Effects of a high-sucrose diet and systemic inflammation on Alzheimer's disease-related processes in reproductively normal female wild-type mice

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ABSTRACT

Alzheimer's disease (AD) incidence is expected to double by 2038. This coincides with similar trends in obesity and chronic inflammation. It is known that insulin resistant and chronic inflammatory conditions can increase the risk of AD-like neurodegeneration, likely through mechanisms involving induction of brain insulin resistance. Insulin resistant brain states are associated with increased activity of glycogen synthase kinase-3β (GSK-3β), whose constitutive activity is inhibited, in part, by activity within the insulin pathway. Aberrant GSK-3β signaling contributes to increased amyloid-β production (senile plaques) and Tau protein hyperphosphorylation (neurofibrillary tangles), hallmarks of AD-like neurodegeneration. In addition, nearly two-thirds of AD patients are female, which strongly suggests a role for the post-menopausal loss of the female sex hormone, estrogen, in the pathogenic events associated with AD. Estrogen is known to diminish neurodegenerative processes, such as β-amyloidopathy, mitochondrial dysfunction and oxidative stress, in a variety of animal models. A reduction in estrogen levels following menopause has also been associated with increased risk of insulin resistance and inflammation, thus necessitating exploration of the neurodegenerative potential of these conditions in a female animal model.

As a first step, by combining a high-sucrose diet (20% of the drinking water) with intraperitoneal LPS injections (0.1 mg/kg; once/month for 3 months) over seven months in reproductively normal female wild-type mice (C57Bl/6; n=10/group), a protective effect of low-dose LPS on high-sucrose diet-induced pathology was demonstrated. Results from the high-sucrose group confirmed that a high-sucrose diet is a suitable model of neurodegeneration, as evidenced by exaggerated glucocorticoid expression, spatial learning deficits, irregularities within the insulin pathway, and increased β-amyloid production and Tau phosphorylation. Interestingly, while LPS had little to no effect in isolation, it exerted a protective influence when added to animals sustained on a high-sucrose diet. Corticosterone homeostasis, Aβ and pTau levels, and insulin pathway second messenger expression were all rescued following addition of LPS.

Given the hypothesized role of increased GSK-3β activity in neurodegeneration, mice following the combined treatment regimen were supplemented with lithium orotate (1 mg/L in the drinking water), a potent inhibitor of GSK-3β, to assess its prophylactic potential against dietary- and-inflammatory insult-mediated neurodegeneration. As the addition of LPS to animals on a high-sucrose diet proved to be protective rather than aggravating, I was unable to assess lithium for prophylaxis against neurodegeneration. However, antagonistic interactions between LPS and lithium were observed (lithium blocked the effects of LPS). When added to mice following the combined regimen, lithium returned corticosterone and Aβ levels to those observed in animals sustained on high-sucrose alone, while completely abolishing spatial learning deficits and anxiety-like behavior.

To sum, the work presented confirms a 1) high-sucrose diet as a model of neurodegeneration, 2) supports a protective role for transient inflammation against dietary-insult, and 3) suggests an antagonistic interaction between lithium and LPS.

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LIST OF ABBREVIATIONS

Aβ – amyloid-β
$Aβ_{42}$ – amyloid-β 42
$Aβ_{40}$ – amyloid-β 40
AD – Alzheimer's disease
AChE - acetylcholinesterase
Akt – also known as protein kinase B
***p*Akt** – phosphorylated Akt
APO ε4 – apolipoprotein ε-4
APP – amyloid precursor protein
BBB – blood brain barrier
BDNF – brain-derived neurotrophic factor
Cdk5 – cyclin dependent kinase-5
CLi – renal clearance of lithium
CNS – central nervous system
COX-2 – cyclooxygenase-2
CSF – cerebrospinal fluid
Dvl - disheveled
Fz – frizzled
Grb2 – growth factor receptor protein 2
GSK-3β – glycogen synthase kinase 3-β
***p*GSK-3β** – phosphorylated GSK-3β
HPA – hypothalamic-pituitary-adrenal axis
hS – high-sucrose
hSL – high-sucrose and LPS
hSLLi – high-sucrose, LPS and lithium
IL-1β – interleukin-1β
IL-5 – interleukin-5
IL-6 – interleukin-6
IL-10 – interleukin-10
iNOS – inducible nitric oxide synthase
InsRes – insulin resistance
IRBS – insulin-resistant brain-state
IRS1 – insulin receptor substrate -1
***p*IRS1** – phosphorylated IRS1
IRS2 – insulin receptor substrate -2
LC – locus coeruleus
Li – lithium
LPS – lipopolysaccharide
LTD – long-term depression
LTP – long-term potentiation
MCI – mild cognitive impairment
mTOR – mammalian target of rapamycin
***p*mTOR** – phosphorylated mTOR
NASH –non-alcoholic steatohepatitis
NO – nitric oxide
NF-κβ – nuclear factor-κ-β
OFT – Open Field test
p70S6K – p70 ribosomal protein S6 kinase
***p*p70S6K** – phosphorylated p70S6K
PDK – phosphoinositide-dependent kinase
PHF – paired helical filament
PI3K - phosphoinositide 3-kinase
PIP2 - Phosphatidylinositol bisphosphate
PIP3 – Phosphatidylinositol trisphosphate
PS1 – presenilin-1
RNS – reactive nitrogen species
ROS – reactive oxygen species
SH2 – Src homology 2
SMase – sphingomyelinase
SOS – son-of-sevenless
TGF-β – transforming growth factor-β
TLR – toll-like receptor
TNFα – tumor necrosis factor-α
Wnt - wingless

CHAPTER 1: INTRODUCTION

1.1 THE RISE OF ALZHEIMER'S DISEASE

1.1.1 The Alzheimer's disease problem

Neurodegenerative diseases are an escalating concern for our aging population. AD is perhaps the most problematic of these conditions, with over 300,000 Canadians estimated to be afflicted with the disease. These numbers are expected to more than double by 2050[1]. Given the rate of increase, and that fewer than 10% of all AD cases are exclusively genetic in origin, it seems unlikely that genetic mutations are to blame for this crisis. While aging is the greatest risk factor for development of late-onset AD, it is possible that changing dietary and lifestyle factors such as 1) increased carbohydrate consumption (obesity, insulin resistance) and 2) chronic inflammatory conditions (arthritis, cardiovascular disease) are involved. Some authors have proposed the idea of AD as being type-3 diabetes (*i.e.* diabetes of the brain[2]), and high-sucrose diets and stress are known to promote both systemic and central inflammation[3–5]. Neuroinflammatory processes are believed to be at the root of many neurodegenerative coniditions[6–12]. Considering the association between obesity/insulin resistance/hyperglycemia, chronic inflammation, and neurodegeneration, it seems possible that changing lifestyle and dietary patterns are contributing to the surge in AD prevalence.

1.1.2 Clinical characteristics of Alzheimer's disease

Neurocognitive disorder is an umbrella term that encompasses a host of neurodegenerative conditions sharing common severe decline in cognitive ability. AD represents the most common form of neurocognitive disorder, accounting for nearly two thirds of cases in the 65 and over population[1]. AD is a progressive neurodegenerative disease characterized by impairment of comprehension, memory, language (production and processing), attention, and judgment[13]. As noted prior, early onset of AD is unusual (prior to age 65), representing just 10% of all cases. Thus, most AD patients demonstrate the sporadic late-onset form of the disease. Early stages of sporadic AD are highlighted by disturbances in short-term memory formation and recall with relative sparing of long-term memory. As the disease progresses, executive functions such as problem solving, judgment, visuospatial skills and organization deteriorate. Mid-to-late stages of AD are characterized by psychiatric symptoms (*i.e.* apathy, disinhibition, psychosis) and a progressive decline in motor skills and sleep quality. Incontinence, loss of primitive reflexes and complete reliance on caregivers exemplify the closing stages[13–16]. The neuropathological features

of AD are senile plaques, neurofibrillary tangles, and subsequent neuronal loss, with the severity of each correlating to the degree of disease progression[16].

