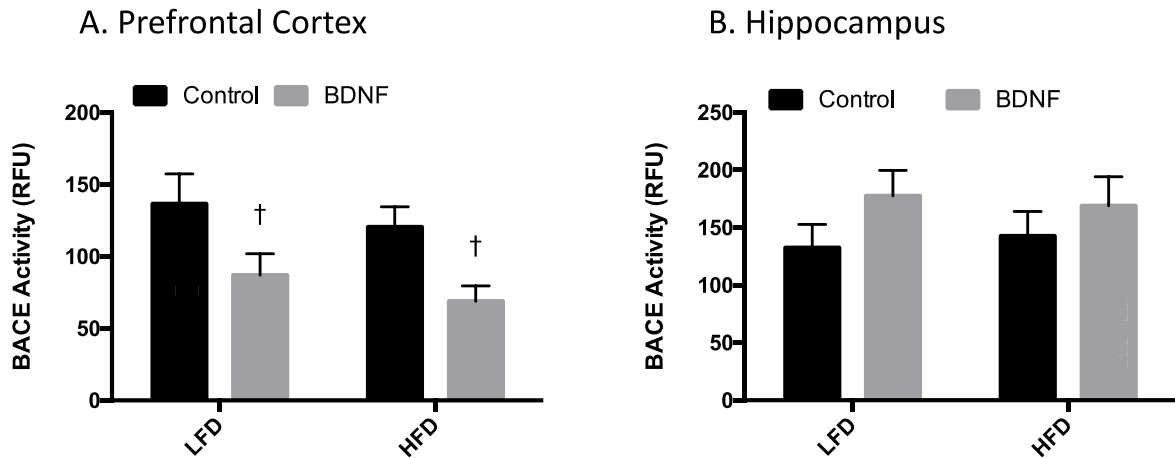


Figure 12: Beta-secretase activity assay. A) BDNF treatment significantly reduced BACE activity in the prefrontal cortex B) there were no detectable changes in BACE activity in the hippocampus. Values are represented as mean  $\pm$  SEM. Significance indicated by ( $\dagger$ ) =  $p < 0.05$  compared to sedentary.

### 5.2.1 Western Blotting

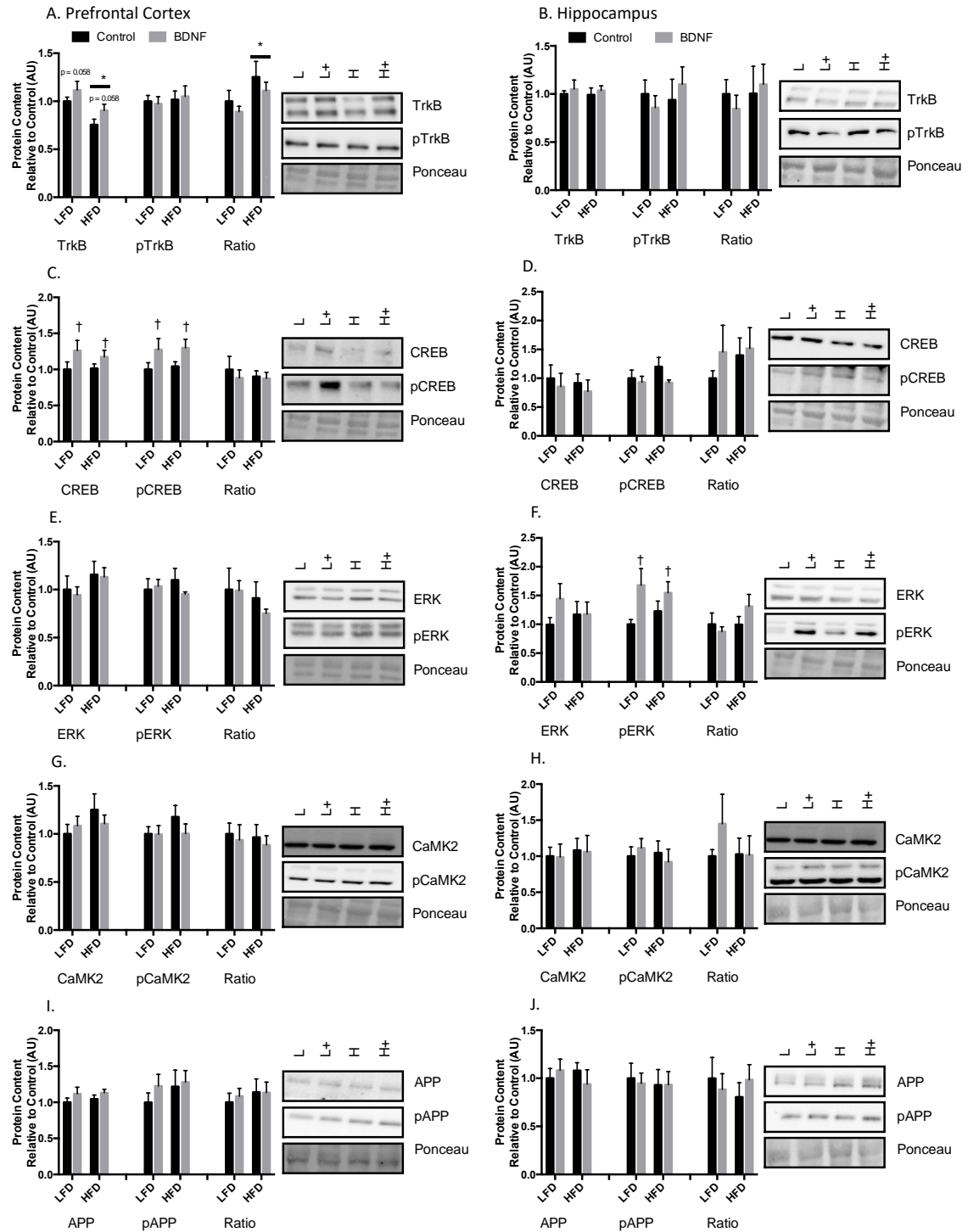
To further explore the mechanisms behind the BDNF-induced reductions in BACE1 activity, Western blotting was performed on downstream targets of BDNF and targets associated with the amyloidogenic pathway. In the prefrontal cortex, TrkB content was reduced with the HFD (Figure 13a;  $p = 0.001$ ) and treatment with BDNF

