Low-dose lithium supplementation influences GSK3β activity in the brains of an early, diet-induced sporadic Alzheimer’s disease mouse model

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by neurofibrillary tangles, amyloid-beta plaques, and cognitive decline. Research supports that key highlights of Type-2 Diabetes Mellitus (T2DM; i.e. obesity and insulin resistance) are significant risk factors for AD. At the forefront of both AD and T2DM pathologies is increased glycogen synthase kinase 3-β (GSK3β) activity. Targeting the inhibition of GSK3β has thus been suggested as an AD prophylactic. Lithium (Li), is a well-known natural GSK3β inhibitor, but is associated with dose-dependent adverse side effects. I thus aimed to examine the effects of low-dose Li supplementation on brain GSK3β activity, the development of AD pathologies, and markers of insulin signaling in a diet-induced insulin resistant mouse model. Male C57BL/6J mice were divided into either a 6-week (n = 24) or 12-week study (n = 72). In the 6-week study, mice were fed a chow diet (CON; n = 12) or a chow diet with Li-supplemented drinking water (Li; 10 mg / kg / day; n = 12) for 6 weeks. Alternatively, in the 12-week study, mice were fed a chow diet (CON; n = 24), a high-fat diet (HFD; 60% fat; n = 24), or a HFD with Li-supplemented drinking water (HFD+Li; n = 24) for 12 weeks. Prefrontal cortex and hippocampal tissues were collected for analysis. HFD and HFD+Li mice experienced significant weight gain and had impaired glucose and insulin tolerance compared to CON mice. Furthermore, HFD+Li mice had reduced caloric efficiency and rescued insulin degrading enzyme content compared to HFD mice. With respect to GSK3β activity, increases in inhibitory p-GSK3β Ser9 were not observed until 12 weeks in the HFD+Li compared to CON mice, however actual activity was reduced after only 6 weeks in the Li compared to CON mice. Collectively, these data provides evidence for low-dose Li supplementation to improve diet-induced impairments that can otherwise contribute to AD. Moreover, these results indicate that GSK3β activity can be inhibited despite any changes in
phosphorylation. These findings contribute to an overall greater understanding of low-dose Li’s ability to influence GSK3β activity in the brain and its potential as an AD prophylactic.
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List of Abbreviations:

- AD - Alzheimer’s disease
- Aβ - amyloid-beta
- NFT - neurofibrillary tangles
- APP - amyloid precursor protein
- sAPPα - soluble APPα
- sAPPβ - soluble APPβ
- CTF - C-terminal fragment
- AICD - APP intracellular domain
- GSK3β/α - glycogen synthase kinase-3β/α
- BACE1 - beta-secretase-1
- ERK - extracellular signaling-regulated kinase
- JNK - c-Jun N-terminal kinase
- Cdk5 - cyclin-dependent kinase 5
- HIP - hippocampus
- PFC - prefrontal cortex
- PET - positron-emission tomography
- MoCA - Montreal Cognitive Assessment
- MCI - mild cognitive impairment
- fAD - familial AD
- sAD - sporadic AD
- PSEN1/2 - presenilin ½
- APOE - Apolipoprotein
- T2DM - Type-2 Diabetes Mellitus
- MAPK - mitogen-activated protein kinase
- HFD - high-fat diet
- LFD - low-fat diet
- BBB - blood-brain barrier
- IR - insulin receptor
- IRS - insulin receptor substrate
- PI3K - phosphoinositide 3-kinase
- PIP2 - phosphatidylinositol 4,5-bisphosphate
- PIP3 - phosphatidylinositol 3,4,5-triphosphate
- PDK1 - phosphoinositide-dependent kinase-1
- Akt - protein kinase B
- AS160 - Akt substrate of 160kDa
- CNS - central nervous system
- IDE - insulin degrading enzyme
- Li - lithium
- IPGTT - intraperitoneal glucose tolerance test
- IPITT - intraperitoneal insulin tolerance test
- AUC - area under the curve
- BCA - bicinechonic acid
- Sgg - Shaggy
- UCP1 - uncoupling protein 1
- PP2A - protein phosphatase 2A
- PHLPP - PH domain leucine-rich repeat protein phosphatase
- EGFR - epidermal growth factor receptor
- SIRT2 - Sirtuin 2
Chapter 1: Literature Review

1.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is a neurodegenerative disease highlighted by three main characteristics: the accumulation of extracellular amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles (NFT) and progressive cognitive decline (91). AD is the most prevalent form of dementia, accounting for 50-75% of all dementia cases (120, 190). Currently, it is estimated that there are 44 million dementia cases worldwide, with expectations of this number to more than triple by 2050 (120). This statistic poses a major public health concern and economic burden in the future as the need for support and care increases. While there are pharmaceutical treatments for patients with AD, they mainly target merely the symptomology of the disease and not the underlying cause. A lack of effective prophylactics for AD demonstrates a significant need for an alternative approach and for successful preventative treatments to be developed.

Aβ and APP Processing

It is hypothesized that Aβ plaques accumulate through a combination of increased activity of the amyloidogenic pathway as well as decreased clearance of Aβ peptides. These Aβ peptides originate from amyloid precursor protein (APP), a type I transmembrane protein abundantly present in brain tissue (199). APP undergoes several cleavage events by various secretases leading to the production of different molecular products (134, 199). Many APP isoforms of varying lengths exist, however it is known that APP 695 is predominantly expressed in neurons while APP 751 and 770 are known to be expressed in glial cells (137, 199).

There are two competing pathways generally associated with APP processing and its relevant cellular products: 1) the amyloidogenic and 2) the non-amyloidogenic pathway (Figure
In the non-amyloidogenic pathway, APP is first cleaved by $\alpha$-secretase at the Leu17 site, releasing soluble APP$\alpha$ (sAPP$\alpha$) into the extracellular space (61, 199, 210). sAPP$\alpha$ is known to have beneficial properties, such as its role in neuronal plasticity and survival as well as neural stem cell proliferation (173). Additionally, when administered intracerebroventricularly in the brains of rodents, sAPP$\alpha$ has shown to enhance learning and memory (139, 189). Initial cleavage of APP by $\alpha$-secretase ultimately prevents A$\beta$ production as its cleavage site lies within the A$\beta$ domain (199, 211). A second cleavage event is then performed by $\gamma$-secretase to the remaining membrane bound C-terminal fragment (CTF83), producing the small peptide, p3 or p83, into the extracellular space (199). p3 is readily degraded and causes no known cellular harm (173). However, the exact physiological role of the p3 protein fragment remains unclear (43). Finally, this cleavage event also results in the production and release of the APP intracellular domain (AICD) into the cytoplasm of the cell (199). The role of AICD is also elusive, as most studies have attempted to look at its function in vitro with limited experiments performed in vivo in the brain (43). What is thought, however, is that AICD mainly functions by binding to the adaptor protein, Fe65, and initiating downstream effects including the transcriptional activation of target genes relevant to AD pathologies such as glycogen synthase kinase 3-β (GSK3$\beta$) and neprilysin (43).

In the amyloidogenic pathway, APP is instead first cleaved by $\beta$-secretase or BACE1, at the Asp1 site producing and releasing sAPP$\beta$ into the extracellular space (199, 210). The role of sAPP$\beta$ is known to be much more detrimental, involved in processes such as axonal pruning and neuronal cell death (156, 173). The subsequent cleavage by $\gamma$-secretase of the remaining CTF99 results in the production and release of A$\beta$ peptides and the AICD in the extracellular space and cytoplasm, respectively (173, 199). Accumulation of these detrimental A$\beta$ peptides in the
extracellular space leads to its eventual oligomerization and plaque formation. Plaque formation will ultimately impair neuronal health and signaling, leading to cell death and tissue atrophy.

**Figure 1.1: APP Processing.** A depiction of APP processing and cleavage events in both the non-amyloidogenic and amyloidogenic pathways.

BACE1 has the ability to cleave APP at two different sites- Asp1 and Glu11 (50). Predominantly, BACE1 will cleave APP at the Glu11 site, producing the alternative CTF89, while Asp11 cleavage is considered the minor cleavage site (50, 210). γ-secretase cleavage is less precise, typically cleaving CTFs between the amino acids residues 37 to 43 (116). The majority of Aβ peptides have been found to end at the amino acid residue 40 (i.e. approximately
50%; Aβ1-40), while roughly 16% ends at the residue 38 (i.e. Aβ1-38) and 10% at the residue 42 (i.e. Aβ1-42) (26). One of the longer forms of Aβ, Aβ1-42, is associated with greater aggregatory properties due a greater number of hydrophobic amino acids on the C-terminal end (98, 184). Thus, Aβ1-42 peptides are more likely to oligomerize and lead to plaque formation compared to its more common and shorter counterpart, Aβ1-40 (116).

BACE1, the major β-secretase involved in APP processing, is thought to be the rate-limiting factor in Aβ production along the amyloidogenic pathway (211). Thus, increased activity of the amyloidogenic pathway of APP processing, and in particular, β-secretase or BACE1 activity, results in the increased production of Aβ peptides in the brain. Further impairments can ensue when APP is phosphorylated at its Thr668 within the cytoplasmic domain. This event increases proteolysis of APP by β-secretase (39, 122), encouraging amyloidogenic pathologies. Several kinases are capable of phosphorylating APP at this site including extracellular signaling-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), cyclin-dependent kinase 5 (Cdk5), and importantly GSK3β (109).

**Tau and NFT**

The second pathological characteristic of AD, NFT, is composed of hyperphosphorylated tau proteins which accumulate in the cytoplasm of neurons (63). Tau is a microtubule-associated protein, which plays an important role in the structural stability as well as function of axonal microtubules of neurons (71). Under healthy, well-functioning conditions, tau will associate with microtubules, increasing their stability for neuronal communication and vesicular transport along the axon. However, the level of which tau is phosphorylated is critical, as too much phosphorylation leads to a reduction in tau’s affinity for microtubules (166, 168). This change
will ultimately decrease the structural stability of the microtubules, eventually forming tangles within the cell.

There are a total of 85 residues on tau which may be phosphorylated by various kinases, and 40 of these sites have been recognized to be modified in the brains of patients with AD (i.e. 28 serine, 10 threonine, and 2 tyrosine residues) (86, 91). With so many possible residues, approximately 20% of the tau molecule has the potential to become phosphorylated (99). This factor makes measuring the phosphorylation status of tau difficult. While some residues may be altered in vivo, others may not and quantifying phosphorylation at only a few sites may not be an accurate reflection of tau’s actual phosphorylation status. Furthermore, tau phosphorylation has been shown to be induced by several different kinases in vivo, including Cdk5, protein kinase A (PKA), and GSK3β (99).

Functionally, NFT cause several detriments including impaired vesicular transport, cellular communication, and eventual cell death (91). Relevant to the progression of AD, NFT are not able to be removed by autophagic and other proteasomal mechanisms within the brain (35), remaining as is and contributing to further impairment.

Cognitive Decline

As a consequence of these microscopic physiological changes in the brains of patients with AD, cognitive deficits appear overtime due to impairments such as reduced neuronal communication, neural death, and tissue atrophy (96, 171). The first forms of behavioural changes which begin to surface are short-term memory deficits (63, 100, 193). This is thought to occur as one of the brain regions shown to be first affected by AD is the hippocampus (HIP) (28, 51, 96, 160), a region responsible for learning and memory consolidation (51). The second brain region which is typically affected in the later stages of the disease is the prefrontal cortex (PFC)
The PFC is responsible for higher-order processes and executive functions such as critical thinking, decision-making, and cognitive flexibility (72, 73). As a result, these types of behaviours tend to become impaired following memory deficits.

The HIP and PFC are strongly connected in both animals and humans via bidirectional neuronal circuitry (167). A previous study used positron-emission tomography (PET) imaging to measure blood flow in the brains of patients with AD compared to healthy subjects during the performance of a memory task (75). Results suggested that the early memory impairments experienced by patients with AD were due to reduced integrated network activity, particularly between the HIP and PFC. The use of optogenetics has also helped to demonstrate the relevance of specific connections in the brain with respect to behavioural output (167). For example, direct neuronal connections between the ventral HIP and medial PFC have shown to be important for encoding spatial cues during spatial working memory tasks (182).

As AD progresses, and Aβ plaques and NFT become more widespread throughout various brain regions and structures, increasing behavioural deficits affect the individual. It is important to note, however, the high degree of individual variability for AD progression with respect to the order which various brain regions are affected, the time this takes to occur and the degree to which the individual experiences clinical behavioural changes and deficits (100). AD’s physiological detriments as they relate to clinically relevant behavioural deficits in a temporal and spatial manner is still not completely understood and remains to be elucidated (100).

It is thought that cognitive decline more accurately reflects the progression of NFT development compared to Aβ pathologies, and cognitive decline can thus be considered tau-dependent (34, 52, 100). Contrarily, Aβ is argued to be associated with the preclinical stages of AD, prior to behavioural deficits surfacing and tau pathologies developing (34, 52, 100). Thus, it
is understood that Aβ pathologies precede NFT development, as NFT would develop alongside of cognitive impairments while Aβ peptide production and plaque formation would correlate with the progression of preclinical AD. Furthermore, phosphorylated tau is also linked to a decrease in the accumulation of Aβ peptides (91), such that as NFT continue to develop later on in the disease, the formation of Aβ plaques begins to plateau (96).

**Diagnosis and Treatment**

The diagnosis of AD has previously focused on the recognition of changes to cognition and behavioural symptoms (63) as well as confirmation via post-mortem brain tissue analysis. However, it is now well-understood that the physiological detriments of AD begin to develop decades before behavioural symptoms become recognizable (35, 63, 172, 178, 199), making diagnosing the disease at a critical time very difficult. A shift in diagnosis criteria to recognizing preclinical AD has thus occurred as to target disease progression during its earlier stages (63, 172).