1.1.3 Neuropathology of Alzheimer's disease

Senile plaques and neurofibrillary tangles are considered two of the hallmark biomarkers of AD. Senile plaques consist primarily of insoluble deposits of amyloid-β (Aβ) peptides. Aβ oligomers are fragments of amyloid precursor protein (APP) generated during β-amyloidogenesis: proteolytic cleavage by the membrane-bound endoproteases β- and γ-secretase. Alternatively, non-pathogenic amyloid species can be formed when α-secretase acts within the Aβ domain of APP[17]. While multiple fragment lengths of Aβ are known to exist due to the imprecise activity of γ-secretase[18], $A\beta_{42}$ and $A\beta_{40}$ appear to be two of the most abundant and influential. $A\beta_{42}$ is more hydrophobic and fibrillogenic than shorter variants, establishing it as particularly neurotoxic[19,20]. While it is unclear whether senile plaques are a causative factor or endpoint of AD, a growing body of data suggests that the presence of the soluble Aβ species is chiefly responsible for driving pathogenesis of the disease[16–18].

Fibrillization of Aβ is preceded by formation of intermediate Aβ species collectively referred to as 'soluble Aβ'. Interestingly, expression of soluble Aβ species has been shown to correlate better than plaque formation with severity of disease progression. These soluble Aβ variants have been linked to neurotoxicity, reduced synaptic density, aberrant activation of adhesion signaling pathways, and cognitive dysfunction in rodent models[20–22]. Aβ oligomers, particularly of the $A\beta_{1-42}$ variety[23], are also involved in the hyperphosphorylation of the microtubule-associated Tau protein, a key event in the formation of neurofibrillary tangles[24,25]. This promotion of Tau phosphorylation may be the result of increased cyclin-dependent kinase-5 (Cdk5) activity induced by $A\beta_{42}$-mediated alterations in lipid second messenger expression[26].

Neurofibrillary tangles are characterized by Tau proteins wrapped around one another in a helical fashion to form insoluble paired helical filaments (PHF). Hyperphosphorylation of Tau proteins is believed to prime Tau for assembly into PHFs. These accumulated PHFs are prone to aggregation, thus setting the stage for development of neurofibrillary tangles[27,28]. In addition to insoluble variants, misfolded Tau proteins may aggregate without forming PHFs, leading to soluble pathogenic Tau clusters[28]. Hyperphosphorylated Tau filaments demonstrate 'prion-like' properties in their ability to induce tangle formation in normal Tau proteins[27]. Both soluble and insoluble hyperphosphorylated Tau proteins have been shown to correlate with severity of cognitive decline in AD[29,30].

1.1.3 Role of sex hormones in Alzheimer's disease

Women will represent nearly two thirds of new AD patients over the coming generation, which strongly suggests a role for the post-menopausal loss of sex hormones (*i.e.* estrogen) in the etiology and/or pathogenesis of the disease, at least in females[1]. Sex hormone loss with aging in men or menopause in women correlates with increased incidence of AD[31,32]. Estrogen and testosterone are considered to be neuroprotective against CNS insults involved in the pathogenesis of AD[33]. Estrogen and its receptor regulate Alzheimer's disease pathology through promotion of non-amyloidogenic APP processing[34] and decreased hyperphosphorylation of Tau[35] (fig. 1.1), while testosterone acts through androgen receptor-mediated increases in the endopeptidase neprilysin responsible for clearing Aβ levels in the brain[36], and inhibition of calpain-mediated tau cleavage known to play a role in Aβ-induced toxicity[37]. Interestingly, estrogen appears to confer greater protection against inflammatory insult in women than is observed for their male counterparts. Female animal models of lipopolysaccharide (LPS)-induced inflammation display subdued inflammatory responses relative to males[38]. While both male and female sex hormones decrease with age, post-menopausal women experience a greater age-related loss of estrogen than is observed for testosterone in like-aged men. Thus, although loss of testosterone or estrogen in males and females both increase risk for AD, the earlier and more rapid decline in estrogen in females associated with menopause likely contributes to the increased incidence of AD in the female population[1].

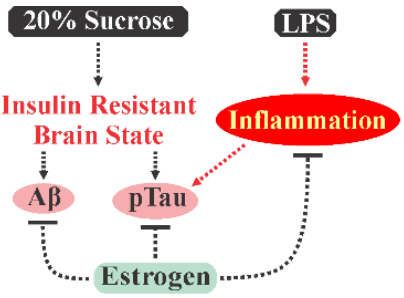

Fig 1.1. Estrogen antagonizes inflammatory and neurodegenerative processes associated with AD. Females demonstrate attenuated LPS-mediated inflammatory responses relative to males. Estrogen promotes non-amyloidogenic APP processing and suppresses Tau phosphorylation.

1.2 THE OBESITY EPIDEMIC

1.2.1 Agriculture and evolutionary discordance

According to the theories of natural selection (survival of the fittest) and punctuated equilibrium (evolution is driven by dramatic environmental changes), evolution represents a constant interaction between the genome of a species and the environment in which it resides. Genetic traits are positively or negatively selected in accordance or discordance with constraints applied by a given environment. When environmental pressures remain relatively consistent, genetic traits come to reflect an optimal pool for survival of the population[39,40]. When rapid and permanent environmental changes occur, individuals within the population experience

evolutionary discordance; *i.e.* failure of the genotype to match the requirements of the environment. Evolutionary discordance has been proposed to manifest phenotypically as disease[40–42].

Prior to the advent of agricultural practices, human dietary choices were limited to wild plant- and animal-based foods. In contrast, the post-agricultural (particularly post-Industrial Revolution) diet is rich in cereals, refined flour products, dairy, alcohol, and added sugars[43–45]. These food sources, which were largely unavailable in pre-agricultural societies, account for the majority of the daily energy consumed by most modern humans. It has thus been proposed that the current human genetic-makeup is ill-suited to the present environment. In other words, modern dietary choices may have placed present day individuals in a state of evolutionary discordance that has manifested in obesity, insulin resistance, cardiovascular diseases, and neurodegenerative conditions[40,41,46–48].

1.2.2 Recent trends in the Western Diet

Following the introduction of high-fructose corn-syrup additives and sucrose-based sweeteners to the western diet, average consumption of dietary and added sugar has increased with each passing year[40,43–45]. Furthermore, three-quarters of the North American population consumes an amount of fruit and vegetables below the recommended daily quantities. As dietary fiber has been suggested as a means to ward of the deleterious effects of excess caloric intake, the lack of fiber-rich vegetable consumption is concerning[49–51]. These dietary trends coincide with similar escalations in the prevalence of obesity. At present, nearly 50% of North Americans are estimated to be clinically obese and while genetic factors are known to contribute to susceptibility to the condition, the majority of cases can be primarily attributed to excess caloric intake, poor dietary composition, and lack of physical activity[48,52,53]. Obesity has been linked to insulin resistance (InsRes), inflammation/neuroinflammation, and cardiovascular impairment, all of which are known risk factors for AD-associated neurodegeneration[6–8,54–57].

1.2.3 Insulin signaling in the brain

Insulin is a key hormone involved in regulating microvascular blood flow, glucose uptake, cell survival, neurogenesis and memory. Most insulin within the CNS is produced locally (of central origin), as evidenced by the abundance of insulin mRNA found within numerous brain regions (hypothalamus and hippocampus, primarily)[58–61]. However, insulin may also enter the brain from the periphery via saturable transport systems. It has been proposed that insulin binds to its receptor resident in the BBB, triggering subsequent transcytosis of the hormone through

endothelial cells[62,63]. Regardless of origin, appropriate insulin activity is required for effective cognition, memory formation, and maintenance of neuronal integrity.