resulted in higher TrkB phosphorylation but did not reach significance ( $p = 0.058$ ). In the hippocampus, there were no changes in total or phosphorylated TrkB content (Figure 13b).

Total CREB content was significantly higher with BDNF treatment in the prefrontal cortex regardless of diet (Figure 13c;  $p = 0.045$ ), which was also accompanied by higher phosphorylated CREB ( $p = 0.025$ ). In the hippocampus, there were no changes in total or phosphorylated CREB content (Figure 13d).

In the prefrontal cortex, there were no changes in total or phosphorylated ERK with BDNF treatment (Figure 13e), however, BDNF treatment significantly increased phosphorylated ERK in the hippocampus regardless of diet (Figure 13f;  $p = 0.003$ ) with no changes in total ERK content. In both prefrontal cortex and hippocampus, there were no changes in total or phosphorylated CaMK2 with a HFD or BDNF treatment (Figure 13g; Figure 13h).

In both prefrontal cortex and hippocampus samples, there were no significant changes in phosphorylated or total APP content with a HFD or BDNF treatment (Figure 13i; Figure 13j).



**Figure 13: Explant Western blotting.** A) There is a significant decrease in total TrkB content in the HFD, while treatment with BDNF increased but not statistically significant in the prefrontal cortex B) There are no detectable changes in total or phosphorylated TrkB in the hippocampus C) BDNF treatment resulted in significantly higher total and phosphorylated CREB in the prefrontal cortex D) there were no detectable changes in CREB with the hippocampus E) No detectable changes in ERK in the prefrontal cortex F) BDNF treatment increased pERK content in the hippocampus G) there were no detectable changes in CaMK2 in the prefrontal cortex H) there were no detectable changes in CaMK2 in the hippocampus I) there were no detectable changes in APP in the prefrontal cortex J) there were no detectable changes in APP in the hippocampus. Values are represented as mean  $\pm$  SEM. Significance indicated by (\*) =  $p < 0.05$  compared to LFD, (†) =  $p < 0.05$  compared to sedentary.



## **Chapter 6: Discussion**

This thesis provides a novel examination of the potential link between exercise-induced BDNF and BACE1 regulation. In the first study, the prefrontal cortex and hippocampus were examined in response to chronic high fat feeding and an acute bout of exercise. The aim of this study was to explore the efficacy of using exercise to increase BDNF protein content and to blunt or reverse the potential impairments in BDNF signalling from HFD-induced obesity. In the second study, explants of prefrontal cortex and hippocampus tissue were treated with BDNF, in the absence of exercise intervention, in order to determine whether or not a direct link between BDNF and BACE1 regulation exists. To the best of our knowledge, this is the first study to demonstrate a direct effect of BDNF treatment on BACE1 activity and provides further novel insight into the potential mechanisms responsible for previously shown exercise-induced reductions in BACE activity.

### **6.1 Exercise Study**

#### ***6.1.1 HFD Impairments***

The exercise study was conducted to determine the efficacy of using an acute bout of exercise to increase BDNF content and signalling, as well as to confirm the effects of HFD-induced obesity on BDNF signalling and markers of early AD progression. High fat feeding resulted in numerous impairments that are associated with early progression of AD. In our model, we demonstrated that chronic high fat feeding resulted in whole body glucose intolerance and insulin resistance as well as impairments in cognitive ability/spatial memory. This is in agreement with previous literature showing that a HFD

can lead to insulin resistance and is linked to AD (11, 12, 30-33), as well as HFD-induced impairments to cognitive ability and memory (99, 107, 116). Furthermore, recent work in our lab has shown comparable results with female C57BL/6J mice (Hayward et al. manuscript in preparation), which displayed glucose and insulin intolerance and impaired in spatial memory indicated by increased latency to correct zones through a Barnes maze. Interestingly, HFD-induced obesity led to impairments in A $\beta$  degradation via reduced levels of IDE, a key enzyme responsible for the clearance of A $\beta$ . Other findings from our lab (Hayward et al. manuscript in preparation) support this result by demonstrating that HFD-induced obesity in female mice led to reduced IDE content in the hippocampus and that there was reduced IDE activity. HFD-induced obesity also led to reductions in brain glucose transporters, neuroinflammation, and perturbed insulin signalling. These findings are in agreement with previous work that has highlighted obesity/high fat feeding as a significant risk factor for neuroinflammation, brain hypometabolism and cognitive impairments, all of which have been attributed to neurodegenerative progression (215).