Various cognitive tests exist to screen for an individual’s current degree of cognitive impairment. The Montreal Cognitive Assessment (MoCA) is a popular tool used to assess patients who show symptoms of mild cognitive deficits (146). MoCA is thus sensitive to detecting mild cognitive impairment (MCI), a condition that is clinically defined as being in between normal cognitive aging and dementia, and typically later develops into dementia (146). Various cognitive skills are tested such as memory recall and executive function (146), with a lower overall score indicative of greater cognitive impairment.

With respect to the physiological changes in the brain, a previous post-mortem analysis of 83 brains from patients with diagnoses of dementia or AD determined discrete stages which clearly define the pathological progression of AD, known as Braak Staging (33). Based on
observed patterns in the brain, 3 stages of AD progression were defined based on Aβ peptide accumulation and location in the brain (Stages A-C) as well as another 6 stages based on NFT and neuropil thread development and location (Stages I-VI) (33).

Currently, no effective treatment or prevention method is available that protects against the development of both the physiological pathologies and behavioural symptoms of AD (16, 153). The treatments currently available to AD patients, rather, are mostly to help manage disease symptoms (153, 208). Furthermore, trials searching for potential therapeutics often encounter many difficulties, particularly difficulty with long-term follow up in older-aged populations (208). Trials to date have explored, and continue to explore, the potential of BACE1 inhibitors to alleviate Aβ peptide production (47, 58). However, many of these trials have been unsuccessful due to patients experiencing adverse side effects or a lack of beneficial results (155). Tau immunotherapy has also been explored which attempt to clear phosphorylated tau content from the brain, decreasing the accumulation of NFT (97, 176). While some tau immunotherapies have been deemed efficacious (31, 32), many considerations (e.g. antibody affinity and clearance location) are still needed to be explored for this therapeutic to identify both its safety and overall efficacy (176). Thus, the need for more long-term investigations exploring various potential therapeutics for AD pathologies is important in order to find successful treatments for patients.

Forms of AD and Associated Risk Factors

There are two main forms of AD: 1) familial and 2) sporadic. Firstly, familial AD (fAD) is the much rarer form of the disease, accounting for less than 5% of all AD cases. fAD is represented by autosomal dominant mutations on either presenilin 1 (PSEN1), presenilin 2 (PSEN2), or APP, three genes associated with AD pathologies (106, 187). PSEN1 and PSEN2 comprise the enzymatic site of γ-secretase (106), altering its enzymatic activity and thus APP
processing if mutated. The symptoms of fAD appear much earlier than sporadic AD (sAD), typically becoming prevalent before the age of 65 (190) and thus is also known as early-onset AD.

sAD makes up the majority of AD cases (>90%) and is developed later in life (i.e. >65 years old) (17, 120, 190). This form of AD is arguably the more complex form, influenced by a combination of genetic and environmental factors (120). In sAD, it is likely that multiple pathways and cellular processes are impaired that contribute to the overall disease progression (16). With respects to genetics, the Apolipoprotein (APOE) gene is recognized as the biggest risk factor for developing sAD (45). APOE encodes the lipoprotein, APOE, which is greatly expressed in the CNS. Astrocytes are the main source, although microglia and neurons are also capable of producing APOE (10, 206, 209). APOE’s role is to transport lipids within the CNS that have been made after neuronal degeneration to cells which require them for proliferation and repair (88, 209, 212).

There are three major, naturally-occurring alleles of the APOE gene in humans; APOE2, APOE3 and APOE4 (148). Each corresponding protein encoded by these alleles differ by one amino acid at residues 112 and 158 (i.e. APOE2Cys,Cys, APOE3Cys,Arg and APOE4Arg,Arg) (10). Various combinations of these alleles have been associated with different risks of developing AD with APOE4 carrying the greatest risk. One copy of APOE4 increases one’s risk of developing AD 4 times that of an APOE3/APOE3 carrier, while a carrier of two copies of APOE4 has a 12-fold increased risk (106, 195). It is estimated that the worldwide prevalence of individuals with AD who are APOE4 carriers is 48.7% and 9.6% for homozygous carriers (198). However, the specific mechanism by which APOE4 contributes to an increased risk of developing AD is less clear (159, 195). It is thought that APOE4 may influence Aβ oligomerization (159).
Alternatively, the difference in amino acid residues and thus protein structure compared to the other alleles alters APOE4’s function with respect to lipid regulation in the CNS, potentially having a negative effect on lipid redistribution and neuronal plasticity (136, 159).

Environmental factors influencing the development of sAD are vast and complex. Many have been identified to date, including physical activity, diet, and associated metabolic diseases (23, 185). A previous analysis of existing meta-analyses estimated the population-attributable risk of AD with seven modifiable risk factors known to be associated with disease (i.e. diabetes, midlife hypertension, midlife obesity, physical inactivity, depression, smoking, and low educational attainment) (151). The results predicted that, when combined, the seven individual risk factors account for approximately half of all AD cases worldwide (151). This analysis highlights the significant role various lifestyle factors play in the pathogenesis of AD and how making modifications to such factors can prevent or slow its development.

1.2 Type-2 Diabetes Mellitus and AD

Type-2 Diabetes Mellitus (T2DM) is a metabolic disease often developed later in life alongside the adherence to a poor diet, a sedentary lifestyle, and an obesogenic status (15). Like AD, the worldwide incidence of T2DM is expected to increase significantly in the coming decades with the aging population (36). It is mainly characterized by insulin resistance, a condition in which tissues of the body become less responsive to circulating insulin and is unable to produce the normal physiological responses it would otherwise be responsible for. More specifically, in healthy populations, insulin is released from pancreatic β cells in response to a postprandial rise of blood glucose levels (79). Insulin binds to its receptor on various insulin-sensitive tissues (i.e. skeletal muscle and adipose tissue), initiating a cascade that influences the uptake of blood glucose into the corresponding tissue for metabolic use or storage. In an insulin
resistant individual, this mechanism is impaired and blood glucose levels remain abnormally high. Furthermore, under such conditions, insulin is constantly released in attempt to fight against the constantly high concentrations of glucose in the blood. This constant stimulation of the insulin receptors will influence its decreased sensitivity as well as its potential down-regulation on the cell membrane (79), ultimately causing insulin resistance. An increase in inflammatory processes, which is often associated with obesity, can also induce insulin resistance. The activation of serine kinases within the mitogen-activated protein kinase (MAPK) signaling pathway (e.g. JNK and ERK) can directly act on the insulin cascade to aberrate its function (95).

A multitude of human studies support a connection between T2DM and AD pathologies. A previous analysis has shown that sAD patients with an additional diagnosis of diabetes had lower cognitive scores than patients with AD pathological changes or diabetes alone (1), suggestive of diabetes influencing the progression of AD or having the ability to worsen symptoms. Furthermore, whole brain glucose uptake has been shown to be lower in patients with both MCI and diabetes compared to patients with MCI alone (125). These results support a strong connection between T2DM and AD, such that diabetes has the ability to influence the brain’s metabolic activity early on in the disease (174). In a longitudinal analysis, older men and women were categorized into either a normoglycemic or impaired glycemic group based on their glycemic status at baseline (142). Compared to the normoglycemic group, individuals in the impaired glycemic group had a significantly greater loss in whole brain volume, global cognition, and a greater rate of change from MCI to AD diagnoses after 2 years (142).

In animals, rhesus monkeys with T2DM had significantly greater Aβ as well as p-tau Ser202 and Thr231 content in whole brain samples as compared to control monkeys (165),
demonstrating the pathological differences in the brain under peripheral insulin resistant states. In another analysis, C57BL6/J mice fed a high-fat diet (HFD) for 7 weeks experienced significantly increased β-secretase activity in the cortex as compared to mice fed a low-fat diet (LFD) (132), suggestive of increased Aβ peptide production via APP processing. Similarly, male mice fed a HFD as well as sugar water for 14 weeks had not only reduced glucose and insulin tolerance, but also significantly greater BACE and phosphorylated tau content in whole brain samples as compared to control mice fed a chow diet (114). It was also shown that having the ApoE4 genotype, the greatest genetic risk factor for sAD, further influenced weight gain and impairments to glucose metabolism in mice fed a high-fat and sugar diet (143). Thus, the influence of disease progression and aggravation may be bidirectional between AD pathologies and T2DM and insulin resistance.

**Insulin Signaling and the Brain**

While insulin resistance and insulin signaling has been classically studied and is well-understood in peripheral tissues such as the liver or skeletal muscle, more recent work has suggested that the brain is also an important insulin-sensitive tissue (11, 111). Importantly, however, in order to enter the brain and have an effect, insulin must first cross the blood-brain barrier (BBB). It is hypothesized that this occurs through a saturable transport process (11, 29, 193) where peripherally circulating insulin binds to a saturable transporter on the endothelial cells lining the BBB. The insulin is then internalized and translocated to the opposite side of the BBB which faces brain interstitial fluid. There is evidence to suggest endogenous sources of insulin exist (29, 76), however the majority of insulin in the brain appears to originate from the periphery.
Once insulin has passed the BBB, it is then able to bind to insulin receptors found at high concentrations on neurons in areas like the HIP and cortex (194). In a healthy, well-functioning neuron, the insulin cascade begins with the membrane-bound insulin receptor (IR), a tyrosine-kinase receptor, via binding of available insulin (Figure 1.2A) (111). This event will allow for autophosphorylation of the IR on various tyrosine residues (2, 121). Following activation of the IR, downstream insulin receptor substrate (IRS) is phosphorylated, allowing for phosphoinositide 3-kinase (PI3K) to be recruited to the membrane and subsequently activated (54, 144). PI3K is then responsible for phosphorylating phosphatidylinositol 4,5-bisphosphate (PIP2) to produce phosphatidylinositol 3,4,5-triphosphate (PIP3) (188). An increase in intracellular PIP3 concentrations will signal the translocation of downstream proteins, phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (Akt), to the membrane (188). PDK1 can then phosphorylate Akt at one of its activating residues, Thr308 (83). It is important to note that Akt is also able to be phosphorylated at its other activating residue, Ser473, by another kinase associated with the insulin cascade (i.e. mTOR complex 2) (83, 87, 169). Following its activation, Akt is then able to phosphorylate important downstream targets including GSK3β and the Akt substrate of 160kDa (AS160) (188).

In the periphery, the phosphorylation of AS160 by Akt is critical for the translocation of the glucose transporter, GLUT4, from basal storage in intracellular vesicles to the cell membrane, allowing for increased glucose uptake into the cell. However, in the central nervous system (CNS), while there are insulin sensitive GLUT4 transporters in neurons (19, 78), glucose uptake via this mechanism is limited. Instead, glucose uptake mainly occurs through the process of facilitated diffusion via the insulin-independent glucose transporters, GLUT1 in glial and endothelial cells as well as GLUT3 in neurons (18).
Because there is limited insulin-dependent glucose uptake in the brain, insulin signaling in the brain has different functional outcomes. One of insulin’s main effects in the brain is related to feeding control mechanisms (11), such that insulin administration in the brain supresses food intake (65, 67). However, region-specific identification of IRs beyond areas responsible for feeding control (i.e. the hypothalamus) such as the hippocampus, cortex, and thalamus support that insulin’s role is much more complex and multifaceted within the CNS (64). For example, insulin has been linked to benefits in learning and memory. Administration of intranasal insulin in mice improved both their short and long-term object recognition (11). Training rats in water maze tests has also shown to change the patterns of IRs in regions of the hippocampus, suggestive of altered insulin signaling and activity during memory consolidation (11, 53, 213). Various other important roles of insulin signaling in the brain have also been identified, including promoting neuronal survival (140), the formation of neuronal circuity (41) and the maintenance of synaptic function and neurotransmission (11, 196). Taken together, insulin’s role in the brain is vastly important and crucial to neuronal health and function.

Impairments to the insulin signaling cascade can prevent the aforementioned cellular events from occurring properly (Figure 1.2B). In the periphery, a dysfunctional insulin cascade would result in decreased GLUT4 translocation and subsequent glucose uptake, leaving blood glucose levels abnormally high. In the brain, insulin resistance can lead to further impairments such as decreased learning and memory, impaired cognition, and neuronal death (49, 115, 131).
Brain insulin resistance has been demonstrated in humans as well as in various animal models. In a study comparing the effects of insulin in the brain of lean versus obese men and women, cortical activity was increased with peripheral insulin infusion in the lean, but not in the obese participants (192). Moreover, the effect of insulin on cortical activity was negatively correlated with body mass index and percent body fat, and positively correlated with insulin sensitivity (192). These results demonstrate an overall reduced response from the brain when exposed to insulin. Similarly, peripherally insulin resistant men had reduced glucose metabolism
in the PFC and striatum in response to insulin as compared to peripherally insulin sensitive men (6). In a post-mortem analysis, the brains of AD patients had significantly greater IRS1 Ser636/639 phosphorylation than the brains from control subjects (30), suggestive of aberrant brain insulin signaling.

In male mice fed a HFD and sugar water for 14 weeks, multiple perturbations in insulin signaling within the brain were noted including significantly reduced IR phosphorylation and increased IRS1 phosphorylation at multiple sites as compared to control mice (114). Similarly, in a transgenic mouse model of AD, signaling through IRS1 was impaired in the hippocampi (30). These studies exemplify impairments at the beginning of the insulin signaling cascade, which would inevitably cause reduced signaling downstream. In support of this, C57BL/6J male mice fed a HFD (60% kcal from fat) for 17 days experienced no changes in Akt Thr308 or Ser473 as well as GSK3β phosphorylation in frontal brain tissue in response to insulin, while mice fed a control diet did (7).