Brain insulin resistance is a condition in which neurons fail to respond to circulating insulin in an appropriate manner. Under normal conditions, insulin will bind to the insulin receptor - a tyrosine kinase - leading to its dimerization and the autophosphorylation of component tyrosine residues. Insulin receptor substrates 1 (IRS1) and 2 (IRS2) will then associate with the receptor, leading to phosphorylation of IRS by the receptor kinase. IRS phosphorylation creates a docking site for the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). PI3K leads to downstream activation of protein kinase B (PKB/Akt). Activated Akt is responsible for the phosphorylation of numerous homeostatic targets involved in glucose balance, cytoskeletal remodelling, cell cycle regulation, cell proliferation/survival, autophagy, and apoptosis[64,65]. Akt and its downstream targets, such as mTORC1, and S6K, engage in negative feedback via delayed inhibitory phosphorylation of IRS1/2 at a variety of serine residues, including serine 302, 307, 522, 612, and/or 635[66]. This inhibitory phosphorylation prevents interaction with the insulin receptor (fig. 1.2). Dysregulation of Akt activity is associated with reduced cell survival, impaired cognition, reduced cytoskeletal stability, and accumulation of cytotoxic misfolded protein aggregates. Regarding neurocognitive disorders, insulin-resistant brain-states (IRBS) have been observed in post-mortem hippocampal tissue collected from AD patients, with the degree of insulin dysregulation correlating positively with intensity of antemortem cognitive dysfunction[67]. Also of note, Willette *et al* (2015) observed that insulin resistance predicts amyloid deposition in late middle-aged adults considered at risk for AD[68]. While mal-activity within the IRS-Akt branch has been well characterized for its role in neurodegeneration, it should be noted that an alternative route within the insulin pathway exits. Adaptor molecules such as growth factor receptor-bound protein 2 (Grb2) contain SH2 domains that allow for interaction with the activated insulin receptor and associated phosphorylated IRS proteins. Activated Grb2 interacts with proteins such as Grb2-associated binding protein 1 and son-of-sevenless (SOS) through resident Src homology 3 domains. SOS catalyzes the transition of

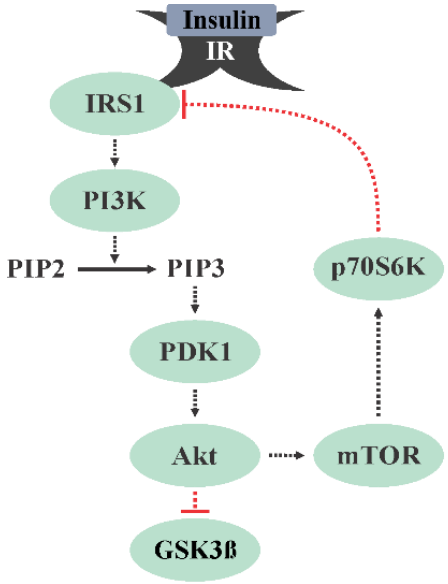

Fig 1.2. Brain insulin signaling cascade. The brain insulin pathway is extensive and cross-talks with numerous other pathways that regulate neuroplasticity, cognition, and neuronal survival. This diagram outlines only those second messengers relevant to the work discussed in this thesis.

Ras from a GDP-bound (inactive) to GTP-bound (active) state. GTP-bound Ras leads to downstream activation of extracellular signal-regulated kinase-1 and -2, which are involved in control of cell proliferation and differentiation[69]. Aberrant Erk signalling has been shown to induce hyperphosphorylation of microtubule-associated Tau proteins[70], a process linked to many of the pathologies characteristic of AD.

The role of obesity in predisposition to AD - noted above - may be mediated by InsRes. An extensive body of literature suggests insulin dysregulation as a prominent risk factor for development of the disease[2,56,71–76], with some authors proposing that IRBS may precede peripheral establishment of insulin insensitivity[67]. Evidence from animal models further supports the link between diet-induced InsRes and AD, as insulin dysregulation precipitated through a high-sucrose diet (caloric excess) has been shown to promote behavioral and physiological pathologies (increased Tau phosphorylation and β-amyloidogenesis) consistent with AD-like neurodegeneration[77,78].

1.2.4 Glucocorticoids, ceramides, and brain insulin dysregulation

Glucocorticoids, named for their actions on glucose metabolism, are stress hormones that function in the maintenance of homeostasis. Glucocorticoid expression is increased via two primary pathways, 1) stress-induced release from the adrenal cortex following activation of the hypothalamic-pituitary-adrenal (HPA) axis[79,80], and 2) tissue specific (*i.e.* adipose tissue, liver) conversion of glucocorticoids from an inactive to active state by 11-β hydroxysteroid dehydrogenase-1[81,82]. The effects of glucocorticoid signaling are widespread and multifaceted, including regulation of immune function, glucose metabolism, cognition, behavior, cell proliferation and cell survival[83]. The integrity of the stress response demands efficient induction of the HPA axis paired with subsequent downregulation of activity via hippocampal glucocorticoid receptor-mediated negative feedback. Once a threshold of interaction between glucocorticoids and their receptors in the hippocampus has been reached, activation of the HPA will be suppressed, allowing for robust yet transient glucocorticoid activity in response to stress[84]. Consequently, any disturbance in ordinary glucocorticoid function and/or HPA axis-feedback will have profound impact on several organs, the brain included.

Excess sucrose intake - an increasingly common occurrence, as noted prior - is associated with increased glucocorticoid levels and exaggerated glucocorticoid-mediated responses to other stressors[85]. Chronic elevations in glucocorticoid activity demonstrate several damaging effects on the brain, including quenched antioxidant capacity (increased oxidative damage)[86], potentiation of neuorinflammation[87], and induction of IRBS[88,89]. In animal models, corticosterone (analogue of the cortisol found in humans) has been shown to inhibit activation of the insulin

receptor while simultaneously reducing expression of the protien[88,90]. Furthermore, glucocorticoids oppose insulin on a functional level; *i.e.* catabolism versus anabolism[91]. Glucocorticoids also slow the movement of insulin into the CNS from the periphery through inhibition of insulin receptors within the BBB[62]. Corticosterone/cortisol may thus antagonize the action of insulin on both functional and molecular levels while simultaneously quenching its availability in the brain.

Peripherally, exaggerated glucocorticoid activity can lead to steatosis (fat accumulation) and inflammation of the liver, both of which are implicated in the development of the insulin resistance- and neurodegeneration-associated condition of non-alcoholic steatohepatitis (NASH). Glucocorticoids and steatohepatitis/liver damage are known to promote production of neurotoxic ceramides through induction of the stress-sensitive salvage pathway[74,92–94] (fig. 1.3). Ceramides share the general form of a sphingosine bound to a fatty acid, with the length of the fatty acid chain expressing a degree of variability[95]. They are produced through two primary mechanisms: the *de novo* synthesis pathway, involving the condensation of palmitate and serine, and the salvage pathway, involving the re-acylation of sphingosine[96]. Over-expression of ceramides can disrupt insulin signaling[97,98] and promote pro-apoptotic pathways[99,100]. The evidence supporting ceramide-induced insulin resistance is compelling. Chitturi *et al* (2002) demonstrated that NASH is comorbid with insulin resistance in approximately 98% of patients[101]. These findings are further supported by the work of others, such as Straczkowski *et al* (2007), who noted a significant correlation between muscle ceramide expression and decreased insulin sensitivity in obese and overweight individuals[102]. *In vitro*, ceramides have been found to decrease insulin signaling via inhibition of Akt signaling. Ceramides promote phosphatase 2A-mediated dephosphorylation of Akt, dampening the activity of the kinase[92,103–108]. As previously discussed, Akt is a central mediator of insulin activity, so any disruption of this system will invariably lead to dysregulation of the insulin pathway. Interestingly, some studies have even shown that ceramide levels are increased in the CNS of AD patients[109–113].