Our findings also indicted that there were impairments in BDNF content and signalling, specifically we saw reductions in total BDNF content, its receptor TrkB and downstream targets CREB, ERK and CaMK2. BDNF, TrkB and CREB content reductions have also been demonstrated in previous work done in our lab (181). Growing evidence suggest that decreases in BDNF and defects in its signalling pathway via its receptor, TrkB, are associated with neurodegeneration and progression of AD (139). Collectively, these findings suggest that the consequences of HFD-induced reductions in BDNF could be an early contributor to AD progression and interventions of recover

BDNF signalling could be used as a therapeutic target.

#### *6.1.2 Increased BDNF Content with an Acute Bout of Exercise*

Previous work in our lab has shown that an acute bout of exercise significantly reduced BACE activity in the prefrontal cortex in a C57BL/6J obesogenic mouse model (111), however the mechanism behind this remained elusive. In the current study using the same model, we have demonstrated that a single bout of exercise results in higher levels of BDNF total protein content in both LFD and HFD groups in the prefrontal cortex. To strengthen the argument that there may be a potential link between BDNF and BACE1 regulation, it was imperative to show, in the same model, that exercise elicited increases in BDNF levels (current study) and reductions in BACE activity (111)). Using exercise to increase BDNF content is heavily supported in the literature (139, 153, 181, 185-188), however, it was important to validate this in our model to show BDNF signalling was still recoverable despite the detriments from chronic high fat feeding. Our work not only highlights that an acute bout of exercise can increase BDNF content but also that it can recover ERK phosphorylation, GLUT1 content and reduce sAPP $\alpha$  content in an obesogenic model.

HFD-induced decreases in ERK and pERK have been previously noted (215), while dysregulation of the ERK pathway has been linked to AD progression (163, 216). ERK is downstream of BDNF and plays a role in synaptic plasticity, memory, and is thought to facilitate these effects via the phosphorylation of CREB (41). ERK plays a pivotal role in BDNF signalling and eliciting its neuroprotective effects, thus it is promising to show that an acute bout of exercise is able to recover ERK phosphorylation

in the prefrontal cortex.

As previously mentioned, brain hypometabolism, specifically brain insulin resistance and impaired glucose uptake, is a characteristic of AD (215). Glucose uptake into the brain requires a transporter to pass the blood brain barrier, this transporter is primarily GLUT1 (38). However, GLUT1 has been found to be downregulated with AD and with high-fat feeding (39, 215). Interestingly, our results demonstrate that high fat feeding-induced reductions of GLUT1 levels can be recovered with a single bout of exercise (Figure 10a,b). Although this is unlikely facilitated by the increased BDNF seen with exercise, it does further highlight exercise as a therapeutic intervention at preventing/combating early AD progression.

A recent study demonstrated that 3 weeks of voluntary exercise resulted in a significant increase in sAPP $\alpha$  content, suggesting that exercise can drive APP processing towards the non-amyloidogenic pathway (217). sAPP $\alpha$  is putatively neuroprotective and lower levels of it have been observed in AD patients (218, 219). Contrarily, several lines of evidence have shown that AD patients have elevated levels of sAPP $\alpha$  and that it may contribute to disease progression via microglia activation (220-222). Additionally, over-abundance of sAPP $\alpha$  has been shown to lead to tumorigenesis and neuroinflammation through activation of the MAPKs pathway (222-224). Neuroinflammation has been shown to lead to AD progression through increased neuronal apoptosis, increased activation of BACE1, and phosphorylation of APP and NFTs (26-28, 212). Contrary to Nigam et al. our results demonstrate that an acute bout of exercise lowers sAPP $\alpha$  content in an obesogenic model, highlighting an ability to alter sAPP $\alpha$  content, however, given

these varying results, it can be speculated that time and duration may play a large role in the effect of exercise on sAPP $\alpha$  content.

Our lab has demonstrated that an acute bout of exercise is able to reduce BACE activity (111), here this study highlights the capability of exercise to increase BDNF content using the same model. Collectively, to our knowledge, these are the first studies to show increases in BDNF content (current study) and reductions in BACE activity (111), in the same model, as a result of an acute bout of exercise. Despite encouraging associations, further work was needed to determine if there was a direct link between the exercise-induced increases in BDNF content and the reductions in BACE activity.

## 6.2 Explant Experiment

### 6.2.1 Treatment with BDNF Reduces BACE Activity

The explant study was performed to further expand our previous findings and determine if there was a direct link between BDNF and BACE1 regulation. We found that direct treatment with BDNF resulted in a significant reduction in BACE1 activity in the prefrontal cortex. This novel finding demonstrates the effect of BDNF on reducing BACE1 activity, independent of other alterations from an exercise stimulus. Moreover, this finding shows for the first time, that there is a direct link between BDNF signalling and BACE1 regulation in this region of the brain. Being the rate-limiting enzyme in the amyloidogenic pathway and playing a large role in the production of the neurotoxic intermediate, A $\beta$ , aberrant BACE1 activity is considered pivotal to AD progression (17, 18, 71-73). Due to the normal physiological roles of BACE1 in the brain, inhibiting BACE1 results in fatal phenotypes and outcomes, thus an approach to modulate BACE

activity, rather than inhibiting it, is crucial. Our study highlights that 1) exercise is a valid intervention for elevating brain BDNF content and 2) perhaps more interestingly, that the mechanisms behind exercise-induced reductions in BACE1 activity are possibly due to elevated BDNF content, as we have shown treatment with BDNF results in reductions of BACE1 activity similar to what is observed post-exercise (111).