Important to note, is that insulin competes with Aβ peptides for its degradation by the same enzyme, insulin degrading enzyme (IDE) (46). In an insulin resistant state when insulin levels are chronically high, IDE will preferentially degrade insulin over Aβ peptides, influencing greater accumulation and eventual plaque formation. The function of IDE is also impaired as one becomes older, worsening the brain’s ability even more so to clear Aβ content with age, the biggest risk factor for AD (64, 119). Finally, Aβ will also compete with insulin for binding to the IR, reducing its overall activation and signaling through important, protective downstream targets (46, 205).
Due to the important role that GSK3β has in both insulin signaling as well as the development of AD pathologies, GSK3β has become an attractive target to prevent or reduce disease progression. GSK3β is a serine/threonine kinase (127) involved in a wide range of cellular processes across various tissues including glycogen synthesis, embryonic patterning, and as discussed above, insulin signaling (158). While there are two isoforms of GSK3 (i.e. α and β), the latter is more abundantly present in the adult mammalian brain (123) and more relevant to human neurodegenerative processes (4). GSK3β is highly expressed in the brain (134, 203) and remains constitutively active in its resting state (21). When phosphorylated at its Tyr216 residue, GSK3β’s activity is enhanced, however the mechanisms which contribute to these changes are less clear (105). Phosphorylation of GSK3β at the Ser9 residue within the N-terminus, however, typically via stimulation by insulin and Wnt signaling pathways, results in the inhibition of its activity (21, 101). Specifically, upstream of GSK3β in the insulin signaling cascade is Akt. When activated via insulin stimulation, Akt phosphorylates GSK3β at its inhibitory Ser9 residue. There are over 40 GSK3β substrates, making GSK3β a multi-target kinase (4, 101).

GSK3β substrates must be primed prior to its recognition by GSK3β and subsequent phosphorylation (Figure 1.3). Ser9 phosphorylation induces GSK3β inhibition, as it allows for autorecognition of GSK3β’s primed substrate binding domain, preventing interactions of primed substrates with the adjacent kinase domain (21, 89). In the absence of Ser9 phosphorylation, primed substrates are able to bind to the primed substrate binding domain, placing the substrate in close proximity to GSK3β’s kinase domain for its phosphorylation. The typical pre-phosphorylated sequence that is recognized by GSK3β is S/T-X-X-X-S/T, where a serine or threonine residue is phosphorylated four residues N-terminal to the pre-phosphorylated serine or threonine residue.
threonine residue (21). Additionally, GSK3β requires free magnesium ions as a cofactor necessary for binding and subsequent activation (102, 164). GSK3β activity can also be modulated via its cellular location (105). For example, localization to the nucleus and mitochondria increases GSK3β activity (27), whereas it is inhibited within growth cones (59).

Figure 1.3: GSK3β’s Primed Substrate Binding Domain and Mechanism of Inhibition by Ser9 Phosphorylation (Beurel et al., 2015). A) Crystal structure of GSK3β with its kinase domain and primed substrate binding domain. B) Ser9 phosphorylation on the N-terminus binds to the primed substrate binding domain. C) A primed substrate binds to the primed substrate binding domain in the absence of Ser9 phosphorylation. D) A primed substrate is blocked from binding to the primed substrate binding domain by Ser9 phosphorylation.

When active, GSK3β is capable of directly phosphorylating targets relevant to AD including tau and APP (37). Of the tau phosphorylation sites shown to be affected in the brains of AD patients, GSK3β is able to phosphorylate 23 of these sites (i.e. 17 serine and 6 threonine
residues) (86, 91). Thus, when consistently active, GSK3β can induce tau hyperphosphorylation and produce NFT. GSK3β is also known to modulate the expression and activity of the APP-cleaving enzymes, β-secretase and γ-secretase, influencing greater production of Aβ peptides (127, 129). Taken together, the activity of GSK3β in the brain is detrimental and encourages the development of AD pathologies.

Previous research has found that the activity of GSK3β in the brain is increased in patients with AD (124, 157). In animals studies, overexpression of GSK3β has been linked to increased hyperphosphorylated tau and impaired performances on cognitive tests (90, 128). Conversely, restoring the activity of GSK3β to normal levels has been shown to reduce tau hyperphosphorylation and lead to improvements in cognitive analyses (60). In vitro, increased tau phosphorylation and reduced cell viability induced by Aβ toxicity was improved following the application of a GSK3β inhibitor (i.e. GSK3β inhibitor VIII) (113), suggestive of the therapeutic benefits of reducing GSK3β activity at the cellular level. In cultures of rat hippocampal neurons, it has also been shown that exposure to Aβ activates GSK3β via the inhibition of PI3K/Akt signaling (186). These results suggest the potential positive feedback on GSK3β activity driven by increased Aβ concentrations, leading to progressively worsened AD pathologies.

1.3 Lithium

Lithium (Li) is an effective mood-stabilizing drug most commonly known for its use in the treatment of bipolar disorder (147). Its use in patients was shown to reduce the risk of developing AD in comparison to the use of other mood-stabilizing medications (147). Potentially connecting the use of Li with a reduced risk of AD is GSK3β. Li is a well-known inhibitor of GSK3β both in vitro and in vivo (70) acting both indirectly and directly to reduce its activity.
Specifically, Li can directly inhibit GSK3β by acting as a competitive inhibitor to magnesium, a cofactor of GSK3β which is necessary for its activation (102). That is, Li competes with magnesium for its binding to GSK3β, blocking its access for subsequent activation. It is also proposed that Li exerts its effects indirectly on GSK3β activity by increasing inhibitory Ser9 phosphorylation via stimulation of associated cellular pathways (i.e. upstream PI3K/Akt activity) (70) as well as inhibition of relevant phosphatases (i.e. protein phosphatase 1 and protein phosphatase 2A (PP2A)) (102, 141).

Figure 1.4: Li’s Therapeutic Potential in AD. Li’s proposed mechanism of action in relation to GSK3β inhibition and AD. A) GSK3β remains active in an unphosphorylated state, contributing to the production of NFT and Aβ peptides. B) Li prevents these pathological events from happening by inhibiting GSK3β activity.

Previous *in vitro* and *in vivo* analyses have demonstrated the therapeutic potential of Li treatment for AD. Older adults (i.e. 60 years and older) diagnosed with MCI receiving a daily
dose of 150 mg Li for 12 months experienced a relatively slowed cognitive decline as well as reduced phosphorylated tau content in CSF samples compared to individuals receiving a placebo treatment (68). Similarly, AD patients treated with a microdose of Li (i.e. 300 µg Li daily) for 15 months experienced no further cognitive decline as measured via changes in mini-mental state examination scores as compared to AD patients in the control group who experienced worsened scores overtime (153). In a recent correlational study, trace levels of Li in the drinking water throughout the majority of Texas counties was negatively correlated with changes in AD mortality (62). This result is important as it suggests that the greater amount of Li consumed is related to lesser rates of AD mortalities, indicating a possible therapeutic connection.

Li treatment has also been shown to increase GSK3β phosphorylation, improve cognitive impairments, and improve physiological detriments including tau phosphorylation and Aβ peptide concentrations in various animal models (16, 22). For example, transgenic mice overexpressing mutant tau protein that received intraperitoneal (i.p.) injections of 10 µL / g /day LiCl for 30 days experienced reduced GSK3β activity (149). In addition to changes in activity, GSK3β Ser9 phosphorylation and Akt Ser473 phosphorylation were both increased in the LiCl treated mice, suggested of Li’s indirect role via Akt activation which influences such results (149). In another study, adult male rats received an acute i.p. administration of 100 mg / kg LiCl 30 minutes prior to task-training in order to study the effects of Li treatment on sevoflurane-induced memory impairments and associated GSK3β phosphorylation status (126). Results found that the acute LiCl treatment prevented sevoflurane-induced memory impairments as well as increased GSK3β Ser9 phosphorylation in the HIP (126), suggestive of both GSK3β’s potential role in memory as well as the use of Li as a successful therapeutic to help with such impairments. Finally, a study examining APP transgenic mice also treated with daily i.p.
injections of LiCl (20 mg/kg) for 3 months found reduced Aβ and phosphorylated tau content in both the frontal cortex and HIP as compared to saline-treated mice (161). Importantly, these changes co-occurred alongside of increased GSK3β Ser9 phosphorylation and decreased GSK3β activity in the frontal cortex in the Li-treated mice compared to saline-treated mice (161).

Similar results have also been observed in vitro. Li treatment has also demonstrated its ability to restore inhibitory GSK3β Ser9 phosphorylation in PC12 cells (204) and alleviate Aβ-induced neurotoxicity in cultured rat cortical neurons (5). Furthermore, in SH-SY5Y cells, both 5 and 10 mM LiCl treatment for 3 hours significantly reduced tau phosphorylation at multiple sites (71).

While the scientific evidence supporting Li for the treatment of neurodegenerative symptoms is abundant and promising, continued research is needed to clearly identify its true potential in the prevention of AD pathologies. Furthermore, Li has been associated with adverse side effects that are mainly dose-dependent such as weight gain, cognitive dullness, tremors, and renal dysfunction. Therefore, a low-dose lithium treatment is instead suggested to avoid such negative health-related outcomes (74, 154). Thus, the need for understanding the efficacy of specifically a low-dose Li treatment for AD is essential.

Safety and Therapeutic Dosing of Li

The therapeutic window of Li for the treatment of affective disorders like bipolar disorder is between 0.5 and 1.5 mM in humans (93, 130). Thus, to examine the therapeutic potential of a relative low-dose Li treatment, resulting serum concentrations below the low-end of this therapeutic window are ideal (i.e. <0.5 mM serum Li). However, it is also very important to consider great individual variability in tolerating Li. For example, while serum Li concentrations as high as 4.5 mM have been reported without any symptoms of neurological impairment (202),
serum Li concentrations within and below the therapeutic range were associated with neurotoxic symptoms and other adverse side effects (8, 130, 175). Factors influencing such individual variability may include age, exposure to other drugs, and pre-existing health of an individual (8, 130, 147). Furthermore, it was previously shown that Li uptake in the brain is not the same within every region, with higher levels found in the HIP as compared to other regions such as the stratum and cerebellum in mice (92). Thus, regional differences in the brain may also be present with respect to Li uptake and accumulation overtime. Finally, it must also be considered that Li can have multiple effects within the body and not does not exclusively act on GSK3β activity in the brain (130). These are all important considerations that need to be made during future investigations regarding Li therapy.

A low-dose Li treatment (0.25-0.50 mmol / L) in patients with MCI was reportedly well-tolerated with a high adherence rate by participants of 91% (68). Another observational study examined both the feasibility and tolerability of a long-term low-dose Li treatment in elderly adults with mild to moderate AD, with a targeted steady-state serum Li concentration of 0.3-0.8 mM (130). Of the 22 participants who commenced the Li treatment, only 8 completed the study or continued to receive Li treatment beyond 1 year. However, only 3 of the reported reasons for incompleteness of the study were due to side effects that were relieved when the Li treatment was stopped and no symptoms were seriously adverse or irreversible (130). Other reasons for treatment incompleteness included unrelated deaths or illnesses or the inability to assure correct administration of Li. The results from this study suggest the potential difficulties that may arise with long-term treatment and maintenance feasibility in elderly AD patients, however the lack of reported symptoms which required cessation of the treatment hints to Li’s relative safety and tolerability in most patients at a low-dose. Continued research is necessary to further understand
both the potential of low-dose Li as an AD therapeutic and its feasibility as a long-term treatment in the targeted elderly population.
Chapter 2: Thesis Aims

2.1 Statement of the Problem

AD, the most common form of dementia, is characterized by the development and accumulation of Aβ plaques, NFT, and progressive cognitive decline. The already high prevalence of AD is expected to worsen within the coming decades, with no effective treatment or prophylactic interventions available. A number of modifiable risk factors for AD have been identified, with an abundance of evidence supporting T2DM and insulin resistance as significant risk factors for developing related pathologies. GSK3β, a serine/threonine kinase involved in the insulin signaling cascade, is abundantly present in the brain and remains active under insulin resistant conditions. With respect to AD, GSK3β overactivity is known to be detrimental as it has the ability to phosphorylate tau and APP, influencing NFT development and Aβ peptide production, respectively. Thus, targeting the inhibition of GSK3β activity in the brain for the prevention of AD is promising. Li, a mood-stabilizer and the gold-standard treatment for bipolar disorder, is a well-known inhibitor of GSK3β. Individuals who take Li have been shown to have a lower risk of developing AD as compared to individuals taking other mood-stabilizers. Li has also reduced both tau phosphorylation and Aβ production \textit{in vitro} and \textit{in vivo}. However, Li has also been associated with several dose-dependent adverse side effects. The need to evaluate the potential and efficacy of low-dose Li therapy for the purpose of inhibiting GSK3β activity in the brain and preventing AD pathologies while avoiding negative side effects is thus evident.

2.2 Purpose

The purpose of the present investigation is to examine the effects of low-dose Li prophylactic treatment in mice on:

1) GSK3β activity in the brain.
2) the development of AD pathologies (i.e. tau phosphorylation, APP phosphorylation, and Aβ peptide production) alongside HFD-induced obesity and insulin resistance.

2.3 Hypotheses

The overall hypotheses of this thesis are that:

1) the low-dose Li treatment will increase inhibitory GSK3β Ser9 phosphorylation and decrease GSK3β activity in the brain.

2) HFD-induced obesity and insulin resistance will impair mice’s brain insulin signaling, resulting in decreased inhibitory GSK3β Ser9 phosphorylation and GSK3β hyperactivity in the brain.