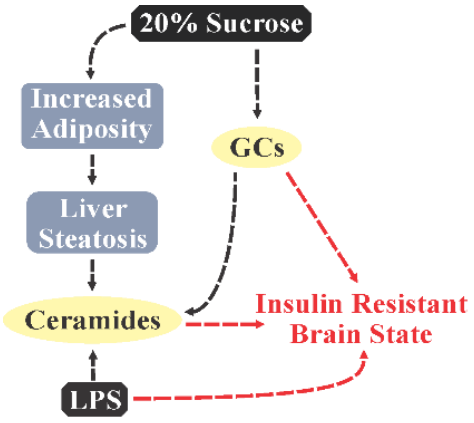

Fig 1.3. Glucocorticoid- and ceramide-induced insulin resistance. Glucocorticoids and ceramides are capable of precipitating insulin resistance. As both are blood-brain-barrier soluble, this diagram proposes a ceramide- and glucocorticoid-dependent mechanism for high-sucrose diet- and LPS-mediated brain insulin resistance.

As glucocorticoids and ceramides are BBB permeable, it is reasonable to propose that they may act as mediators of diet-induced dysregulation of the brain insulin pathway (fig. 1.3). Irregular brain insulin signaling, irrespective of cause, results in extensive tissue injury through

formation of reactive oxygen species (ROS), reactive nitrogen species (RNS), dysfunction of mitochondria (promotes apoptosis), and induction of proinflammatory cytokine release[2,92,114–116]. Furthermore, diet-mediated dysregulation of brain insulin signaling has been linked to β-amyloidogenesis and Tau protein hyperphosphorylation, the hallmark processes of AD[77,92,114,117].

1.2.5 The Glycogen Synthase Kinase-βeta hypothesis of Alzheimer's disease

Glycogen synthase kinase-β (GSK-3β), a serine/threonine kinase, is an integral component of the insulin pathway that is negatively regulated by Akt. While two primary isoforms of GSK-3 exist – alpha (α) and beta (β), the β variant demonstrates increased expression in the CNS, implicating it as the isoform of interest when discussing brain insulin activity[118]. When active, GSK-3β is involved in such essential processes as cell cycle regulation, glucose homeostasis, glycogen synthesis, and cell proliferation. However, GSK-3β also plays a role in the phosphorylation of Tau, induction of inflammation, and regulation of β-secretase activity (increased processing of APP to amyloid-β)[118,119]. Over-activation of GSK-3β has thus been associated with AD-related neurodegenerative processes and phenotypes such as memory impairment, hyperphosphorylation of Tau[120], increased β-amyloidogenesis[121], and increased microglial-mediated inflammatory responses[122].

Given the role of Akt in regulating GSK-3β activity, it is of no surprise that brain insulin resistance/dysregulation leads to hyperactivation of GSK-3β; less Akt activity results in disinhibition of the GSK-3β enzyme. Considering the impact of GSK-3β on neurodegeneration-, abnormal signaling activity may represent a mechanism linking obesity and InsRes to AD pathogenesis. This notion has led to the 'GSK-3 hypothesis of AD'[122] which suggests that the amyloid-β and Tau hypotheses are the consequences of an overactive GSK-3β signaling pathway. As the insulin-signaling pathway is heavily involved in suppressing constitutive GSK-3β activity[123], even a modest irregularity in the insulin pathway may induce GSK-3β-dependent pathology.

In addition to accelerating the formation of Aβ oligomers and neurofibrillary tangles (misfolded aggregate Tau proteins), GSK-3β hyperactivity slows the removal of aggregate proteins via suppression of the autophagy-lysosomal pathway[124–126]. Interestingly, inhibition of GSK-3β has been shown to restore lysosomal function in murine models of AD, with subsequent amelioration of Aβpathology[127,128]. While other factors are almost certainly involved, GSK-3β dysregulation seems to represent a possible mechanistic explanation for the diet-induced neurodegeneration observed in animal models of AD[78,122].

1.3 INFLAMMATION AND NEURODEGENERATION

1.3.1 Peripheral lipopolysaccharide as a model of neuroinflammation

Neuroinflammation is a shared feature of neurodegenerative diseases. It is widely accepted that microglia, the resident sentinel cells of the CNS, are principal effectors of the inflammatory response in the brain. When in the presence of appropriate triggers, such as proinflammatory cytokines, free fatty acids, ROS, or endotoxins, microglia undergo a process deemed M1 polarization. Polarization refers to the transition between classical proinflammatory (M1) and alternative anti-inflammatory (M2) activation states in response to environmental stimuli, though microglia are known to frequently demonstrate characteristics of one state while occupying the other (*i.e.* polarization is rarely complete)[129]. M1 activation is characterized by a retraction of microglial processes and swelling of cell bodies accompanied by subsequent release of proinflammatory mediators, including interleukin-6 (IL-6), interleukin-5 (IL-5), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNFα)[12,129,130]. Conversely, M2 polarization is associated with increased expression of anti-inflammatory mediators such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and enhanced phagocytosis of foreign pathogens, misfolded proteins, and aggregate proteins[131].

LPS is a structural component of the outer membrane of gram-negative bacteria. LPS binds to toll-like receptors (TLRs) in numerous cell types, microglia (central) and macrophages (peripheral) included. The interaction of LPS with microglia/macrophage resident TLR4 induces secretion of proinflammatory cytokines via the nuclear factor-κβ (NFκβ) pathway while increasing expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)[132]. While microglial activation is necessary for defense of the host, hyperactivation can lead to release of cytotoxic factors such as superoxide[133] (a potent ROS) and nitric oxide[134] (via increased iNOS activity). In addition, proinflammatory cytokines themselves can become neurotoxic. Excess cytokine signaling is associated with exacerbation of inflammation, induction of neuronal insulin resistance[135–137], formation of cytotoxic kynurenine, and promotion of excitotoxicity[138–140].

Peripherally administered LPS has been shown to induce central inflammation[141]. While LPS may interact directly with microglial TLR4 in the brain, peripherally induced inflammatory mediators (of macrophage origin) are also capable of propagating into the CNS. Three immune-to-brain communication pathways (among others) that monitor peripheral immune responses have been proposed. First, proinflammatory cytokines engage in neuronal communication with the CNS through activation of afferent vagal nerves forming synapses in the nucleus tractus solitarius. Afferent vagal stimulation is associated with elevated levels of glucocorticoids in the brain, the exaggerated activity of which may suppress antioxidant capacity, increase local

proinflammatory cytokine production, and heighten sensitivity to stress. Second, peripheral cytokines interact with TLRs on macrophage-like cells within the circumventricular organs and choroid plexus of the brain. Resultant pro-inflammatory cytokines then enter the brain through volume diffusion. Third, select cytokines such as TNF-α and IL-6 express saturable transport systems resident in the BBB, allowing for direct passage into the CNS[141]. Further, TNF-α has been shown to disrupt BBB permeability, thereby accelerating immune cell infiltration into the brain.