Surprisingly, there were no changes in BACE activity with BDNF treatment in the hippocampus. This result is supported by the Western blot analysis of the explant tissue showing no detectable changes in TrkB, CREB and pCREB. In addition to these findings, the exercise study that showed no changes in BDNF content and signalling with exercise in the hippocampus. These findings indicate that for an unknown reason, hippocampal TrkB was unresponsive, at this time point, to explant BDNF treatment and with exercise intervention. Preliminary work in our lab has shown that TrkB total protein content is significantly lower in the hippocampus when compared to the prefrontal cortex (data not shown;  $p = 0.008$ ). The reduced content of TrkB in the hippocampus may have influenced the responsiveness to BDNF treatment and explain the lack of response to exercise. The exercise and explant results highlight the importance of studying brain regional differences. It is possible that the hippocampus needed either a longer intervention, incubation time or recovery time to elicit a response, however, because the mechanisms that link BDNF to BACE1 remain elusive, it is difficult to explain this particular difference in brain region response.

The prefrontal cortex findings are complimentary to a recent study by Choi et al. (225) who explored the benefits of exercise in an AD model on cognition. Choi et al. found that by combining the promotion of neurogenesis and elevating BDNF levels in 5x

FAD mice (a familial model of AD), they were able to mimic the beneficial effects of exercise with regards to the improvement of cognitive ability and spatial memory (225). These results in conjunction with our findings that direct BDNF treatment mimics exercise-induced reductions in BACE1 activity, suggests that BDNF is an apparent player behind the therapeutic effects of exercise on BACE1 regulation and impaired cognition. Furthermore, these findings indicate that BDNF may be a promising avenue in the development of drug therapies in the prevention or treatment AD.

#### *6.2.1 Treatment with BDNF Increased Downstream Targets*

Now that a direct link between BDNF treatment and reductions in BACE1 activity has been established, it is necessary to understand how BDNF may mediate these effects on BACE1. In efforts to address this, Western blotting was performed using the remaining explant samples. In the prefrontal cortex, the region BACE1 activity was reduced with BDNF treatment, we observed elevated levels of TrkB content ( $p = 0.058$ ) despite initial impairments by the HFD. Additionally, BDNF treatment resulted in significant increases in CREB and pCREB content, however, there were no changes in BDNF's downstream effectors ERK and CaMK2. This suggests that BDNF downstream signalling potentially plays a role in BACE1 modulation but not through its downstream targets ERK and CaMK2. Interestingly, there were no changes in APP or pAPP content with treatment of BDNF, which may indicate that BDNF is not altering BACE1 activity via modification to the precursor protein but rather acting on BACE1 directly.

Complimentary to the activity assay results, there were no changes in BDNF signalling, TrkB and CREB, with BDNF treatment in the hippocampus, however there

was a significant increase in pERK. Despite ERK being downstream of TrkB signalling, ERK has been shown to be activated by BDNF via mechanisms other than this (226). Specifically, Easton et al. demonstrated that blocking TrkB did not eliminate BDNF-induced activation of ERK. Furthermore, they showed that BDNF induced phosphorylation of fibroblast growth factor receptor substrate 2, which in turn associates with growth factor-receptor binding protein 2. This interaction has been shown to be able to activate the Ras/MAPK pathway (226), thus explaining the increase in pERK seen with BDNF treatment in the hippocampus, despite no detectable changes in TrkB.

These results in the hippocampus may help explain why BDNF treatment did not reduce BACE activity. For reasons unknown, TrkB was seemingly unresponsive to BDNF treatment. Although it remains elusive as to why there are brain regional differences to BDNF treatment, this finding is valuable as it indicates that activation of downstream signalling targets may be required to mitigate BACE1 activity. Taken together, Western blot results reveal that treatment with BDNF in the prefrontal cortex elicits an increase in the content and phosphorylation of its downstream target CREB and subsequent activation of this pathway may be required to mitigate BACE activity. On top of this, our results suggest that BDNF signalling is likely acting on BACE1 directly, rather than altering the phosphorylation of APP.

Given this, understanding the mechanisms behind BACE1 regulation is key in elucidating how BDNF signalling modifies this pathway. BACE1 can be regulated through various post-translational modifications including: palmitoylation, acetylation, as well, through phosphorylation (227). Palmitoylation of BACE1 occurs at cysteine residues and results in the localization of BACE1 to lipid rafts (227, 228). Although



palmitoylation is seemingly required for BACE1 localization to lipid rafts, it is suggested that BACE1 cleavage of APP occurs mainly in nonlipid rafts (227). Indicating that palmitoylation likely regulates BACE1 activity with regards to substrates other than APP (229, 230) and therefore is unlikely the link between BDNF and BACE1. BACE1 has also been shown to be acetylated on seven lysine residues of the N-terminal region mediated through two acetyltransferases: ATase1 and ATase2 (231). These acetyltransferases are thought to regulate the level of BACE1 and interestingly, are upregulated in the brain of AD patients (232). Ding et al. demonstrated that by inhibiting ATase1 and ATase in neurons and glial cells, there was a significant downregulation in expression and activity of BACE1. Although these findings highlight ATase1 and ATase2 inhibition as being a potential therapeutic target for downregulating BACE1, to the extent of our knowledge, there is no known connection between ATase1/ATase2 and BDNF signalling.