3) the low-dose Li treatment will prevent the development of AD pathologies in the brain otherwise caused by GSK3β hyperactivity and decreased inhibitory Ser9 phosphorylation.
Chapter 3: Methods

3.1 Materials

The chow diet (2014 Teklad diet; Appendix II) was purchased from Envigo (Huntingdon, UK). The HFD (60% kcal fat; Appendix III; cat#D12492) was purchased from Research Diets Inc. (New Brunswick, NJ). Specific nutrient information of the chow diet and HFD are compared below (Table 3.1). Insulin was purchased from Eli Lilly and Company (Indianapolis, IN).

<table>
<thead>
<tr>
<th>Nutrient Information</th>
<th>Chow</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>13% kcal</td>
<td>60% kcal</td>
</tr>
<tr>
<td>Protein</td>
<td>20% kcal</td>
<td>20% kcal</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>67% kcal</td>
<td>20% kcal</td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
<td>2.9</td>
<td>5.21</td>
</tr>
</tbody>
</table>

Table 3.1: Nutrient information for the chow diet and HFD.

Antibodies against Akt (cat# 4685), pAkt Ser473 (cat# 4058), pAkt Thr308 (cat# 9275), pAPP Thr668 (cat# 6986), BACE (cat# 5606), GSK3α (cat# 9338), pGSK3α Ser21 (cat# 9316), GSK3β (cat# 9315), pGSK3β Ser9 (cat# 5558), SAPK/JNK (cat# 9252), pSAPK/JNK Thr135/Tyr138 (cat# 4668), Tau (cat# 4019), pTau Ser396 (cat# 9632), pTau Ser404 (cat# 20194), β-catenin (cat# 9562), and pβ-catenin Ser33/37/Thr41 (cat# 9561) were purchased from Cell Signaling (Danvers, MA). Antibodies against APP (cat# 825001) and sAPPβ (cat#813401) were purchased from BioLegend (San Diego, CA). Antibodies against pGSK3β Tyr216 (cat# ab75745) and IDE (cat# ab32216) were purchased from Abcam (Cambridge, UK).
3.2 Animals and Design

Ninety-six C57BL/6J male mice (The Jackson Laboratory) arrived at the laboratory at 16 weeks of age. The mice were acclimatized to the animal facility for 1 week prior to experimental interventions beginning. The mice were then randomly separated into two groups: 1) a 6-week study \((n = 24)\) and 2) a 12-week study \((n = 72)\).

Mice assigned to the 6-week study were further separated into one of two other groups: 1) a control group \((\text{CON}; n = 12)\) and 2) a Li-supplemented group \((\text{Li}; n = 12)\) (Figure 3.1A). \(\text{CON}\) mice were fed a standard chow diet and drinking water for 6 weeks while \(\text{Li}\) mice were fed a standard chow diet and Li-supplemented drinking water \((10 \text{ mg Li/kg/day in the form of LiCl})\) for 6 weeks.

Mice assigned to the 12-week study were further separated into one of three other groups: 1) a control group \((\text{CON}; n = 24)\), 2) a HFD group \((\text{HFD}; n = 24)\), and 3) a HFD and Li-supplemented group \((\text{HFD+Li}; n = 24)\) (Figure 3.1B). \(\text{CON}\) mice were fed a standard chow diet and drinking water for 12 weeks. \(\text{HFD}\) mice were fed a HFD \((60\% \text{ kcal from fat})\) and normal drinking water for 12 weeks. Finally, \(\text{HFD+Li}\) mice were fed the same HFD as the \(\text{HFD}\) group, but with Li-supplemented drinking water \((10 \text{ mg Li/kg/day in the form of LiCl})\) for 12 weeks.

All mice were housed individually with a 12:12 hour light:dark cycle. Body weights and food intake were monitored weekly, whereas water intake was monitored twice weekly. Drinking water and added Li supplementation was changed twice weekly according to current body weights and average daily water intake to ensure that the Li dosage of \(10 \text{ mg/kg/day}\) was continuously provided to the mice as accurately as possible. Access to food and drinking water was \textit{ad libitum} for all groups for the entire studies’ durations. All experimental procedures were
approved by the Brock University Animal Care Committee (Appendix I; AUP# 19-04-01) and followed guidelines of the Canadian Council on Animal Care.

Figure 3.1: Timeline of experimental design. Timeline of the A) 6-week study and B) 12-week study.

3.3 Intraperitoneal Glucose and Insulin Tolerance Tests

Intraperitoneal glucose and insulin tolerance testing (IPGTT and IPITT, respectively) was performed on the mice for the purpose of measuring whole-body glucose tolerance and insulin sensitivity, respectively. Both IPGTT and IPITT were performed after 6 and 11 weeks of the diets and treatments for the 6- and 12-week studies, respectively. IPGTT and IPITT were performed 48 hours apart and mice had free access to their respective diets and treatments in between testing.
For the IPGTT, animals were fasted for 6 hours prior to the IP glucose injection (2 g / kg body weight). Blood samples were taken from the tail vein at 0, 15, 30, 45, 60, 90, and 120 minutes post-injection and analyzed using a hand-held glucometer (Freestyle Lite, Abbott). Similarly, for the IPITT, concentrations of blood glucose were determined by sampling blood from the tail vein at 0, 15, 30, 45, 60, and 90 minutes following the IP insulin injection (0.75 U / kg) and analyzed using a hand-held glucometer (Freestyle Lite, Abbott). Plots of the average changes in glucose over time were made for each group and the average total area under the curve (AUC) was calculated. AUC is presented in mmol / L * time and baseline values are set to Y=0.

3.4 Barnes Maze Spatial Memory Test

The Barnes Maze is a circular, dry-land maze that was first developed by Dr. Carol Barnes in 1979 to assess hippocampal-dependent spatial learning and memory in rodents (14, 162). The purpose of this test in the current analysis was to determine potential differences in learning and memory between the groups of mice of the 12-week study only. Importantly, its use is considered advantageously less stressful for rodents in comparison to common, similarly functioning water mazes such as the Morris Water Maze (94, 162).

The Barnes Maze consisted of three phases: 1) the habituation phase, 2) the training phase, and 3) the probe phase. The habituation phase first took place to introduce and habituate the mice to both the room and the maze which would be later used for testing and data collection. The maze used was a circular platform with holes outlining its perimeter (Figure 3.2). With the exception of one, all holes were covered with paper to prevent access by the mice and are considered the incorrect zones. The hole which was not covered (i.e. the correct zone or escape hole) allowed the mice to enter into and access a small cage underneath. Each mouse was
individually placed inside an upside clear beaker and gently guided around the maze for 30 seconds. At the end of the 30 seconds, the mouse was guided over top of the escape hole and left there. After 3 minutes, if the mouse did not enter the escape hole on its own, it was gently nudged into the escape hole and allowed 1 minute to explore the escape cage underneath.

The second phase, or the training phase, took place 24 hours after the habituation phase. During this phase, the mice were again placed individually inside an upside-down clear beaker in the centre of the maze. The beaker was lifted and the mice were allowed 2 minutes to freely explore the maze. If the mouse found and entered the escape hole within the 2 minutes, it was allowed 1 minute to explore the cage underneath before returning to its cage. Only one trial took place for each mouse during this phase.

The third and final phase, the probe phase, took place 48 hours after the training phase. During this phase, the mice were individually placed in the centre of the maze and given 3 minutes to find the escape hole. If the mouse did not find and enter the escape hole within the 3 minutes, the trial ended, and they were returned to their cage. The movements of the mice were tracked and recorded with attention to the amount of time spent in the incorrect and correct zones as well as the general area outside of the outlining zones on the platform. Trials were tracked and recorded using the SMART video tracking software (Panlab, SL, Barcelona, Spain).
3.5 Tissue Collection

Following 6 and 12 weeks of the diets and treatments, mice were anesthetized with sodium pentobarbital (5 mg / 100 g body weight, IP injection). Collection of specific brain regions (i.e. left and right PFC and HIP) were dissected as described previously (183). To determine if Li treatment alters brain insulin sensitivity, brain tissue from mice in the 6-week study were all collected 10 minutes following an IP insulin injection (0.5 U / kg). Alternatively, brain tissue from mice in the 12-week study was either collected under anesthesia alone (n = 36) or 10 minutes following an IP insulin injection (0.5 U / kg; n = 36). Samples were snap frozen in liquid nitrogen and stored at -80°C for later analyses.

3.6 Western Blotting

Brain tissues were homogenized using a FastPrep-FP120 Tissue Homogenizer (Savant) with a 1:20 ratio of mg of sample:µL of lysis buffer (9 mL distilled H₂O, 1 mL 10x RIPA Lysis Buffer, 34 µL phenylmethane sulfonfyl fluoride, and 50 µL protease inhibitor). Homogenized
samples were then centrifuged at 4°C (5 minutes at 10,000 g) and resulting supernatants
collected. Homogenized brain tissues were used for protein quantification via a bicinchoninic
acid (BCA) assay (179). The samples were prepared to contain equal concentrations of protein in
2x Laemmli buffer and placed in a dry bath at 100°C for 5 minutes. 20 µg of protein was loaded
per sample and separated on 7.5-10% SDS-PAGE gels for 90 minutes at 120 V and subsequently
wet transferred onto nitrocellulose membranes. Resulting membranes were blocked in 5% non-
fat dry milk-TBST (tris-buffered saline / 0.1% tween 20) for 1 hour and incubated overnight at
4°C with the appropriate primary antibody. Membranes were then rinsed with TBST and
incubated with the appropriate secondary antibody for 1.5 hours at room temperature.
Membranes were imaged with a ChemiDoc Imager (Bio-Rad Laboratories; Hercules, CA).

3.7 GSK3 Activity Assay

PFC and HIP homogenates were used to measure the activity of GSK3 activity in the
brain. This assay allowed for a more direct measurement of activity in comparison to changes in
relative GSK3β phosphorylation via western blotting and thus provided a greater overall picture
of potential differences in brain GSK3 activity amongst the different groups of mice. The assay
indirectly measured GSK3-specific activity by linking GSK3-induced ATP hydrolysis to NADH
oxidation and depletion by lactate dehydrogenase (Figure 3.3).

10 µL of sample homogenate was loaded into a 96 well plate in triplicate, with each
sample loaded twice in subsequent rows (i.e. 6 wells loaded per homogenate sample). Following
this, 5 µL of GSK3 substrate was added to each well. The GSK3 inhibitor was warmed in a dry
bath at 50°C for approximately 5 minutes and 0.25 µL was then added to each well of only one
of the two triplicate rows of homogenate sample. This allowed for a comparative, indirect
analysis of GSK3 activity by subtracting the average overall activity (i.e. the sample rows
without added inhibitor) by the average overall activity with GSK3 inhibition (i.e. the sample rows with added inhibitor). Following this, 100 µL of a reaction buffer (i.e. 7.5 mL ATPase buffer, 28 µL lactate dehydrogenase [18 U’ml⁻¹], and 36 µL pyruvate kinase [18 U’ml⁻¹],) was added to each well. Lastly, 4 µL of NADH (1.9% w/v) was added to each well and the plate was read at an absorbance of 340 nm for 30 minutes.

**Figure 3.3: Enzyme-linked reaction used to indirectly quantify GSK3 activity.** ATP hydrolysis via GSK3 substrate phosphorylation is linked to the depletion of added NADH in each sample measured at an absorbance of 340nm.

3.8 Statistical Analysis

A two-way repeated measures ANOVA was used to analyze changes in body weights, food intake, and water intake over time as well as the IPGTT and IPITT results. For the IPGTT and IPITT results, the AUC was also calculated and analyzed using a one-way ANOVA. A relationship was considered significant when p<0.05. Data are presented as mean ± the standard error of the mean (SEM).

A t-test analysis was used to determine differences in western blot and GSK3 activity assay analyses between CON and Li mice of the 6-week study. A one-way ANOVA with Tukey’s post-
hoc analysis was used to determine differences in western blot and GSK3 activity assay analyses between the groups of the 12-week study. A relationship was considered significant when p<0.05. Data are presented as mean ± the standard error of the mean (SEM).
Chapter 4: Results

4.1 6-week Study

Body Weights and Water Intake

CON and Li mice did not differ in body weight throughout the entire 6-week study duration (CON 30.55 ± 0.17 g; Li 29.95 ± 0.19 g; Figure 4.1A). Importantly, there was also no significant difference in average body weight between the CON and Li mice at the start of the study intervention (CON 30.28 ± 0.46 g; Li 29.66 ± 0.57 g).

There were no differences in the average daily water intake between CON and Li mice, except during weeks 4.5, 5.0, and 5.5, during which the Li mice drank significantly more water per day on average (1.18 mL, 1.19 mL, and 2.49 mL more on average, respectively; p<0.01, p<0.05, and p<0.01, respectively; Figure 4.1B). However, the overall average water intake over the 6-week study duration was not significantly different between the two groups (CON 5.88 ± 0.56 mL / day; Li 6.62 ± 0.48 mL / day; Figure 4.1C). Important to note, is that the mice were subjected to the IPGTT and IPITT during the final week of the study duration (i.e. week 6), and likely influenced the relative increase in average water intake observed during this time.
Figure 4.1: 6-week body weights and water intake. A) Weekly average body weights of CON and Li mice. B) Average daily water intake per week and C) average water intake overall of CON and Li mice. Data are presented as means ± SEM. * p<0.05, ** p<0.01 relative to CON.

**IPGTT and IPITT**

IPGTT and IPITT were performed to assess possible changes in whole-body glucose and insulin tolerance respectively between the CON and Li mice at the end of the 6-week study duration. No significant changes in glucose tolerance were observed between the two groups following 6 weeks of the respective treatments as determined by the IPGTT and corresponding AUC (Figure 4.2A & 4.2B). Similarly, no differences in insulin tolerance were observed
between the CON and Li mice as determined by the IPITT and corresponding AUC (Figure 4.2C & 4.2D).