1.3.2 Caloric excess and sensitization of the CNS to inflammatory insult

Western dietary choices may prime the brain for establishment of chronic inflammation through elevated glucocorticoid activity, induction of IRBS, and compromised integrity of the BBB. As previously discussed, excess sucrose consumption is positively correlated with exaggerated glucocorticoid expression/activity and glucocorticoid-mediated responses to stressors[85]. While ordinarily anti-inflammatory during transient stress responses[142,143], chronic glucocorticoid signaling can exacerbate inflammatory cascades via several mechanisms (fig. 1.4). First, glucocorticoids quench the antioxidant capacity of the brain, which augments the oxidative damage resultant of neuroinflammation[86]. Second, chronic glucocorticoids may contribute to activity-mediated 'burn-out' of noradrenergic locus coeruleus (LC) neurons by increasing their basal firing rate via interaction with glucocorticoid receptors located on the cell body[144]. As noradrenaline suppresses microglial activation (anti-inflammatory)[145,146], loss of LC neurons would reduce the anti-inflammatory capacity of the brain. Of note, loss of LC neurons is a shared characteristic of neurodegenerative conditions[147–149]. Third, a western diet can increase BBB permeability in the hippocampus after 90 days[150]. Taken together, caloric excess-induced impairments of antioxidant-, anti-inflammatory-, and BBB-mediated defense mechanisms may sensitize the brain to damages resultant of neuroinflammation while simultaneously enhancing propagation of inflammatory mediators into the CNS.

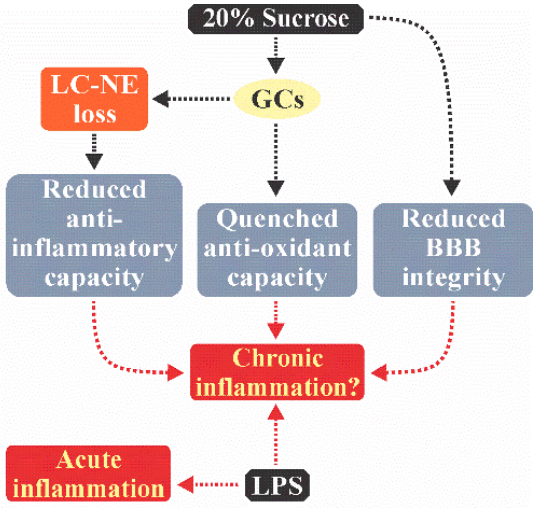

Fig 1.4. Western Diet-mediated sensitization of the CNS to inflammatory insult. While low-dose LPS may be incapable of inducing neuroinflammation on its own, reduced antioxidant and anti-inflammatory capacities associated with high-sucrose feeding may lower the threshold for development of central inflammation. High-sucrose diet-induced impairments of BBB integrity may also enhance propagation of LPS-induced inflammatory mediators into the brain.

Alternatively, the anti-inflammatory activity of glucocorticoids[142,143] establishes the possibility that events involving induction of robust glucocorticoid responses could attenuate

neuorinflammation. Thus, whether high-sucrose diets and inflammatory events prove to be synergistic in their promotion of neurodegenerative processes may depend on the strength of the event in question (*i.e.* potent inflammatory events promote acute glucocorticoid release)[151].

1.3.3 The inflammatory cascade and neurodegeneration

Neuroinflammatory processes are believed to be at the root of neurodegenerative diseases[6–12]. As discussed previously, inflammation is typically short-lived and beneficial: the stressor - be it pathogen, foreign protein, cellular debris, or toxic metabolite - is eliminated and the immune response is terminated. In moderation, inflammation can even serve to attenuate AD pathology, as identified in a study by DiCarlo *et al* (2001) who found that intracranial LPS-injections in aged APP/PS1 transgenic mice lowered amyloid-β (Aβ) deposition within 7 days[152]. However, inflammatory immune responses are dysregulated in cases of neurodegeneration, resulting in collateral damage and loss of neuronal function. Perhaps the best example of this occurs in the hippocampus, where chronic inflammation has been shown to lead to reduced neuronal plasticity and neurogenesis[153], and increased apoptosis[11]. Disruption of regular inflammatory cascades occurs during all stages of disease progression, spanning from the prodromal mild cognitive impairment (MCI) stage through the early and late stages of AD[154–156]. Interestingly, the ratio of proinflammatory to anti-inflammatory gene expression has been observed to dampen as the disease progresses, suggesting inflammation as an early event in AD as opposed to a late consequence of the disease[154,155]. In support of this notion, Tarkowski *et al* (2003) observed that patients with mCI exhibited increased cerebrospinal fluid (CSF) levels of TNFα – an inflammatory cytokine known to propagate inflammation into the CNS[12] – before later progressing to full AD[156].

Inflammation exacerbates accumulation of Aβ-plaques through modulation of amyloid precursor protein (APP) processing and iNOS expression. A study conducted by Semmler *et al* (2005) involving male Wistar rats injected with LPS found a marked increase in the activation of microglia and the expression and immunoreactivity of iNOS[11]. Elevated iNOS expression leads to increased nitric oxide (NO) production and nitrosative activity, both of which are known to regulate β-secretase[157] and γ-secretase[158] in such a manner as to potentiate formation of Aβ peptides. Dysregulation of APP processing invariably leads to AD; Down syndrome patients with trisomy of chromosome 21, the location of APP, develop AD with regularity[159]. Inflammation thus appears to be a potent factor in the progression of AD pathology, as it not only induces Aβ deposition, but appears to shift APP processing to favorably express the splice variants that result in Aβ production[17,160].

Aβ accumulation leads to sustained inflammatory activation of microglia[161], a phenomenon known to promote neurodegeneration[132,161–163]. Aβ binds to several microglial immune receptors, including TLR2, TLR4, TLR6 and CD14[132,161,163], which results in elevated production of inflammatory factors. The production of these mediators appears to be self-perpetuating: inflammation results in increased APP production, APP cleavage and Aβ aggregation[17,160], and elevated Aβ deposition results in increased inflammation[131,164]. This cycle has thus been coined the 'inflammatory cascade hypothesis' of AD[164].

1.4 A NEW ROLE FOR LITHIUM

1.4.1 Lithium is a potent inhibitor of GSK-3β

Lithium salts have a well-established role in the treatment of affective disorders, most notably mania. Intriguingly, lithium may also have use in the prevention of neurodegeneration. At present, lithium administration has demonstrated little to no capacity as a primary treatment for AD. Trials administering lithium carbonate[165] and lithium sulfate[166] have failed to generate either significant reductions in AD biomarkers or notable benefits to cognitive performance. However, lithium may slow the development of AD when administered during prodromal stages. A study by Forlenza *et al* (2011) found that long-term lithium treatment in 45 individuals with amnestic mild cognitive impairment (MCI), a state often prodromal to AD onset, yielded a significant decrease in CSF concentrations of phospho-Tau and a marked increase in cognitive performance relative to placebo groups[167]. Furthermore, a comparison by Nunes *et al* (2007) of the prevalence of AD in elderly bipolar disorder patients found that AD was diagnosed in just 5% of patients undergoing lithium therapy, in contrast to a diagnosis rate of 33% in those not on the medication[168].

It has been suggested that lithium owes its potential as a prophylactic agent against neurodegeneration to its ability to attenuate GSK-3β activity. Lithium and the GSK-3β cofactor magnesium share similar ionic radii, allowing lithium to act as a competitive inhibitor for the binding of Mg^{2+} at the catalytic core of the enzyme[169]. It is through this mechanism that lithium appears to maintain signaling through of the canonical Wingless/int (Wnt)/β-catenin pathway, itself a regulator of GSK-3β activity. The Wnt/β-catenin pathway is essential for hippocampal health. Expression of Wnt3 is associated with increased adult hippocampal neurogenesis, while blockade nearly abolishes it[170]. Reduced hippocampal neurogenesis is symptomatic of numerous conditions, such as depression, often prodromal to AD. Therefore, protection and/or rescue of hippocampal neurogenesis may attenuate progression of AD-like neurodegeneration[171–175]. Wnts are glycoproteins responsible for activating developmental and pro-proliferative signaling

pathways through interaction with several distinct receptors, including the anti-GSK-3β Frizzled (Fzd)-mediated cascade. When Wnt binds to Fzd, the protein Dishevelled (Dvl) is recruited. Activation of Dvl leads to downstream inhibition of GSK-3β, preventing the phosphorylation of the GSK-3β substrate β-catenin, thereby sparing β-catenin from degradation via the proteasomal pathway[176–178]. Increased endogenous β-catenin expands the population of dividing adult hippocampal progenitor cells, enhancing neurogenesis[179,180]. Lithium may thus potentiate the pro-neurogenesis activity of the canonical Wnt signalling pathway by increasing the pool of active β-catenin through inhibition of GSK-3β[179]. In support of this notion, a study by Gould *et al* (2007) found that transgenic mice overexpressing a constitutively active form of β-catenin in the brain exhibited behaviours similar to those observed in mice following lithium treatment[180].