Finally, and most relevant to our findings, BACE1 can be phosphorylated at two different phosphorylation sites. One of which is the Ser498 site in the cytoplasmic domain, however, phosphorylation at this site does not appear to influence A $\beta$  production (233) but rather play a role in intracellular trafficking (234). More importantly, the other major phosphorylation site is at Thr252. Recent work has demonstrated that BACE1 can also be phosphorylated at the Thr252 site by the p25/Cdk5 complex (235). Song et al. showed that p25/Cdk5 phosphorylation of BACE1 increased its activity *in vitro*, whereas phosphorylation-defective mutant BACE1 resulted in lower activity and A $\beta$  production compared to wild-type BACE1 (235). Furthermore, they demonstrated that protein content of phospho-BACE1 was significantly higher in the brains of patients with AD

when compared to control patients, suggesting that elevated levels of phospho-BACE1 are linked to AD pathology. In corroboration of these findings, other studies have shown that Cdk5 and p25 levels are significantly higher in AD brains (236), p25/Cdk5 complex is involved in BACE1 transcription (237) and that it is major tau kinase (238). Collectively, these findings suggest that the p25/Cdk5 complex plays a fundamental role in BACE1 regulation and could be a key target for therapeutic approaches and could perhaps provide a link between BDNF and BACE1.

Cdk5 is thought to be the master regulator of synaptic plasticity, having a plethora of functions/roles including: neuronal migration, neuronal differentiation, synapse development and function neuronal survival, learning and memory formation, pain signalling, drug addiction and long-term behavioral changes (239-242). Cdk5 is able to elicit these neuroprotective effects by the binding to its protein partner, p35, however this interaction is tightly regulated as dysregulation of Cdk5 has been linked to several neurodegenerative diseases (243). More specifically, an issue arises when the truncated form of p35, p25, binds to Cdk5 instead. This interaction creates several neurotoxic conditions, but of particular interest, the p25/Cdk5 complex is able to phosphorylate BACE1. Due to its vital physiological roles, inhibition of Cdk5 is not an option, however, a recent paper has identified a specific fragment of p35 referred to as the Cdk5 inhibitory peptide, which displayed an inhibitory effect of p25/Cdk5 activity without impairing normal p35/Cdk5 activity (244). Thus, upregulation of p35 could possibly provide a feasible means of inhibiting p25/Cdk5-induced phosphorylation of BACE1.

Cdk5 and p35 are upregulated by BDNF and BDNF signalling (245, 246), which provides us with a potential mechanism to explain how BDNF treatment is reducing

BACE1 activity. It is conceivable that BDNF is upregulating Cdk5 and more importantly, p35, thus leading to the inhibition of p25/Cdk5 activity, which then prevents the phosphorylation of BACE1. However, this is just speculation and future work should further examine these particular pathways to determine if this has any merit.

### 6.3 Conclusions

In conclusion, this thesis provided novel information regarding the mechanistic link between BDNF and BACE1 regulation. 1) This study demonstrated high fat feeding-induced impairments to BDNF signalling as well, cognition, brain metabolism and glucose homeostasis, which have all been shown previously (215). 2) Furthermore, this study highlights that BDNF levels are elevated with a single bout of exercise in an obesogenic model and that exercise can recover HFD-induced impairments to BDNF signalling. The exercise study demonstrated exercise-induced increases in BDNF within the same model that showed exercise-induced reductions in BACE activity (111).

3) More interestingly, this is the first study to elucidate a direct link between BDNF and BACE1. We have shown that with BDNF treatment, in the absence of exercise, BACE activity is significantly reduced. The information provided by our study contributes to our understanding of the underlying mechanisms behind APP processing and more specifically the mechanisms behind modulating BACE1 activity. This is valuable as it will allow the possibility of designing and developing evidence-based preventative or therapeutic interventions, both by using exercise programs and pharmacologically approaches, for populations that cannot exercise or at a greater risk of AD.

Future research should focus on uncovering the exact mechanisms behind BDNF-induced reductions in BACE activity. Of particular interest is to further explore the Cdk5 pathway and its role in BACE1 regulation. Cdk5 presents a potential link between BDNF signalling and BACE1 regulation with its roles in BACE1 phosphorylation and being a major tau kinase, as well, being regulated by BDNF and BDNF signalling. Elucidating this mechanism is crucial as it will further our knowledge on BDNF's neuroprotective effect and its role in BACE1 regulation and allow the development of future therapeutic interventions.

## Chapter 7: Appendix

### Appendix A



Animal Care Committee (ACC)  
Chair - Paula Hunter, PhD 905.688.5550 ext 5884  
Council Members - Dr. Alissa Kar  
Animal Care Committee Coordinator - Doree Carbone, PhD 905.688.5550 ext 5820

Date: June 22, 2017

Dear Dr. MacPherson,

The Animal Care Committee has approved your "Animal Use Protocol (AUP)" entitled:

**The effects of a high fat diet and acute exercise on brain health in male C57BL/6J mice.**

This approval expires in one year on the last day of the month.