![Figure 4.2: 6-week IPGTT and IPITT results and AUC. A) IPGTT results and B) corresponding AUC for CON and Li mice. C) IPITT results and D) corresponding area under the curve for CON and Li mice. Data are presented as means ± SEM.](image)

**Markers of GSK3β Signaling**

GSK3β signaling in the brain was next examined to assess if the 6-week low-dose Li supplementation provided to the Li mice altered the activity of this pathway compared to the CON mice. Phosphorylation of Akt at two of its activating residues, Ser473 and Thr308, was first measured due to the ability of Akt to directly alter GSK3β phosphorylation and activity. In the PFC, relative phosphorylation of Akt at the Ser473 site remained unchanged between the
groups (Figure 4.3A). However, relative Akt Thr308 phosphorylation was significantly increased in the Li group compared to CON mice (p<0.05; Figure 4.3A). In the HIP, relative phosphorylation of both Akt residues were not significantly different between the two groups (Figure 4.3B). No differences were observed between groups for total Akt content in either the PFC or HIP (p>0.05).

Figure 4.3: Relative Akt Ser473 and Thr308 phosphorylation in Li and CON mice post-insulin injection. A) Akt phosphorylation in the PFC and corresponding representative blots. B) Akt phosphorylation in the HIP and corresponding representative blots. Data are presented as mean ± SEM. * p<0.05 relative to CON.
Immediately downstream of Akt, relative inhibitory GSK3β Ser9 phosphorylation was unaltered between the Li and CON groups in both the PFC and HIP (Figure 4.4A & 4.4B). Similarly, relative Tyr216 phosphorylation of GSK3β, an activating residue, remained unchanged between the groups in both the PFC and HIP (Figure 4.4A & 4.4B). No differences were observed between groups for total GSK3β content (p>0.05).

Figure 4.4: Relative GSK3β Ser9 and Tyr216 phosphorylation in Li and CON mice post-insulin injection. A) GSK3β phosphorylation in the PFC and corresponding representative blots. B) GSK3β phosphorylation in the HIP and corresponding representative blots. Data are presented as mean ± SEM.
Markers of AD Pathology

Tau and APP phosphorylation was measured to assess potential changes in markers of AD pathologies in the brain between the CON and Li mice. Relative tau Ser396 phosphorylation was unaltered between the CON and Li groups in both the PFC and HIP regions (Figure 4.5A & 4.5B). Similarly, relative APP Thr668 phosphorylation was unchanged between the two groups in both regions of the brain (Figure 4.6A & 4.6B). No differences were observed between groups for total tau or APP content (p>0.05).

Figure 4.5: Relative Tau Ser396 phosphorylation in Li and CON mice post-insulin injection. A) Tau phosphorylation in the A) PFC and B) HIP and corresponding representative blots. Data are presented as mean ± SEM.
Figure 4.6: Relative APP Thr668 phosphorylation in Li and CON mice post-insulin injection. A) APP phosphorylation in the A) PFC and B) HIP and corresponding representative blots. Data are presented as mean ± SEM.

GSK3 Activity Assay

To be able to compare GSK3β’s phosphorylation status to its activity, the specific activity of GSK3 was next assessed. In the HIP, GSK3 activity was relatively increased in the Li treated mice compared to CON mice, however statistical significance was not reached (p = 0.06; Figure 4.7).
Figure 4.7: GSK3-specific activity in the HIP of Li and CON mice. Data are presented as mean ± SEM.

4.2 12-week Study

Body Weights, Food Intake, and Water Intake

For the 12-week study, the mice’s food intake was measured once weekly to assess any potential differences in food intake between groups over time. HFD and HFD+Li mice had an overall greater daily average food intake compared to CON mice (CON 5.60 ± 0.56 g/day; HFD 8.00 ± 0.74 g/day; HFD+Li 9.55 ± 1.48 g/day; p<0.05; Figure 4.8A). Average daily food intake for each group per week is depicted in Figure 4.8B. HFD+Li mice ate significantly more than HFD mice during weeks 2, 3 and 4 (p<0.05, p<0.05, and p<0.01, respectively). From weeks 3-6, HFD mice ate more than CON mice (p<0.0001, p<0.01, p<0.05, and p<0.05, respectively), as did the HFD+Li mice (p<0.0001, p<0.0001, p<0.001, and p<0.01, respectively). After week 6, there were no differences in the amount of food consumed between groups each week.

As in the 6-week study, average water intake was also monitored twice weekly to ensure
accurate Li dosages were provided and as well as to assess any potential differences in average water consumed between groups. Overall, there were no differences in average daily water intake between the three groups (CON 7.75 ± 0.42 mL/day; HFD 7.75 ± 0.51 mL/day; HFD+Li 8.22 ± 0.47 mL/day; Figure 4.8C). Average daily water intake for each group per week is depicted in Figure 4.8D. Importantly, similar to the 6-week study, mice in the 12-week study were subjected to the IPGTT and IPITT during the final week, and likely influenced the relative increase in average water intake during this time.
Figure 4.8: 12-week average food and water intake. A) Overall average daily food intake and B) average food intake per week between groups. C) Overall average daily water intake and D) average water intake per week between groups. Data are presented as means ± SEM. * p<0.05 relative to CON; # p<0.05 relative to HFD; ~ p<0.05 relative to HFD+Li.

Lastly, body weights were monitored once weekly to assess potential differences in body weights between groups over the 12-week study period. HFD and HFD+Li mice weighed significantly more than CON mice as early as the second week of their respective treatments (CON 30.18 ± 0.34 g; HFD 32.51 ± 0.50 g; HFD+Li 32.64 ± 0.55 g; p<0.01; Figure 4.9A). The differences in body weights between these groups continued to increase over the remaining
course of the study duration, reaching a significance of \( p<0.001 \) by week 3 (CON 30.70 ± 0.31 g; HFD 34.34 ± 0.72 g; HFD+Li 33.71 ± 0.68 g) and \( p<0.0001 \) by week 4 (CON 30.81 ± 0.46 g; HFD 37.58 ± 0.68 g; HFD+Li 37.08 ± 0.71 g). HFD and HFD+Li mice did not differ in body weight throughout the 12 weeks. Importantly, there were also no differences in body weights between groups at the start of the study duration (CON 30.33 ± 0.34 g; HFD 29.62 ± 0.34 g; HFD+Li 30.41 ± 0.25 g).

To further analyze potential effects of the Li supplementation on body metrics alongside the HFD, differences in total body weight gained, total kcals consumed, and overall caloric efficiency was calculated between the HFD and HFD+Li mice. While there were no differences between total body weight gained (HFD 21.45 ± 0.74 g; HFD+Li 20.80 ± 0.46 g; Figure 4.9B), the HFD+Li mice consumed slightly more kcals compared to HFD mice, although non-significant (HFD 2857 ± 362.4 kcal; HFD+Li 3508 ± 330.0 kcal; \( p=0.19 \); Figure 4.9C). However, the caloric efficiency, defined as grams of body weight gained per kcal consumed, was significantly less in the HFD+Li mice compared to HFD mice (HFD 0.01 ± 0.001 g / kcal; HFD+Li 0.007 ± 0.0007 g / kcal; \( p<0.05 \); Figure 4.9D).
**Figure 4.9: 12-week average body weights and caloric efficiency.** A) Average body weights per week between groups. B) Total body weight gained. C) total kcal consumed, and D) overall caloric efficiency between HFD and HFD+Li mice. Data are presented as means ± SEM. *p*<0.05.

**IPGTT and IPITT**

After 11 weeks of the respective treatments, the mice underwent IPGTT and IPITT to analyze the potential effects of the diets and Li supplementation between groups on whole-body glucose and insulin tolerance, respectively. In the IPGTT, HFD and HFD+Li mice had significantly greater blood glucose levels 15, 30, 60, and 120 minutes after the glucose injection compared to CON mice (p<0.05; Figure 4.10A). Additionally, the HFD mice had significantly greater blood glucose levels 15 and 30 minutes after the glucose injection compared to HFD+Li
mice (p<0.05; Figure 4.10A). These differences in blood glucose levels were reflected in the AUC data, with the HFD and HFD+Li group being significantly greater than the CON group (p<0.0001; Figure 4.10B). However, there was no significant difference between the HFD and HFD+Li groups’ AUCs.

For the IPITT, the HFD+Li mice had significantly higher blood glucose levels at all time points compared to CON mice (i.e. 0, 15, 30, 60, and 120 minutes; p<0.05; Figure 4.10C). HFD mice, however, had significantly greater blood glucose levels only at the 15, 60, and 120 minute time points relative to CON mice (p<0.05; Figure 4.10C). Again, this was reflected in the AUCs, such that both the HFD and HFD+Li mice had a significantly greater AUC relative to CON mice (p<0.05; Figure 4.10D).
Barnes Maze Spatial Memory Test

The Barnes Maze was conducted next to assess changes in cognitive performance and, more specifically, spatial memory between the groups during the final week of the study. First, the percentage of mice that passed or failed per group was calculated, with no significant differences in the total numbers of passes or fails between groups (CON 83% pass; HFD 67% pass; HFD+Li 62.5% pass; Figure 4.11A). Secondly, latency time to the correct zone, or the time it took for the mice to reach the correct zone, was measured. However, no significant differences
between groups were observed for this measure as well (CON 77.73 ± 12.20 secs; HFD 76.91 ± 14.86 secs; HFD+Li 102.90 ± 12.70 secs; Figure 4.11B). Permanence time spent in the incorrect zones by the mice was next assessed, although no significant differences were observed between groups (CON 134.90 ± 11.09 secs; HFD 144.30 ± 9.66 secs; HFD+Li 157.00 ± 7.28 secs; Figure 4.11C). Lastly, in order to assess possible effects of the HFD on the overall movements and activity of the mice during the test, average speed and total distance travelled were analyzed. The HFD and HFD+Li mice had a significantly slower speed relative to the CON mice (CON 6.54 ± 0.67 cm / sec; HFD 3.86 ± 0.42 cm / sec; HFD+Li 3.68 ± 0.51 cm / sec; Figure 4.11D; p<0.01). Correspondingly, HFD and HFD+Li mice also travelled significantly less distance in total compared to the CON mice (CON 1622 ± 137.6 cm; HFD 1097 ± 69.75 cm; HFD+Li 1088 ± 49.52 cm; Figure 4.11E; p<0.001).
Figure 4.11: Barnes Maze results. A) Percent of mice that passed or failed the test per group, B) the time it took to reach the correct zone (i.e. latency time), C) total time spent in the incorrect zones, D) average speed of the mice, and E) total distance travelled by the mice. Data are presented as means ± SEM. ** p<0.01, *** p<0.001 relative to CON.

Diet-Influenced Markers

The next aim was to assess potential influences of the HFD on the mice at the protein level, and if the Li supplementation may have altered or protected against such influences.

Previous literature has shown reductions in IDE content in the brains of metabolically distressed animals (i.e. animals with diabetes or fed a HFD) (112, 114), an important enzyme involved in the clearance of both insulin as well as Aβ peptides. Thus, IDE content was first assessed in both the PFC and HIP. In the PFC, HFD mice had significantly less IDE content compared to CON mice (p<0.05; Figure 4.12A). Interestingly, this reduction in IDE was blunted in the HFD+Li
mice, with no statistical difference observed compared to the CON mice. In the HIP, however, no differences in IDE content was observed between any of the groups (Figure 4.12C).

Figure 4.12: IDE content in CON, HFD, and HFD+Li mice post-insulin injection. A) Relative IDE content in the PFC and C) HIP. Corresponding representative blots are depicted in B) and D), respectively. Data are presented as mean ± SEM. * p<0.05 relative to CON.

Previous work has also demonstrated increases in pJNK content in the brains of mice fed a HFD (114). Importantly, JNK can also be phosphorylated by active GSK3β. Thus, pJNK Thr135/Tyr138 content was next assessed. However, no differences in total or pJNK was
observed between groups in the PFC nor the HIP (Figure 4.13A & 4.13B and Figure 4.14A & Figure 4.14B, respectively).

Figure 4.13: Total JNK content and relative Thr135/Tyr138 phosphorylation in the PFC of CON, HFD, and HFD+Li mice. A) Total JNK content, B) relative pJNK content, and C) corresponding representative blots. Data are presented as mean ± SEM.

Figure 4.14: Total JNK content and relative Thr135/Tyr138 phosphorylation in the HIP of CON, HFD, and HFD+Li mice. A) Total JNK content, B) relative pJNK content, and C) corresponding representative blots. Data are presented as mean ± SEM.
Markers of GSK3β Signaling

To assess the effects of both the diets and the Li supplementation on GSK3β signaling in the brain, relative Akt phosphorylation at both the Ser473 and Thr308 sites was analyzed. In the PFC, both total and pAkt Ser473 remained unchanged (Figure 4.15B & 4.15C). However, pAkt Thr308 was significantly increased in the HFD mice compared to CON mice (p<0.01; Figure 4.15D).

In the HIP, total Akt content was, again, unchanged between groups (Figure 4.16B). Akt Ser473 phosphorylation, however, was significantly increased in the HFD+Li mice compared to CON mice (p<0.05; Figure 4.16C), and pAkt Thr308 in the HFD+Li mice approached significance relative to CON mice (p = 0.09; Figure 4.16D).
Figure 4.15: Total Akt and relative pAkt content in the PFC post-insulin injection. B) Total Akt, C) relative pAkt Ser473, and D) relative pAkt Thr308 content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM. ** p<0.01 relative to CON.
GSK3β phosphorylation in the brain was next examined. In the PFC, total GSK3β content was reduced in both the HFD and HFD+Li mice compared to CON mice (p<0.05; Figure 4.17B). Furthermore, inhibitory GSK3β Ser9 phosphorylation was significantly increased in the HFD+Li mice relative to CON mice (p<0.05; Figure 4.17C). Phosphorylation at GSK3β’s activating residue, Tyr216, was also significantly increased in both HFD and HFD+Li mice relative to CON mice (p<0.05 and p<0.01, respectively; Figure 4.17D).
In the HIP, however, total GSK3β content was not different between the three groups (Figure 4.18B). Inhibitory GSK3β Ser9 phosphorylation was also significantly increased in the HFD+Li mice relative to CON mice (p<0.01; Figure 4.18C). Interestingly, HFD mice also had relatively increased Ser9 phosphorylation compared to CON mice (p<0.05; Figure 4.18C). Lastly, GSK3β Tyr216 phosphorylation was also increased, but only in the HFD+Li mice relative to CON mice in the HIP region (p<0.05; Figure 4.18D).