1.4.2 Modern dietary choices and lithium deficiency

Given the integral role played by GSK-3β in the pathogenesis of AD and the inhibitory effects of lithium on its action, it is reasonable to question whether the growing AD concern can be partially accounted for by a lack of dietary lithium. Considering that lithium is a trace mineral found in both drinking water and plant matter, it is fair to assume that mammalian species evolved with lithium in the environment and thus developed some use for it in complex signaling pathways. Should factors arise that limit the availability of lithium or disrupt our handling/retention of it, it is possible that the resultant loss of the neuroprotective benefits[181] of the element would leave us susceptible to neurodegeneration.

As lithium is primarily removed from the body via renal clearance, it is sensible to presume that factors which increase the renal clearance of lithium (CLi) are capable of contributing to a state of lithium deficiency. Lithium is an alkali metal and monovalent cation that directly competes with sodium for transport across epithelial membranes on account of similar ionic radii[182,183]. As a consequence, lithium and sodium share an intriguing inverse relationship: as the degree of sodium intake increases, so too does the renal clearance of lithium. When sodium concentrations become excessive, transport systems resident within the epithelium become saturated and lithium resorption decreases[184]. It is thus of note that fast food, processed food and home flavorings rich in sodium have taken on a progressively larger role in the western diet[185]. In addition to salt, consumption of caffeinated beverages also contributes to lithium excretion, as indirectly demonstrated in a study by Shirley *et al* (2002) who found that males given a 400 mg daily oral dose of caffeine showed a marked increase in renal Li clearance relative to placebo control groups[186]. The dose of caffeine administered is the rough equivalent of 4 cups of coffee.

Intriguing observational evidence concerning the deleterious effects of insufficient lithium can be found within western populations. In normal and criminal populations, the concentrations of lithium found in the drinking water demonstrates a negative correlation with suicidal and aggressive behaviors[187,188]. In Texas, mental hospital admission and readmission rates in 27 communities were inversely proportional to the lithium content of residential drinking water[189]. Scalp hair analyses yield similar results. Both children with autism and their mothers demonstrate markedly reduced hair lithium concentrations relative to the general population[190], while a study of American and German adults found that roughly 20% of all individuals have low scalp hair lithium levels, with the lowest concentrations occurring in individuals with learning impairments, cardiovascular disease, and violent criminal behavior[191]. While these associations are intriguing, they do not necessarily suggest a connection between a lack of dietary lithium and AD; however, they do provide evidence of widespread lithium deficiency.

1.4.3 A case for lithium orotate

Given the reported capacity of lithium to attenuate the progressive cognitive decline seen in AD, the lack of scientific research surrounding the element as a prophylactic agent is truly perplexing. Much of the hesitation surrounding the use of lithium salts stems from the narrow therapeutic index for lithium carbonate, a common treatment option in bipolar disorder. While these concerns are certainly valid, they operate under the incorrect assumption that all lithium salt compounds exert equivalent effects in the human body. In the late 1970's, Kling *et al* (1978) noted that lithium orotate injections resulted in greater serum and brain concentrations of elemental lithium than did equivalent lithium carbonate dosages[192], perhaps as a result of reduced kidney filtration rate[193] and/or enhance delivery of lithium across cell membranes as a neutral non-dissociated lithium orotate complex[194,195]. As such, lithium orotate can theoretically achieve therapeutic brain lithium concentrations at markedly reduced dosages relative to traditional lithium compounds. Why then hasn't this compound been more heavily studied? Perhaps the reticence to explore lithium orotate as a treatment option can be traced back to a study conducted by Smith *et al* (1979), who compared the resultant renal concentrations of lithium following administration of various salt compounds. Compared to lithium carbonate, lithium orotate treatment resulted in reduced glomerular filtration rate and urine flow, and increased renal toxicity; however, the study used identical amounts of carbonate and orotate, thus negating the original reason for using lithium orotate over alternative salt compounds in the first place[193]. While others have seemingly confirmed the toxic nature of lithium orotate, they failed to demonstrate severe adverse effects despite the dose used being 18 times greater than what is recommended[196]. Considering the potential reduced dose requirements of lithium

orotate - an over the counter supplement - in comparison to lithium carbonate, it is clear that the compound warrants further study as a potentially safe treatment for a host of neurological illnesses, AD included.

1.5 AIMS AND HYPOTHESES

Aim 1 evaluated the ability of a high-sucrose diet combined with repeated bouts of systemic inflammation to generate AD-like pathology in female wild-type mice. Three-month old female C57/BL6 mice were randomized into four groups of ten in a two-by-two design. Controls, hS (20% in the drinking water), LPS (0.1 mg/kg IP), and hSL (concurrent hS and LPS treatment) constituted the four study groups. Mice were tested behaviorally after 6 months, and their brains and livers were harvested for biochemical/histological analysis at the end of the 7th month.

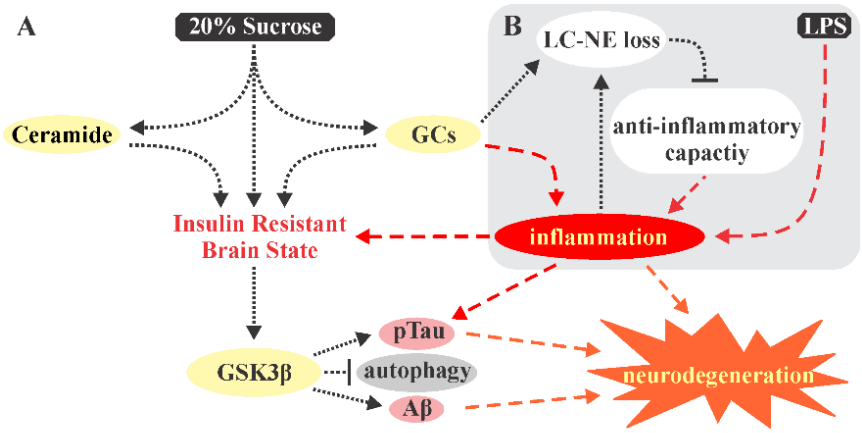

Fig 1.5. The 'perfect storm' hypothesis of sporadic late-onset AD. **A**. A high sucrose diet forms the basis of this study. We will characterize changes in insulin signaling and the downstream autophagic pathways in addition to the hallmark pathological determinants of AD (Aβ, pTau). **B**. The novelty of this work concerns the addition of repeated bouts of low-dose LPS-induced systemic inflammation to help drive neuroinflammation and neurodegeneration.

Chronic stress, fast food (high in carbohydrates) and sedentary lifestyles are fast becoming the norm in Canadian society. At great cost to our medical establishment, AD and neurocognitive disorder incidence is estimated to more than double over the coming generation. Women are expected to account for nearly two-thirds of these new patients[1]. As the rise in AD prevalence coincides with similar trends in insulin resistant and inflammatory conditions, known risk factors for AD[2,8,12,75,197], the goal of the work presented in this thesis was to combine dietary and inflammatory factors to accelerate AD-like neurodegenerative processes in female wild-type mice. These factors are a high-sucrose diet-induced insulin resistant brain state and intermittent mild systemic inflammatory events. Both a high-sucrose diet (10-20% of the drinking water)[197] and LPS can induce mild neurodegenerative phenotypes in isolation within 7-10 months[12]. Inflammation is known to contribute to neurodegeneration and brain-insulin resistance[135–137], while high-sucrose diets may exacerbate inflammation while sensitizing the brain to inflammatory insult through glucocorticoid- and ceramide-mediated mechanisms[85,86,144,147–150]. Thus, the addition of intermittent LPS injections

to animals sustained on a high-sucrose diet may prove synergistic in the pathogenesis of AD-like neurodegeneration, as outlined in figure 1.5.