The number for this project is **AUP # 17 - 06 - 02**.

This number must be indicated when receiving animals for this project.

#### ANIMALS APPROVED:

Mouse	C57BL/6J	Wild type	72 M
Mouse	B6.129Sv-Bal <sup>c</sup> /J	BDNF heterozygote mice	48 M

#### REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

  
Jim Wilkwerth, Chair of ACC

**THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY  
AND IS SUBJECT TO POST APPROVAL MONITORING.**

**ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO  
ANIMAL CARE SERVICES STAFF IMMEDIATELY.**

## Appendix B



### Animal Facility

*For Internal Use*

*Only*

<b>Rodent Glucose Tolerance Test</b>		Document No: <i>SOP-</i> _____	
		New <u>  X  </u> Revised <u>    </u>	Version No. <u>    </u>
Issuing Date: dd/mm/yy	No. of attachments: <u>    </u>	Effective Date: dd/mm/yy (updated with revisions)	

### 1.0 BACKGROUND

The glucose tolerance test measures the clearance of an intraperitoneally injected glucose load from the body. It is used to detect disturbances in glucose metabolism that can be linked to human conditions such as diabetes or metabolic syndrome. Animals are fasted for approximately 6 hours, fasted blood glucose levels are determined before a solution of glucose is administered by intra-peritoneal (IP) injection. Subsequently, the blood glucose level is measured at different time points during the following 2 hours.

### 2.0 PURPOSE

This procedure is being standardized and implemented to achieve the following purposes:

1. to determine clearance of an intraperitoneally injected glucose load from the body.
- This SOP discusses the materials required and the method used to ipGTT in the rodent. This is a survival procedure.

### 3.0 APPLICABILITY

This procedure is applicable to mice and rats.

### 4.0 DEFINITIONS (Optional)

### 5.0 RESPONSIBILITIES

Rebecca MacPherson has extensive experience with this procedure and will perform and train her students in the procedure. It is the responsibility of the researchers to seek out proper training through the Animal Care Committee (ACC) in the techniques described in this Standard Operating Procedure (SOP). The researchers and the animal health care staff share the responsibility of post-testing monitoring and ensuring the animal's well-being.

## 6.0 MATERIALS AND SUPPLIES (Optional)

- 6.1 A pair of scissors
- 6.2 Glucometer
- 6.3 Timer
- 6.4 Insulin injection syringes (Note: for rats or mice weighing 30g or more a 1ml syringe is needed as the glucose dose will bypass the volume of the 300U insulin syringes)
- 6.5 Table to record values and a pen

## 7.0 PROCEDURES/FOCUS OF SOP

- 7.1 Give the mice a **clean** cage (HARDWOOD BEDDING), and begin fasting them (**6 hour fast**) with water ad lib.
- 7.2 Weigh each mouse (weight will determines the amount of glucose to inject).
- 7.3 Glucose solution: (\*for rats 50% glucose solution and multiply body mass by 4 in ul)
  - 7.3.1 20% glucose : 9 mL H<sub>2</sub>O + 2g D-glucose. Then QS to 10 mL with H<sub>2</sub>O. This dilution will get 0.2g/ml.
  - 7.3.2 Multiply the body weight in g by 10 to obtain a desired dose of 2g/kg in µl volume. (i.e. For a mouse weighing 30g, need to inject glucose: 30g x 10 = 300ul.). **Need 1ml syringe**, bypass the volume of 300U insulin syringes.
- 7.4 Set up your bench with the followings:
  - A pair of scissors to cut the tails;
  - 2 glucometers; timers; insulin injection syringes;
  - Table of mice for recording keeping of values and pens;
- 7.5 Calculate the dose of glucose required for each mouse.
- 7.6 At the end of the 6 hour fast, when you are completely set up and ready to begin, cut the tip of the tail, **measure blood glucose** (0), and then inject the mouse with the diluted glucose following the steps below:
  - Fill the syringe with the correct dose of diluted glucose. Fill your syringe for **ALL** cages first (1 person can safely handle 12 mice or 8 rats for one ipGTT at once).

7.7 Glucose injection intraperitoneal: Grab the mouse by the neck (skin) and hold it tight with the tail wrapped around the small finger from front to back. Have the mouse tilted towards lying position (the mouse relaxes more in this position). Have an insulin syringe with glucose solution ready and inject 2g/kg body weight. Hold the needle parallel to the lower abdomen (right or left) and place it at a flat angle through the abdominal wall into the intraperitoneal space. **Slowly** inject the glucose solution. Remove the needle and let the mouse go back into the cage. Measure the blood glucose after 15, 30, 45 and 60 min, 120min.

7.8 As soon as you inject the mouse start your timer counting up from 0:00 and record the time and blood glucose.

7.9 Continue these steps until you have taken blood glucose and injected all of your mice.

**Make sure you record all injection times and the blood glucose for each mouse.**

## 8.0 Revision History

Revision	Reason(s) for Revision	Date	Action by

## 9.0 References / Associated Materials (Optional)

Frendo-Cumbo S, **MacPherson REK**, Wright DC. Beneficial effects of resveratrol and metformin combination therapy in treating diet-induced insulin resistance. *Physiological Reports*; 2016 Aug;4(15).