**Figure 4.17:** Total GSK3β and relative pGSK3β content in the PFC post-insulin injection. B) Total GSK3β, C) relative pGSK3β Ser9, and D) relative pGSK3β Tyr216 content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM. * p<0.05, ** p<0.01 relative to CON.
As Li may also have effects on the other GSK3 isoform, GSK3α, GSK3α’s phosphorylation status was next measured and compared between groups. Phosphorylation at the Ser21 site is mechanistically similar to Ser9 phosphorylation of GSK3β, allowing for inhibition through physically blocking primed substrates from binding to GSK3α’s primed substrate binding domain (21). Total content and relative GSK3α Ser21 phosphorylation remained
unchanged in the PFC (Figure 4.19A & 4.19B). In the HIP, total GSK3α content also remained unchanged (Figure 4.20A), however, pGSK3α Ser21 was significantly increased in HFD+Li mice compared to CON mice (p<0.05; Figure 4.20B).

Figure 4.19: Total GSK3α and relative pGSK3α Ser21 content in the PFC post-insulin injection. A) Total GSK3α, B) relative pGSK3α, and C) corresponding representative blots. Data are presented as mean ± SEM.

Figure 4.20: Total GSK3α and relative pGSK3α Ser21 content in the HIP post-insulin injection. A) Total GSK3α, B) relative pGSK3α, and C) corresponding representative blots. Data are presented as mean ± SEM. * p<0.05 relative to CON.
β-catenin is a key marker in the Wnt signaling pathway and is immediately downstream of GSK3β. Thus, potential changes in β-catenin phosphorylation at the Ser33/37/Thr41 residues was also examined in the brain as a way of addressing differences GSK3β activity as a result of the diets and Li supplementation. However, in both the PFC and HIP, total and pβ-catenin Ser33/37/Thr41 content remained unaltered between groups (Figure 4.21A & 4.21B and Figure 4.22A & 4.22B, respectively).

**Figure 4.21: Total β-catenin and relative pβ-catenin Ser33/37/Thr41 content in the PFC post-insulin injection.** A) Total β-catenin, B) relative pβ-catenin, and C) corresponding representative blots. Data are presented as mean ± SEM.
Figure 4.22: Total β-catenin and relative pβ-catenin Ser33/37/Thr41 content in the HIP post-insulin injection. A) Total β-catenin, B) relative pβ-catenin, and C) corresponding representative blots. Data are presented as mean ± SEM.

Markers of AD Pathology

For the 12-week study, two different tau phosphorylation sites were examined- Ser396 and Ser404. Total tau content was significantly reduced in the PFC, but not the HIP, of the HFD mice compared to CON mice (p<0.05; Figure 4.23B & Figure 4.24B). Relative tau Ser396 and Ser404 phosphorylation both, however, remained unchanged between groups in both regions of the brain (Figure 4.23C & 4.23D and Figure 4.24C & 4.24D).
Figure 4.23: Total Tau and relative pTau content in the PFC. B) Total Tau, C) relative pTau Ser396, and D) relative pTau Ser404 content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM. * p<0.05 relative to CON.
Figure 4.24: Total Tau and relative pTau content in the HIP. B) Total Tau, C) relative pTau Ser396, and D) relative pTau Ser404 content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM.

APP phosphorylation as well as sAPPβ content, a by-product of the amyloidogenic pathway, was next measured. No differences in total APP content or relative APP Thr668 phosphorylation was observed between groups in the PFC nor the HIP (Figure 4.25B & 4.25C and Figure 4.26B & Figure 4.26C, respectively). sAPPβ content remained unchanged between groups in both regions of the brain as well (Figure 4.25D & Figure 4.26D).

Lastly, BACE was measured to see if its protein content varied with the mice’s diets and
Li supplementation. However, BACE protein content was not different between groups in the both the PFC nor the HIP (Figure 4.25E & Figure 4.26E, respectively).

![Diagram of protein content](Image)

**Figure 4.25**: Total APP, relative pAPP, and amyloid-related marker content in the PFC. B) Total APP, C) relative pAPP Thr668, D) sAPPβ, and E) BACE content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM.
Figure 4.26: Total APP, relative pAPP, and amyloid-related marker content in the HIP. B) Total APP, C) relative pAPP Thr668, D) sAPPβ, and E) BACE content in the CON, HFD, and HFD+Li mice. Corresponding representative blots are depicted in A). Data are presented as mean ± SEM.
Chapter 5: Discussion

5.1 Summary of Main Findings

GSK3β hyperactivity in the brain has been linked to the development of AD pathologies, and targeting its inhibition has been implicated as a potential AD prophylactic. The current investigation sought to determine if supplementation with Li, a GSK3β inhibitor, at a low-dose would result in 1) GSK3β activity inhibition in the brain, and 2) restore reductions in diet-induced inhibitory GSK3β Ser9 phosphorylation in the brain and consequently prevent the development of AD pathologies in mice. Our results demonstrate that at 6 weeks, GSK3β phosphorylation does not vary with Li supplementation in neither the PFC or HIP, however GSK3-specific activity was decreased in the HIP. These results highlight the potential for low-dose Li supplementation to reduce GSK3 activity in the brain, despite a lack of changes in phosphorylation. Previous studies have also demonstrated diet-induced detriments to brain health and function including increased GSK3β activity and reduced IDE content in the brain. Thus, we next examined GSK3β phosphorylation after 12 weeks of Li supplementation alongside a obesogenic diet. We observed changes in GSK3β’s phosphorylation status, with Li-supplemented mice having significantly greater inhibitory Ser9 phosphorylation compared to CON mice. Despite these changes, phosphorylation of targets downstream of GSK3β including β-catenin and markers specifically related to AD pathologies (i.e. tau, APP, sAPPβ, and BACE) were not influenced by Li. Taken together, these results suggest that while a low-dose Li supplementation does not change pGSK3β Ser9 content in the brain until 12 weeks, a reduction in its actual activity can be observed as early as 6 weeks. This result is important as it implicates that the activity of GSK3β may be altered despite a lack of changes in inhibitory or activating phosphorylation. An even longer supplementation may be needed to observe beneficial
functional changes such as protection from cognitive impairments and reduced AD pathologies in the brain. Overall, these data are important as they demonstrate the ability of a low-dose Li supplementation to effectively increase inhibitory Ser9 phosphorylation and decrease GSK3 activity in various regions in the brain, and moreover, a rough time-course of these cellular events.

5.2 GSK3β and Low-Dose Li Supplementation

Previous work has evaluated the effects of low-dose Li supplementation on GSK3β activity in various tissues. For example, Li supplemented-mice have been shown to have significantly increased inhibitory GSK3β Ser9 phosphorylation in bone tissue, which was associated with Wnt/β-catenin signaling activation and a shift towards bone formation (118). In other work, GSK3-specific activity was significantly decreased in the left ventricle of the heart of Li-supplemented mice, which was ultimately linked to improved calcium regulation in myocytes (85). Lastly, a study investigating the effects of LiCl on HFD-induced artherosclerosis in ApoE-deficit mice showed vascular-related benefits with LiCl supplementation including reduced lipid accumulation and macrophage infiltration rates in the aorta and aortic valve (42).

Each of the above studies had used the same low-dose Li dosage and delivery method (i.e. 10 mg / kg / day in drinking water) and resulted in a serum Li concentration of 0.02mM (85, 118). Based on these beneficial results in other tissues, we chose to utilize this same Li dosage in order to study the effects on GSK3β activity in the brain.

The ability to accurately measure GSK3β activity in the brain has proven difficult and is met with several challenges (56). For example, there are a multitude of phosphorylation sites on GSK3β which each have the potential to influence its activity. However, how the combination of these phosphorylation sites integrate and translate to actual activity is much less clear. It is well
understood that Ser9 phosphorylation allows for GSK3β inhibition, while phosphorylation of the Tyr216 residue increases its activity (56, 69). While these are the main modifiable residues that are recognized and most commonly studied in the literature, there are also a few other phosphorylation sites on GSK3β which influence its activity, although to a much lesser degree (56). To obtain a clearer picture, Duthie and colleagues (2019) provide a list of potential biomarkers of GSK3 activity that can be measured, including both upstream regulatory pathways (e.g. PI3K/Akt and MAPK signaling) and downstream targets (e.g. glycogen synthase, β-catenin, and tau) (56).

We began assessing an overall picture of GSK3β activity by measuring upstream Akt phosphorylation at 6 weeks in CON and Li-treated mice. Our results show that Akt Thr308, but not Ser473, phosphorylation is higher in the Li mice in the PFC, but not the HIP. As it is the Thr308 residue that is directly phosphorylated by upstream PDK1 in the insulin signaling cascade, this result is indicative of increased insulin sensitivity in the Li mice. These results also highlight regional differences, indicating that certain regions of the brain may be more sensitive to insulin stimulation with Li supplementation and/or at different timepoints.

To more directly assess GSK3β activity, we compared Ser9 and Tyr216 phosphorylation between the CON and Li mice. At 6 weeks, GSK3β phosphorylation at both residues was unchanged in the brain between groups. However, GSK3-specific activity was reduced in the Li mice compared to CON mice. This finding is important as it suggests that GSK3 activity does not perfectly correspond to its phosphorylation status, and that it may differ despite a lack of modifications to its key residues. Indeed, alterations in GSK3 activity have been observed previously without any changes in phosphorylation. For example, in a study examining the effects of LiCl supplementation in murine hearts, while GSK3-specific activity in the left
ventricle was significantly reduced compared to control mice, changes in relative inhibitory GSK3β Ser9 nor GSK3α Ser21 phosphorylation were not observed (85).

5.3 Effects of an Obesogenic Diet

Obesity and metabolic disease (i.e. T2DM) is linked to the development of AD and increases the production of its pathologies in the brain including tau hyperphosphorylation and Aβ peptides. We thus used a HFD that has been repeatedly shown to induce obesity and metabolic distress in mice (13, 132, 207) to replicate these effects in the mice in our study, and furthermore, influence a disease profile like that of prodromal or early AD. Significant increases in weight in the HFD-fed mice were observed as early as the second week of feeding and continued to increase steadily over the remainder of the study duration. To assess whole-body metabolism and, more specifically, glucose and insulin tolerance, IPGTT and IPITT were performed. In our 12-week analysis, HFD mice had an increased AUC for both the IPGTT and IPITT compared to CON mice, as anticipated and also seen repeatedly in previous studies (13, 40, 80, 132). Together, these results demonstrate the ability of this HFD to induce whole-body metabolic distress by rapidly causing weight gain as well as a diabetic phenotype (i.e. glucose intolerance and insulin resistance) in the mice.

To observe a more brain-specific effect of the HFD and more particularly, its relation to GSK3β activity, we measured upstream Akt phosphorylation. We found that pAkt Thr308 was significantly increased in HFD mice compared to CON mice in the PFC only. These results conflict with our hypotheses that a HFD would impair insulin signaling (i.e. reduced Akt phosphorylation). While there are studies that have reported increases in Akt phosphorylation in the mouse brain alongside a HFD (12, 114, 132) as well as in the AD brain (77), the mechanistic reasoning for these results are unclear. It has been previously demonstrated that under
hyperglycemic conditions, Akt phosphorylation is increased at both the Thr308 and Ser473 residues in both the cerebral cortex and HIP of streptozotocin-treated mice (44). This could suggest that chronically elevated glucose levels can cause aberrant Akt signaling and activation in the brain. Further investigation is needed to better understand these abnormalities in Akt phosphorylation observed in the brain under chronic metabolic stress.

We also quantified both the inhibitory Ser9 and activating Tyr216 GSK3β phosphorylation in the brain as a measure of its activity. At 12 weeks, Ser9 and Tyr216 phosphorylation was significantly increased in the HFD mice compared to CON mice in the HIP and PFC, respectively. While an increase in Tyr216 phosphorylation, demonstrating an increase in diet-induced GSK3β activity, was anticipated, the increase in Ser9 phosphorylation was not. These results suggest that the HFD provided to these groups of mice has the ability to influence either inhibitory or activating phosphorylation in different regions of the brain. It is possible that these changes do indeed translate to varying levels of GSK3β activity across different areas of the brain. However, a more specific GSK3-activity assay would be needed to verify this hypothesis as well as a further investigation to understand the potential reasoning behind these regional differences in activity alongside a HFD.

Both IDE and pJNK have previously been reported to be altered by a HFD or in an obesogenic state, specifically with decreased IDE content (114, 117) and increased pJNK and JNK activity (12, 108, 181, 207). In our study, no changes in pJNK Thr135/Tyr138 were observed, however IDE content was significantly reduced in the PFC, but not the HIP, of the HFD mice compared to CON mice at 12 weeks. Sufficient IDE levels are critical for the clearance of not only insulin, but also Aβ peptides in the brain (117, 145). Reduced levels caused by the HFD can thus lead to hyperinsulinemia as well as increased Aβ accumulation. This result
is particularly important, as it demonstrates that the HFD used in our analysis has the potential to influence AD pathologies in the brain and resembles what the disease may look like in its early stages.