I hypothesize that the combination of a high-sucrose diet and repeated bouts of systemic inflammation will aggravate and accelerate AD-related pathological processes in female wild-type mice.

Aim 2 evaluated the ability of lithium orotate to act in prophylaxis against neurodegenerative insults induced by the combination of a high-sucrose diet and repeated bouts of mild systemic inflammation. Three-month old female C57/BL6 mice were randomized into four groups of ten in a two-by-two design. Controls, hSL (20% in the drinking water; three 0.1 mg/kg IP LPS injections, delivered once per month), Li (1 mg/L elemental lithium in the drinking water) and hSLLi (Li added to hSL treatment).

The GSK-3β hypothesis of AD proposes a central role for aberrant GSK-3β activity in neurodegeneration[122]. Constitutive GSK-3β is inhibited by activity of the insulin pathway, namely Akt[118]. As GSK-3β has been shown to increase Aβ production and Tau protein hyperphosphorylation, hallmarks of AD-like neurodegeneration[118,120,122,123], it is no surprise that dysfunction of the insulin pathway has been implicated in the pathogenesis of AD[67,68]. High-sucrose diets are known to contribute to dysregulation of brain insulin signaling[85,88,197]. Thus, it is possible that inhibitors of GSK-3β, such as lithium[169,198,199], could attenuate or even prevent neurodegenerative pathologies associated with a high-sucrose diet. Lithium is also a potent inhibitor of LPS-induced inflammation[200], which itself promotes insulin resistance and CNS damage[12,135–137]. Lithium may thus exert two-fold antagonism against insults resultant of the combination of a high-sucrose diet and intermittent systemic LPS injections, as outlined in figure 1.6.

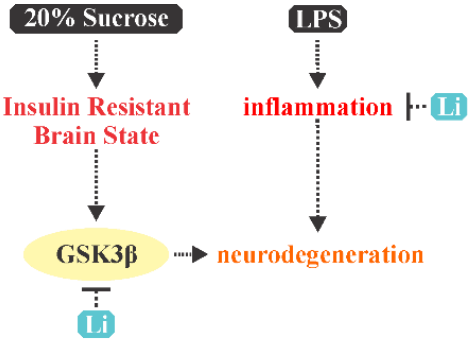

Fig 1.6. Lithium prophylaxis. Trace lithium orotate was added to mice following the hSL combination treatment to assess its prophylactic potential in neurodegenerative conditions. Lithium is a potent inhibitor of GSK3β, an enzyme central to the deleterious effects of brain insulin resistance and neuroinflammation.

I hypothesize that the addition of lithium orotate to mice sustained on a high-sucrose diet and intermittent injections with LPS will prevent development of a neurodegenerative phenotype in female wild-type mice.

CHAPTER 2: GENERAL METHODS

2.1 BEHAVIOR

After six months, mice were subjected to behavioral testing that included the open-field test (OFT) for general locomotion and thigmotaxis (wall-seeking behavior) and the Barnes maze (BM) for spatial memory acquisition and retrieval. Animals were consistently habituated to the testing room for a period of 30 minutes prior to each trial/testing. All testing was video recorded and analyzed offline using Ethovision XT 11.0 software (Leesberg, VA).

2.1.1 Open Field test

Mice were placed in an opaque white box (35 x 35 x 30 cm) under bright white light and allowed to explore for 10 minutes (min). Animals were scored for total distance travelled and time spent within the center of the field defined as nose body and tail-base >10 cm away from all walls.

2.1.2 Barnes maze

The Barnes maze featured a white escape box (placed beneath the escape hole of the stationary platform), white 100 cm (diameter) rotating platform with twenty 5.5 cm (diameter) escape holes evenly distributed about the perimeter, and a white 100 cm (diameter) stationary base with one escape location. The rotating platform was placed on top of the stationary base (itself 12 inches above the ground) (fig. 2). ***Habituation***: mice were habituated to the maze on the first day of testing via placement in the center of the testing field (within a translucent container) for 30 seconds. Animals were then gently nudged toward the escape location over a period of 30 seconds. Upon entry, animals were habituated to the escape box for two minutes. Escape location was rotated every third mouse; assigned holes were held constant throughout the acquisition protocol. ***Acquisition***: mice were placed in the center of the maze under a translucent container. After ten seconds, the container was lifted, and an aversive buzzer was triggered. Animals were allowed 3 minutes to locate the escape hole under duress of the buzzer. If successful, the buzzer was silenced, and mice were held in the escape box

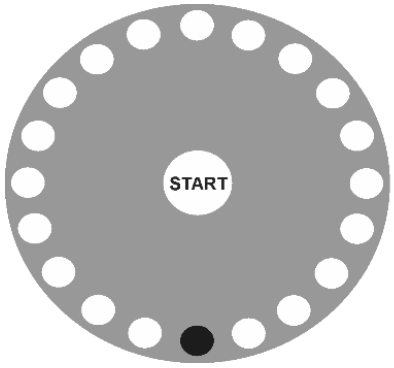

Fig. 2. Barnes maze design schematic. The Barnes maze featured a 100 cm (diameter) rotating platform with 20x 5.5 cm (diameter) holes evenly distributed about the perimeter. The rotating platform was placed over a stationary base of identical size with only one escape hole. A small holding box was placed beneath this escape (affixed to the stationary base). The entire apparatus was situated 12 inches above the ground.

for two minutes prior to return to their home cages. If unsuccessful, mice were guided to the escape location, followed by cessation of the buzzer and two-minute reinforcement in the escape box. This procedure was repeated once daily for seven consecutive days. Animals were scored for latency to escape (entry of the nose into the escape location or holes immediately adjacent) and number of errors (exploration of incorrect holes), which were used to create an acquisition index (time to escape x # of errors averaged over 7 days). High acquisition index suggests impaired spatial learning. ***Probe***: mice were probed for long-term recall of the escape location 48 hours after the final acquisition trial. Animals were allowed 3 minutes to explore the platform with the escape location closed. Animals were scored for latency to escape and number of errors, which were used to create a probe index (time to escape x # of errors). High probe index suggests impaired long-term spatial recall.

2.2 BIOCHEMISTRY

After seven months, mice were weighed and sacrificed for harvesting of liver and brain tissue. One-half of the brain was flash frozen in isopentane and ground into powder for long-term storage at -80° C.

2.2.1 Acetylcholinesterase activity assay

Mouse brain tissue was homogenized in cold 0.1 M phosphate buffered saline (PBS; pH 8.0; 10 µl/mg) and protein content was measured by the Pierce™ Bicinchoninic Acid protein assay (BCA; Thermofisher Scientific). 5 µl (4 µg protein/µl) of the sample was added to the reaction mixture that included 300 µl of 0.1 M PBS (pH 8), 2 µl of 0.075M acetylthiocholine, and 10 µl of 0.01 M DTNB (5,5-dithiobis (2-nitro benzoic acid)) solution for a total volume of 317 µl/well. 96-well plates were measured on a spectrophotometer (SpectraMax M5, Molecular Devices) at 415 nm (25 °C) in 5 min intervals for 40 min. The maximum slopes over a 10 min period were normalized to the average control activity for final comparisons.