**MacPherson REK**, Gamu D, Frendo-Cumbo S, Castellani L, Kwon F, Tupling AR, & Wright DC. Sarcolipin knockout mice fed a high fat diet exhibit altered indices of adipose tissue inflammation and remodelling. *Obesity*: 2016 Jul;24(7):1499-505. (**Editor's Choice**)

Snook LA, **MacPherson REK**, Monaco C, Frendo-Cumbo S, Castellani L, Peppler WT, Andersen ZG, Buzelle SL, Leblanc PJ, Holloway GP, Wright DC. Prior Exercise Training Blunts Short Term High Fat Diet Induced Weight Gain and Glucose Intolerance. *Am J Physiol Regul Integr Comp Physiol*: April 13 2016

**MacPherson REK**, Beaumeister P, Peppler W, Wright DC, Little JP. Reduced cortical BACE1 content with one bout of exercise is accompanied by declines in AMPK, AKT, and MAPK signaling in obese, glucose intolerant mice. *J Appl Physiol* 2015; Nov 15; 119:10; 1097-1104.



## Appendix C



Only

### Animal Facility

*For Internal Use*

<b>Rodent insulin tolerance test (ipITT)</b>	Document No: <u>SOP-TECH17</u>	
	New <u>X</u> Revised <u>    </u>	Version No. <u>00</u>
Issuing Date: 24/01/17	No. of attachments: <u>0</u>	Effective Date: dd/mm/yy (updated with revisions)

#### 10.0BACKGROUND

An insulin tolerance test is designed to determine the sensitivity of insulin responsive tissues by measuring blood glucose values after an injection of insulin.

#### 11.0PURPOSE

This procedure is being standardized to determine insulin sensitivity in mice and rats.

This SOP discusses the materials required and the method used to perform an intraperitoneal insulin tolerance test (ipITT) in mice and rats. This is a survival procedure.

#### 12.0RESPONSIBILITIES

It is the responsibility of the Principal Investigator (PI) to ensure that all students are appropriately trained in the procedure.

The researchers and the animal care staff share the responsibility of post-approval monitoring and ensuring the animal's well-being.

#### 13.0MATERIALS AND SUPPLIES

- a pair of surgical scissors;
- glass bead sterilizer;
- glucometer and test strips;
- timer;

- insulin injection syringes (29 gauge x 1/2" length). Note: for rats or mice weighing 30g or more a 1ml syringe is needed as the glucose dose will exceed the volume of the 300U insulin syringes;
- table to record values and a pen.

#### 14.0 PROCEDURES/FOCUS OF SOP

- 14.1 Weigh each rodent (weight will be used to determine the amount of insulin to inject).
- 14.2 Prepare the insulin injection. The stock solution is 100U/ml bottle of human insulin (Humulin R). Dilute insulin 1 : 400 (25 µl of this stock to 10 ml of saline) in saline or water or PBS to prepare for injection. Prepare the insulin solution **shortly** before the test. This dilution will result in 0.025U/100µl injection.
- 14.3 Calculate the dose of insulin required for each rodent:  
Multiply body weight in grams by 3 to obtain a desired dose of 0.75U/kg in µl volume. e.g. For a mice weighing 30g, need to inject: 30g x 3 = 90 µl. For small lean rodents (<20g) the dose may have to be reduced to 0.5 U/kg.
- 14.4 **Use a U300 insulin syringe:** the total volume of 30 units equals 300 µl.
- 14.5 Make up a 10% glucose solution for injection to treat mice who may develop hypoglycaemia (<20mg/dl). (10% glucose: 9 mL H2O + 1g D-glucose. Then fill to 10 mL with sterile H2O, this dilution will result in 0.1g/ml, to be injected by intraperitoneal route).
- 14.6 Set up the bench with the following:
  - a clean pair of scissors to snip the tails;
  - glass bead sterilizer to sterilize scissors between rodents;
  - 2 glucometers; timers; insulin injection syringes;
  - table for recording keeping of values and pens.
- 14.7 When you are completely set up and ready to begin, snip the tip of the tail (as per **SOPTECH13 Blood withdrawal from the rodent – sequential small volume collection by snipping the end of the tail**), then **measure blood glucose (0)** and then inject the rodent with the diluted insulin following steps 5.8 and 5.9 below:
- 14.8 Fill a new syringe with the correct dose of diluted insulin for **ALL** cages first (1 person can safely prepare and test 12 mice or 8 rats for ipITT in one session).
- 14.9 Administer an intraperitoneal insulin injection:
  - Hold the mouse by the loose skin on the back of the neck and restrain it with the tail wrapped around the small finger from front to back.
  - Tilt the mouse toward a dorsal position (the mouse relaxes more in this position: for clarification see video: <https://www.youtube.com/watch?v=GseebUnPQEec>).
  - Have a new insulin syringe with the insulin solution ready and prepare to inject 0.75U/kg body weight.

- Hold the needle parallel to the lower abdomen (right or left), bevel up, and place it at a flat angle through the abdominal wall into the intraperitoneal space.
  - **Slowly** inject the insulin solution. Remove the needle.
- 5.10** Start the timer at 0:00.
- 5.11** Place the rodent back into its cage.
- 5.12** Measure blood glucose after 10, 20, 30, 45 and 60, 90, 120min.
- 5.13** Sterilize scissors in between rodents as per **SOP STER01 Operation of the glass bead sterilizer – the Germinator 500**.
- 5.14** Continue these steps until all rodents have been injected and blood glucose levels measured.