In further support of the idea that the our HFD model at 12 weeks resembles an early stage of AD, we did not find any significant changes in AD-related pathologies in the brain including phosphorylated tau or APP-related pathologies (i.e. pAPP Thr668, sAPPβ, and BACE content). In the earlier stages of AD, these physiological hallmarks may not yet be altered, but rather preoccurring changes to regular mechanisms and functions (e.g. reduced IDE content or increased GSK3β Tyr216 phosphorylation and activity) are first observed. Thus, it is possible that if our study extended beyond 12 weeks, we would begin to see a more exacerbated AD profile. Furthermore, at the end of the 12-week study duration, the mice were approximately 7 months of age, which roughly correlates to a period equilvant to that between mature adulthood and middle age in humans (57). This time period is fairly early for humans, typically a period before AD pathologies are detectable or are just beginning to develop, particularly in at risk populations (3, 55). Nevertheless, it is a sensitive timeframe for future disease development, and the continuation of the HFD and persistence of whole-body metabolic distress certainly sets the stage for AD later on.

5.4 Effects of Li Supplementation with Obesogenic Diet Markers

To test our hypothesis for low-dose Li to protect against the detriments of an obesogenic diet, we observed and compared HFD-fed mice in combination with Li drinking water. HFD+Li mice had a reduced caloric efficiency compared to the HFD mice, meaning the HFD+Li mice gained less weight per calorie consumed. This result was particularly intriguing, as no significant
differences in body weights or overall food and water intake was observed between these groups. However, the average calories consumed was indeed higher in the HFD+Li mice (3508 ± 330 kcal) compared to the HFD mice (2857 ± 362 kcal). Previous studies have found that Li supplementation in *Drosophila* helped protect against a reduction in lifespan associated with a high-sucrose diet and blocked whole-body triglyceride accumulation (38, 177). These beneficial effects were dependent on increased inhibitory Ser9 phosphorylation of the GSK3 fly ortholog, Shaggy (Sgg) (38). It is possible that the reduction in caloric efficiency observed in the HFD+Li mice is associated with Li-induced GSK3β inhibition at the whole-body level. *In vitro*, GSK3β activity has been shown to be essential for adipogenesis (i.e. the production of new adipocytes), and that the application of a GSK3 inhibitor or knocking-down GSK3 inhibited adipocyte differentiation reduces lipid accumulation (197). Further, the inhibition of GSK3 activity has been linked to an upregulation of genes related to adipose tissue browning (135), and unpublished data from our lab group demonstrates that the same low dose Li supplementation utilized in the current thesis results in an increase in uncoupling protein 1 (UCP1) content in white adipose tissue (Ryan et al. manuscript in preparation). However, the literature regarding Li, GSK3β activity, and whole-body metabolism is lacking and conflicting. Chen and colleagues (2016) reported that PI3K-resistant GSK3 (i.e. both GSK3β and GSK3α were unable to be phosphorylated and inhibited) mice had increased serum levels of adiponectin, a hormone which can help prevent metabolic disease, compared to wild-type mice (40). Their results are thus indicative of GSK3 activity as being beneficial and capable of preventing metabolic impairments. Further studies on this topic are needed to clarify this relationship and the particular role which Li supplementation may play.

Li has also been shown to have insulin-mimetic effects (163). Groot and colleagues
(2017) provided 12-week old female obese mice with 0, 10, and 40 mmol LiCl / kg in their chow diet for 12 weeks and subsequently underwent IPGTT (80). Mice that were provided with the 40 mmol LiCl / kg dose had significantly lower blood glucose levels 15 minutes after the glucose injection compared to the mice given no Li, while there was no difference in the mice provided the 10 mmol LiCl / kg dose. Furthermore, non-fasting blood glucose levels were also significantly lower in the 40 mmol LiCl / kg group compared to the mice given no Li, suggestive of improved glucose tolerance and homeostasis. Despite differences at a few timepoints, overall the HFD+Li mice did not experience any improvements in glucose tolerance compared to the HFD mice according the AUC data. Similar results were also observed for the resulting AUC data from the IPITT. These results might suggest that at the whole-body level, the low-dose Li supplementation may not have been enough to allow for protective, insulin-like effects against the HFD-induced impairments to glucose and insulin tolerance. The aforementioned study by Groot and colleagues (2017) supports this idea, as the higher 40 mmol LiCl / kg dose was enough to relatively improve blood glucose levels during the IPGTT, but the lower dose of 10 mmol LiCl / kg was not.

For Akt phosphorylation, we observed increases only at the Ser473 site in the HFD+Li mice in the HIP compared to CON mice. Again, the increased Akt phosphorylation at the Ser473 site suggests of increased insulin sensitivity in this group. Furthermore, increases in phosphorylation at the Ser473 residue may indicate long-term Akt activation, as it is typically phosphorylated second to Thr308 in a biphasic manner following insulin stimulation (12).

After 12 weeks, as predicted, inhibitory GSK3β Ser9 phosphorylation was increased in the HFD+Li mice in both the HIP and PFC. Tyr216 phosphorylation was also increased in the HFD+Li mice in both regions of the brain compared to CON mice. Mechanisms responsible for
GSK3β Tyr216 phosphorylation are not as well understood as the mechanisms resulting in Ser9 phosphorylation, however some possibilities have been discussed. For example, proapoptotic conditions such as the absence of nerve growth factor and toxicity caused by staurosporine, an alkaloid with anti-cancer and apoptotic properties, have been shown to increase GSK3β Tyr216 phosphorylation in PC12 cells and SH-SY5Y cells, respectively (25, 150). The possibility of autophosphorylation has also been suggested, but remains controversial (25). However, a specific tyrosine kinase or signaling pathway that is responsible for GSK3β Tyr216 phosphorylation has yet to be identified. An interesting finding has also implicated that Li treatment does not have any further effects on Tyr216 phosphorylation that is already induced under apoptotic conditions, as demonstrated in vitro using PC12 cells (25). In this same work, the Li treatment (10 mM for 16 hours) was successful in increasing Ser9 phosphorylation and decreasing GSK3β activity as measured by a kinase assay, indicating the ability of Li-induced Ser9 phosphorylation to effectively override Tyr216 phosphorylation to inhibit GSK3β activity when both are present. Considering this with respect to our current results, it is possible that where both Ser9 and Tyr216 phosphorylation was observed (i.e. in the PFC and HIP of the HFD+Li mice), the effects of Ser9 overpowers that of Tyr216, allowing for overall GSK3β inhibition in Li-supplementated mice across multiple brain regions.

In addition to changes in phosphorylation, total GSK3β content was also reduced in both HFD and HFD+Li mice in the PFC, but not the HIP, at 12 weeks. In a study examining methamphetamine-induced neurodegeneration in adult male rats, daily i.p. administration of Li for 28 days reduced levels of total GSK3β in the HIP (138). Alternatively, studies implementing various HFDs in mice have seemingly only observed changes in relative phosphorylation and not in total GSK3β content (7, 24). In our current study, since there were no differences in total
GSK3β content between the HFD and HFD+Li mice, it is likely that these differences observed relative to CON mice were merely an effect of the HFD provided, and not the Li supplementation. It is possible that this decrease in total content is an initial compensatory mechanism to account for increases in GSK3β activity that are often observed with a HFD or in an obesogenic state, however this hypothesis requires further evaluation.

While GSK3β activity is more commonly evaluated and reviewed, the activity of its isoform, GSK3α, is much less discussed. We measured relative GSK3α Ser21 phosphorylation, the equivalent of GSK3β Ser9 phosphorylation, and observed significant increases in Ser21 phosphorylation in HFD+Li mice in the HIP, but not in the PFC at 12 weeks. Our results here indicate that the low-dose Li supplementation provided to the HFD+Li mice not only had the ability to influence GSK3β inhibition, but also GSK3α inhibition in select regions of the brain. It is interesting to note that the $K_i$ value (i.e. the inhibitor constant that reflects binding affinity and the concentration needed to induce a degree of inhibition) of Li for GSK3α is higher than that for GSK3β (70). Thus, a greater amount of Li would be required to achieve the same degree of inhibition in GSK3α compared to GSK3β. This difference in $K_i$ values may be why both regions of the brain had increased inhibitory phosphorylation for GSK3β with the Li supplementation at the 12-week timepoint, but inhibitory phosphorylation was only increased in one region for GSK3α. Future research should be aware of the importance of analyzing both GSK3 isoforms due to their relevance in different pathways and the varying degree to which Li is capable of influencing their activities.

With respect to IDE, the effect of the HFD-induced decrease in content in the PFC was blunted in the HFD+Li mice, suggestive of protection from diet-influenced detriments in the brain. These results are particularly important as, again, IDE is critical for clearing both insulin
and Aβ peptides in the brain (117, 145). With increased IDE content in the HFD+Li mice compared to HFD mice, approaching levels comparable to that of CON mice, our results suggest the ability of the low-dose Li supplementation used in the current analysis to have a protective effect against these IDE-related impairments, however in a seemingly region-specific manner.

The exact mechanisms as to how Li may protect the mice from diet-induced IDE deficiency is unclear. To date, there are no investigations that have examined this particular relationship. Furthermore, there is limited research looking at a possible role of GSK3β in changes to IDE content and activity. However, the majority of evidence thus far points to an inverse relationship (i.e. decreased GSK3β activity leads to increased IDE expression and activity) (180). It is possible that the blunted decrease in IDE content in the HFD+Li mice is linked Li-induced decreased GSK3β activity in the brain, however further investigation is needed to confirm this and identify a more precise mechanism.

To begin assessing if the changes we observed in GSK3 phosphorylation correlated to changes in downstream targets and thus demonstrating changes in actual activity, we measured phosphorylation of β-catenin at 12 weeks. However, the lack of differences between groups in both regions of the brain suggests that despite the changes in GSK3 phosphorylation, GSK3 activity was not sufficiently influenced. A previous analysis provided a transgenic AD-mouse model with Li (10 μL / g / day) for 5 weeks via i.p. injections and observed increased expression of β-catenin at the nuclear level in neurons in the subgranular zone of the dentate gyrus in the HIP compared to saline-treated mice (66). Translocation of β-catenin to the nucleus occurs during an unphosphorylated state and is enabled by GSK3β inhibition in Wnt signaling, ultimately allowing for the transcription of genes involved in central development and neurogenesis (84, 107). Since GSK3β inhibition was also observed in the above mentioned
analysis, it was suggested that the increased expression of β-catenin was associated with the decreased GSK3β activity observed with Li treatment (66). The delivery of Li via daily i.p. injections may have allowed for a quicker, increased bioavailability of Li in the brain which in turn provided observable changes after only 5 weeks in this downstream marker. However, this application can produce stressful conditions for the mice and the practicality of this method must be considered with respect to clinical application.

It is also important to note, that all of the GSK3β signaling markers measured in our study (i.e. Akt, GSK3α, GSK3β, and β-catenin) are each involved in an array of intricate and complex cell signaling pathways. Thus, there are several other kinases, phosphatases, or other interacting molecules that can affect the overall phosphorylation status of these markers. For example, as mentioned previously, Akt can be phosphorylated at its Ser473 site by mTOR, and dephosphorylated by various phosphatases including PP2A and PH domain leucine-rich repeat protein phosphatase (PHLPP) (133). Interestingly, Akt can also be phosphorylated by GSK3α at the Thr312 residue which lies within the kinase domain and is associated with decreased Akt activity (81, 133). Additionally, besides GSK3β, β-catenin can also be phosphorylated by src and epidermal growth factor receptor (EGFR) family kinases (48). Thus, these markers are not measured in isolation, but rather are apart of dynamic cellular pathways that must be taken into consideration during an analysis.

Besides phosphorylation, other post-translational mechanisms have been identified that modify GSK3β activity. Sarikhani and colleagues (2018) found that deacetylation of GSK3β by Sirtuin 2 (SIRT2) allowed for increased activity (170). Specifically, SIRT2 binds to GSK3β and deacetylates the Lys183 residue. This deacetylation event in turns leads to enhanced adenosine binding for ATP hydrolysis, a necessity for GSK3β activity. In the hearts of SIRT2-deficient
mice, reduced GSK3β activity was associated with lysine acetylation, however not with inhibitory Ser9 phosphorylation (170). This finding is particularly interesting, as it demonstrates once again that GSK3β activity does not perfectly equate to its varying levels of phosphorylation. Future studies must take into account the complexity of GSK3β activity, and the multitude of ways which it can be modified and measured to get a truly accurate depiction of its activity within a physiological setting.

Lastly, except for average speed and total distance travelled, there were no differences between groups for the results of the Barnes maze, suggestive of no differences in cognitive performance and more particularly, spatial memory. Previous analyses have reported various findings in relation to Li consumption and cognitive performances in both humans and animals, which may be attributed to a number of factors. In a human study, patients with AD who were provided with a microdose of Li (300 μg / day) for 15 months experienced slowed cognitive decline compared to patients receiving a placebo (153). To follow up on this finding, a male AD transgenic mouse model was used, similarly, to assess the effects of a chronic microdose of Li (1.2 mg / kg / day in drinking water) on memory and AD pathology overtime (152). While latency times for the Barnes maze were not improved after not only 8 months, but also 16 months of the Li treatment compared to control mice, the time spent in the target quadrant was significantly increased in the mice treated with Li for 16 months. This finding suggests of the potential for a low-dose of Li in drinking water, similar to the method of administration used in our current study, to improve cognitive performances in mice. However, it demonstrates the lengthy period of time that may be required for such changes to occur, as some beneficial results were only observed after 16 months of treatment, and not 8 months. It is possible that if the 12-week study was extended, changes or improvements in learning and memory, according to
results from the Barnes maze, would be observable in the HFD+Li mice.