**Make sure all injection times and blood glucose levels for each rodent are recorded.**

**Note:** The insulin injection can sometimes cause the blood glucose to go so low that some mice can show signs of hypoglycemic shock such as convulsions or coma. This may occur if the blood glucose goes below 20 mg/dl. If the described adverse symptoms are noted, immediately give the mouse an injection of glucose (10% glucose: 9 mL H<sub>2</sub>O + 1g D-glucose, this dilution will result in 0.1g/ml) to reverse the condition. There should be sufficient “pre-loaded syringes” of this glucose solution for each mouse in a “batch” to facilitate “rescue” of any hypovolemic mice without unduly disrupting the process for other mice. These mice will then be removed from the ITT testing and results.

## 15.0 Revision History

Revision	Reason(s) for Revision	Date	Action by

## 16.0 References / Associated Materials (Optional)

Frendo-Cumbo S, **MacPherson REK**, Wright DC. Beneficial effects of resveratrol and metformin combination therapy in treating diet-induced insulin resistance. *Physiological Reports*; 2016 Aug;4(15).

**MacPherson REK**, Gamu D, Frendo-Cumbo S, Castellani L, Kwon F, Tupling AR, & Wright DC. Sarcolipin knockout mice fed a high fat diet exhibit altered indices of adipose tissue inflammation and remodelling. *Obesity*: 2016 Jul;24(7):1499-505. (**Editor’s Choice**)

Snook LA, **MacPherson REK**, Monaco C, Frendo-Cumbo S, Castellani L, Pepler WT, Andersen ZG, Buzelle SL, Leblanc PJ, Holloway GP, Wright DC. Prior Exercise

Training Blunts Short Term High Fat Diet Induced Weight Gain and Glucose Intolerance.  
Am J Physiol Regul Integr Comp Physiol: April 13 2016

**MacPherson REK**, Beaumeister P, Peppler W, Wright DC, Little JP. Reduced cortical BACE1 content with one bout of exercise is accompanied by declines in AMPK, AKT, and MAPK signaling in obese, glucose intolerant mice. J Appl Physiol 2015; Nov 15; 119:10; 1097-1104.

## Appendix D



### Animal Facility *For Internal Use Only*

Use of the Barnes maze to assess learning and memory in aging mice		SOP- Document No: <u>PI09</u> —	
		New <u>√</u> Revised —	Version No. <u>0</u>
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#### 1. BACKGROUND

The Barnes maze was developed in 1979 as a less stressful test of rodent learning and memory than previously developed tests relying on creation of a more acutely stressful condition, such as the Morris water maze. With the Barnes maze, the rodents are placed individually on a circular table-like structure, with holes along the edges. One hole leads to a small dark enclosed space called “the escape cage” positioned underneath the table. The animal is motivated to escape the exposed table top and will explore escape routes until a suitable one (the escape cage) is found. In subsequent repetitions of the experiment the observer records time taken for the mouse to remember the location of the escape cage (which does not move) and enter it. The test assesses spatial reference learning and memory since it assesses the individual’s ability to remember the location of the hole linked to the escape cage.



## **2. PURPOSE**

This procedure is being standardized and implemented to describe the equipment and its use and to help achieve repeatability between researchers/handlers, thus improving the data quality (i.e. reducing variability) and reducing the number of experimental replicates that might be required.

## **3. APPLICABILITY**

This procedure is applicable to the study of learning and memory in mice. A similar approach is applicable to rats but requires appropriate scaling of the maze dimensions.

## **4. RESPONSIBILITIES**

The researcher/research assistant will:

- thoroughly clean the apparatus for each use using Virox®;
- handled mice gently and returned them to home cage immediately at end of the procedure.

## **5. MATERIALS AND SUPPLIES**

The Barnes maze is located in the Discovery Wing either in room CRN164 or CRN 165. A stopwatch, clear beaker, Virox® spray for cleaning, and computer to enter data are required as additional materials.

## **6. PROCEDURES/FOCUS OF SOP**

- 6.1** During the habituation phase, each mouse is put inside an upside down clear beaker and guided around the maze for approximately 30 seconds, ending with the hole to the escape cage. Mice are given 3 minutes to enter the escape hole, and are nudged in if they do not voluntarily enter. They are then given one minute to explore the escape cage.
- 6.2** The training phase is conducted 24 hours after the habituation phase, mice are allowed to explore the maze freely for two minutes, and if the mouse finds the escape cage within that time, they are allowed to explore it for 1 minute before being returned to their respective housing.
- 6.3** The probe phase is conducted 48 hours after training. Mice are given 3 minutes each to explore the maze and find the escape hole. The amount of time spent on the table and in the escape cage are both recorded. In addition, time spent in different quadrants of the maze, i.e. those where the escape cage is found and those where it is not, is also recorded.

## **7. Revision History**

Revision	Reason(s) for Revision	Date	Action by


**9. References / Associated Materials (Optional)**

Attar A, Liu T, Chan W-TC, Hayes J, Nejad M, et al. (2013) A Shortened Barnes Maze Protocol Reveals Memory Deficits at 4-Months of Age in the Triple-Transgenic Mouse Model of Alzheimer's Disease. PLoS ONE 8(11): e80355. doi:10.1371/journal.pone. 0080355

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