It is also important to note that there are multiple ways of assessing cognition in mice, with different tests assessing various particular functions or types of memory. Thus, it is possible that if the mice were tested using other cognitive assessments, changes might be observed with the varying diets and Li supplementation. The same aforementioned study looking at the effects of chronic microdose Li-supplementation in AD transgenic mice used a multitude of cognitive and behavioural tests besides the Barnes maze including the elevated plus maze used to evaluate anxiety and an inhibitory avoidance shuttle box used to evaluate aversive-related memory (152). It may be important to include different types of evaluations such as these in future studies to obtain a more clearer picture of the full cognitive abilities and functions in subjects alongside Li-supplementation.

Finally, the HFD and HFD+Li mice both had reduced average speeds and total distances travelled during the Barnes maze compared to the CON mice. These results are simply likely due to the diet-induced increases in body weight in these mice, particularly at the end of the study duration. Importantly, previous studies have not shown changes in locomotion with Li (152) and there were no differences in speed or distance travelled between the HFD and HFD+Li mice in the current analysis. Thus, it is unlikely that the Li-supplementation in our study alone would have influenced these results.

5.5 Strengths, Limitations, and Future Directions

Our study highlights the effects of a low-dose Li supplementation on brain GSK3β activity in mice alongside an obesogenic diet, a risk factor for AD. This model is fairly unique, as previous research studying low-dose Li supplementation in rodents in relation to AD have almost exclusively been done in AD-transgenic animals (152, 200, 201). While this is certainly a
useful tool for studying AD interventions and treatment methods, the disease often develops much quicker and more aggressively in these animals. Thus, AD-transgenic models can more so resemble the pathology of fAD as compared to sAD. Furthermore, as sAD represents the large majority of all AD cases, it is important to also consider potential prophylactics and therapies in animal models that more closely resemble the sAD pathology and timeline. With our HFD model, we placed the focus on common lifestyle and metabolic risk factors that have the strong potential to increase the likelihood of AD development in aging populations. To this effect, our study provides a novel insight on how Li might work in a pathological setting which more closely resembles sAD influenced by obesity and metabolic disease.

Another key strength of our study is our chosen method of Li administration. When considering the feasibility and overall delivery of a potential Li supplementation in humans, our method has several advantages. First, Li-supplemented drinking water is noninvasive and generally easy to administer in comparison to alternative methods such as regular injections. Furthermore, methods such as taking a supplement in the form a tablet or pill on a regular basis may be less ideal for the targeted aging population where individuals might not remember to take a dose or take the wrong amount, reducing compliance and overall reliability. Lastly, because Li is administered orally, it can become bioavailable to multiple tissues in the body, and not solely to the brain. This chosen method of delivery is important as Li has been recognized to have beneficial effects in various tissues and organs including, but not limited to, the heart, bone, and adipose tissue (9, 82, 85, 118). Taken together, Li-supplemented drinking water is an ideal method of administration that can be relatively easy to transfer into human clinical settings as well as has the potential to benefit other aspects of health besides cognition and AD prevention.
Importantly, recognizing the complexities of how GSK3 functions, we analyzed its activity through a multitude of methods. We measured phosphorylation of GSK3β’s main modifiable residues (i.e. Ser9 and Tyr216) and compared that to its actual activity measured via a GSK3-specific activity assay. To further this, we also measured inhibitory GSK3α Ser21 phosphorylation, understanding that the effects of Li are not limited to that of the GSK3β isoform. Measuring both GSK3 isoforms’ phosphorylation status as well as actual activity is a particular strength, as previous studies have frequently assessed GSK3β using only one or two of the above measurements, thus limiting the results and overall understanding of how GSK3β activity is truly influenced. As our results demonstrate, activity might change despite no changes in phosphorylation. Thus, limiting an analysis in this way may cause one to miss the true depiction of GSK3β activity and function. Future work should bare this in mind and consider multiple avenues of analysis to better the understanding of their results.

At the end of the 12-week study duration, the mice were approximately 7 months of age. Equating this age to humans, the mice were not yet an aged population when the onset of sAD typically becomes observable. While understanding the preclinical stages of AD are important, particularly when considering potential prophylactic therapies, we were unable to observe differences in cognition or AD pathologies likely because of the relatively young age at endpoint. This may be considered a limitation of our study, as it is equally important to understand how Li supplementation would influence disease progression in its later stages. To further our current findings, a longer study duration beginning in mature adulthood and continuing into aging would be necessary to analyze the effects of low-dose Li in the late stages of life and disease. Furthermore, reassessing various cognitive measurements throughout the study and mice’s lifespan would help to obtain a better picture of disease onset and progression alongside Li
supplementation. Similar work was done previously in AD-transgenic mice using a smaller Li dosage than currently used (i.e. 0.25 mg / kg / day) (152). With this microdose Li supplementation beginning at 2 months of age and continuing until 18 months of age, spatial memory in the mice was tested via the Barnes maze every 3 months. Their results showed that the neuroprotective effects of their Li supplementation on spatial memory were not present until the mice were tested at 12 months of age in comparison to controls. These observations suggest that while low-dose Li certainly has prophylactic potential in relation to AD, it may need a long, continuous period of administration to take meaningful effect. Moreover, there may be a particular timeframe during disease progression when the brain is most sensitive to changes and protection from Li. These are critical questions that should be further investigated to better understand Li’s efficacy and how best to use it in AD prevention.

We also observed several regional differences across our results. For example, at 12 weeks, IDE content was reduced in HFD mice in the PFC, but not the HIP. Similarly, changes in GSK3β phosphorylation were not always consistent between the PFC and HIP in each group. Indeed, regional differences in various markers in the brain related to aging and AD have been found previously (20). More specifically, in non-demented subjects, it was shown that changes to synaptic gene expression occurred more in neocortical regions (i.e. superior frontal gyrus and postcentral gyrus) in comparison to limbic regions (i.e. HIP and entorhinal cortex). However, in subjects with AD versus aged-matched controls, synaptic gene expression occurred almost exclusively in the HIP compared to other regions. Why such regional differences exist, however, is much less clear. More research is needed to fully elucidate these differences and how they ultimately translate to functional or behavioural changes in aging.
Lastly, as GSK3β has several functions and is central to a multitude of cellular pathways and processes, consideration of the degree of GSK3β inhibition must be made. Work examining GSK3’s role in cancer, for example, has demonstrated that it can promote intrinsic apoptotic signaling and thus would be detrimental to inhibit in this context (21, 22). Due to its diversity, GSK3β must be carefully regulated to properly balance out its activity, rather than be completely inhibited (103, 105). Li has been suggested to only moderately inhibit GSK3 activity, with both its direct and indirect mechanisms, making it a strong candidate for achieving beneficial effects associated with reduced GSK3 activity while avoiding over-inhibition (105). Furthermore, it was found that the IC50 (i.e. the concentration needed to reach 50% maximum inhibition) of Li for GSK3 is approximately 2 mM (104, 110). This value is greater than the therapeutic concentrations of Li (i.e. 0.5-1.5 mM), and thus a low-dose of Li would likely cause a degree of GSK3 inhibition much less than 50%. Future work is needed to investigate the specific pharmacokinetics and pharmacodynamics of low doses of Li in the brain in order to better understand a precise degree of GSK3 inhibition that can both protect one from detrimental effects as well as contribute to maximum benefits in relation to AD pathologies.

5.6 Conclusion

In conclusion, our findings demonstrate the ability for a low-dose Li supplementation to reduce GSK3β activity in the rodent brain as early as 6 weeks and in the absence of increased inhibitory Ser9 phosphorylation. Furthermore, low-dose Li has the potential to improve HFD-induced impairments such as reducing caloric efficiency and increasing IDE content in the brain. Overall, these results support the potential of low-dose Li to be an effective prophylactic for sAD during its early stages. Future research should investigate the effects of a longer supplementation
on various aspects of cognition and behaviour as well as the development of AD pathologies during aging.
References


47. Cusulin C, Wells I, Badillo S, Pacheco GCD, Baumann K, Patsch C. Gamma secretase modulators and BACE inhibitors reduce Aβ production without altering gene expression in Alzheimer’s disease iPSC-derived neurons and mice.


Appendix

Appendix I: AUP Approval Certificate

Date: May 3, 2019

Dear Dr. Rebecca MacPherson

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

Examination of low dose lithium feeding on markers of thermogenic capacity in adipose and skeletal muscle as well as markers of Alzheimer’s disease in brain tissue from male C57BL6 mice

This approval expires in one year on the last day of the month. The number for this project is AUP # 19-04-01
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 96 males

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

[Signature]
Jim Willwerth, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING (PAM). Arrangements for PAM must be made, for one or more components of your study, when animals are ordered.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.
Appendix II: 2014 Teklad Rodent Chow Diet Information

Teklad Global 14% Protein Rodent Maintenance Diet

Product Description: 2014 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to promote longevity and normal body weight in rodents. 2014 does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical soybean concentrations (diastereom + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosoamines. Also available certified (2014C) and irradiated (2014I). For autoclavable diet, refer to 2014AS (Sterileable).

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Amino Acids

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<tr>
<td>C18:2 Linoleic</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>C18:3 Linolenic</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Total Unsaturated</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Total Monounsaturated</td>
<td>0.7</td>
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</tr>
<tr>
<td>Total Polyunsaturated</td>
<td>2.1</td>
<td></td>
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Other

<table>
<thead>
<tr>
<th>Other</th>
<th>Percentage</th>
<th>mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Appendix III: Rodent HFD #D12492 Information

### Description
Rodent Diet with 60% kcal% fat.

### Used in Research
- Fatty Liver
- Inflammation
- Obesity
- Diabetes

### Packaging
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

### Lead Time
IN-STOCK. Ready for next day shipment.

### Gamma-Irradiation
Yes. Add 10 days to delivery time.

### Form
Pellet, Powder, Liquid

### Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months. Because of the high fat content is best if kept frozen.

### Control Diets
D12450B, D12450J, D12450K

---

### Formula

<table>
<thead>
<tr>
<th>Product #D12492</th>
<th>gm%</th>
<th>kcal%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>26.2</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>26.3</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>34.9</td>
<td>60</td>
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</table>

**Total**

<table>
<thead>
<tr>
<th></th>
<th>kcal/gm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5.24</td>
</tr>
</tbody>
</table>

### Ingredient

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm</th>
<th>kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, 30 Mesh</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>Sucrose</td>
<td>68.8</td>
<td>275.2</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>0</td>
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<tr>
<td>Soybean Oil</td>
<td>25</td>
<td>225</td>
</tr>
<tr>
<td>Last*</td>
<td>245</td>
<td>2295</td>
</tr>
</tbody>
</table>

**Total**

|          | 773.85 | 4057 |


*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 216.4
Cholesterol (mg)/kg = 279.6
Appendix IV: Summary of 6-week Study Western Blot Data

<table>
<thead>
<tr>
<th>Protein</th>
<th>PFC</th>
<th>HIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAkt Ser473</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pAkt Thr308</td>
<td>↑ in Li*</td>
<td>NC</td>
</tr>
<tr>
<td>pGSK3β Ser9</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pGSK3β Tyr216</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pTau Ser396</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pAPP Thr668</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

*p <0.05 relative to CON
NC= no change
## Appendix V: Summary of 12-week Study Western Blot Data

<table>
<thead>
<tr>
<th>Protein</th>
<th>PFC</th>
<th>HIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDE</td>
<td>↓ in HFD*</td>
<td>NC</td>
</tr>
<tr>
<td>JNK</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pJNK Thr135/Tyr138</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Akt</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pAkt Ser473</td>
<td>NC</td>
<td>↑ in HFD+Li*</td>
</tr>
<tr>
<td>pAkt Thr308</td>
<td>↑ in HFD*</td>
<td>NC</td>
</tr>
<tr>
<td>GSK3β</td>
<td>↓ in HFD*</td>
<td>NC</td>
</tr>
<tr>
<td>pGSK3β Ser9</td>
<td>↑ in HFD+Li*</td>
<td>↑ in HFD*</td>
</tr>
<tr>
<td>pGSK3β Tyr216</td>
<td>↑ in HFD*</td>
<td>↑ in HFD+Li*</td>
</tr>
<tr>
<td>GSK3α</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pGSK3α Ser21</td>
<td>NC</td>
<td>↑ in HFD+Li*</td>
</tr>
<tr>
<td>β-catenin</td>
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<tr>
<td>β-catenin Ser33/37/Thr41</td>
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</tr>
<tr>
<td>Tau</td>
<td>↓ in HFD*</td>
<td>NC</td>
</tr>
<tr>
<td>pTau Ser396</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pTau Ser404</td>
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<td>NC</td>
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<tr>
<td>APP</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>pAPP Thr668</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>BACE</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

*p <0.05 relative to CON; **p<0.01 relative to CON
NC= no change
Li mice had increased Akt Thr308 phosphorylation in the PFC. While there were no changes in GSK3β phosphorylation, actual GSK3 activity was decreased in the HIP of the Li mice.
Appendix VII: Summary of Main Findings for the 12-week Study

HFD mice had increased Akt Thr308 phosphorylation in the PFC while HFD+Li mice had increased Akt Ser473 phosphorylation in the HIP. Downstream, GSK3β Ser9 phosphorylation was increased in both the PFC and HIP in the HFD+Li mice. In the HFD mice, GSK3β Ser9 phosphorylation was also increased in the HIP while Tyr216 phosphorylation was increased in the PFC of both the HFD and HFD+Li mice. However, no changes in relative pTau content nor APP-related pathologies (i.e. total APP, relative pAPP, sAPPβ, and BACE) were observed. Similarly, no changes in cognitive decline as per the results of the Barnes Maze were observed as well. HFD+Li mice had rescued IDE content compared to HFD mice in the PFC. As IDE preferentially degrades insulin, this result may represent an increased ability for IDE to clear Aβ content, ultimately protecting against cognitive decline.