2.2.2 Nitrate/nitrite colorimetric assay

Mouse brain tissue was homogenized in cold Tris lysis buffer (TLB; 0.01M, pH 7.4, 3x protease/phosphatase inhibitors) and protein content was measured using the BCA assay. 55 µL of samples (4 µg protein/µL) and standards (Item No. 780014) were incubated with 10 µL each of nitrate reductase cofactors (Item No. 780012; Cayman Chemicals) and enzymes (Item No. 780010; Cayman Chemicals) for 3 hours at room temperature, followed by deproteinization with 1:1 acetonitrile. Samples/standards were vortexed for 1 minute prior to centrifugation (4° C) for 10 minutes at 10,000 rcf. Supernatants (80 µL) were placed into a 96 well plate. 50 µL of Greiss

reagent 1 (Item No. 780018; Cayman Chemicals) and Greiss reagent 2 (Item No. 780020; Cayman Chemicals) were added, followed by 10-minute color development. End Point absorbance was measured on a spectrophotometer (SpectraMax M5, Molecular Devices) at 550 nm (25 °C). Total nitrates are indicative of general NO activity.

2.2.3 Corticosterone ELISA

Fecal pellets were collected from the colon during sacrifice and immediately stored on dry ice for subsequent ethanol extraction (100 µL ethanol/10 mg fecal powder) and equal volume measurement of corticosterone metabolites using an ELISA-based Assay Kit (Arbor Assays). Fecal samples were chosen over serum as they better represent a long-term average less influenced by rapid stress-induced changes in corticosterone expression.

2.2.4 GSK-3β/pGSK-3β, Akt/pAkt, Amyloid-β_{40}/Amyloid-β_{42}, and Total Tau/pTau electrochemiluminescence

Mouse brain tissue was homogenized in cold TLB (0.01M, pH 7.4, 3x protease/phosphatase inhibitors) and protein content was measured using the BCA assay (diluted to 4 µg protein/µL). 20 µg of protein was assessed for total and phospho GSK-3β and Akt protein concentrations, 100 µg of protein for $A\beta_{40}$ and $A\beta_{42}$ expression, and 10 µg of protein for total and phosphorylated Tau, as per individual Assay Kit instructions (Meso Scale Discovery). Plates were read on the MESO QuickPlex SQ 120 instrument and analyzed using the associated Workbench Discovery software (Meso Scale Discovery). Phosphorylation at serine-9, serine-473, and threonine-231 residues was assessed for GSK-3β, Akt and Tau, respectively.

2.2.5 IRS1/pIRS1, mTOR/pmTOR and IRS2 ELISA

Mouse brain tissue was homogenized in cold TLB (0.01M, pH 7.4, 3x protease/phosphatase inhibitors) and protein content was measured using the BCA assay (diluted to 4 µg protein/µL). Tissue homogenates were further diluted to 2 µg protein/µL using TLB without protease/phosphatase inhibitors before being packaged and shipped to EVE technologies (Calgary, Canada) for ELISA-based Assay (EVE Tech). Total and phosphorylated (human $pSer^{636}$, correlates to mouse $pSer^{632}$) IRS1 and mTOR ($pSer^{2448}$) were quantified. Total levels of IRS2 were assayed in-house using an ELISA-based assay kit (Aviva Assays). 200 µg/well of the 4 µg/µL samples were used for analysis of IRS2.

2.2.6 Neutral sphingomyelinase

Mouse liver tissue was homogenized in cold TLB (0.01M, pH 7.4, 3x protease/phosphatase inhibitors) and protein content was measured using the BCA assay (diluted to 4 µg protein/µL). 200 µg of sample were used for analysis of sphingomyelinase activity via ELISA-based assay (Cayman chemicals). Kinetic and Endpoint absorbance were measured on a spectrophotometer (SpectraMax M5, Molecular Devices).

2.3 HISTOLOGY

Mouse livers were fixed in neutral buffered formalin (Thermo Fisher Scientific) prior to long-term storage in PBS (0.01M, pH 7.4). Fixed liver tissues were sectioned on a vibratome (40 µm, Leica VT1200) and stained with hematoxylin and eosin for assessment of fatty liver (ballooning hepatocytes and lipid droplets.

2.4 STATISTICAL ANALYSIS

Two-way ANOVA ($P<0.05$) was performed to assess potential interactions between a high-sucrose diet and repeated lipopolysaccharide challenge (aim 1), and between the combined treatment (hS + LPS) and lithium supplementation (aim 2). Cohen's D values were determined relative to control for all groups to emphasize the size of the difference between means in an effort to avoid confounding strength of effect with sample size[201]. As both the small sample size used (n=10) and the lengthy duration of the study (7 months) likely contributed to increased variation within groups, I decided that a focus on treatment significance (two-way ANOVA with a sample size of 20) along with magnitude of effect (Cohen's D) would provide more reflective data than one-way ANOVA and associated post-hoc tests (which are heavily influenced by sample size). Level of significance does not reflect effect size, and p-values greater than 0.05 are not inherently worthless[202]. In fact, if the effect size is not exactly zero, large enough samples will yield significance for differences that are essentially meaningless[201–203]. For this reason, medium-to-large effects are worthy of consideration even in the face of increased risk of type I error for the potential they may represent when larger samples sizes are used. In this thesis, Cohen's D effects were classified as medium ($D>0.5$) or large ($D>0.8$) [201]. Small effects were excluded as they did not correlate well with reasonably low p-values.

CHAPTER 3: DIET, INFLAMMATION AND NEURODEGENERATION

3.1 INTRODUCTION

The rise in AD incidence corresponds with similar trends in insulin resistant and inflammatory conditions, both of which are known risk factors for neurodegeneration. While aging remains the largest factor in AD pathogenesis, it is possible that dietary and lifestyle choices associated with insulin resistance and inflammation are at play. AD is twice as prevalent in diabetic (insulin resistant) patients, leading some authors to propose AD as type-3 diabetes; *i.e.* diabetes of the brain[2]. A substantial body of evidence also suggest inflammation as causative. In fact, neuroinflammation is considered by many to be at the root of neurodegenerative conditions[6–12]. With two-thirds of new AD patients are expected to be female, the post-menopausal loss of estrogen in women represents an additional underexplored risk factor, especially when considering the neuroprotective capabilities of estrogen[31,33,204,205]. The majority of AD research in animal models relies on humanized genetic mutations that account for less than 10% of all cases, necessitating examination of the role of non-genetic risk factors, such as obesity, insulin resistance and inflammation, in the pathogenesis of neurocognitive disorder in both male and *female* wild-type animal models.

Excess sucrose (20% sucrose in the drinking water) has been shown to induce metabolic, behavioral and pathological changes consistent with AD-related neurodegeneration in male wild-type mice[75,77,197]. While only a mild phenotype is present after numerous months, it should be noted that multiple pathways involved in the pathogenesis of AD are altered. As such, it has been proposed that while insulin resistance alone is insufficient to generate a full AD phenotype, it may serve as a cofactor in the etiology and progression of the disease[2]. Considering the consistent increase in average sucrose consumption over time[43–45], a high-sucrose diet could represent a model of neurodegeneration that reflects human dietary patterns. High sugar intake is associated with elevated glucocorticoid levels and exaggerated glucocorticoid-mediated responses to other stressors[85]. Chronic glucocorticoid activity has been demonstrated to quench CNS antioxidant capacity (increased oxidative damage)[86], potentiate neuorinflammation[87], and induce brain insulin resistance[88]. Caloric excess and chronic glucocorticoid activity can also lead to steatosis of the liver[206–208]. Steatosis, and associated pathology, is linked to increased stress-sensitive production of neurotoxic ceramides[74,92–94]. Over-expression of ceramides and glucocorticoids has been shown to contribute to disruption of insulin signaling[85,97,98,209] and induction of pro-apoptotic pathways[99,100]. Dysregulation of the brain insulin pathway has been

linked to increased β-amyloidogenesis and hyperphosphorylation of the microtubule-associated Tau protein, hallmarks of AD-like neurodegeneration[78,197,210]. This increased phosphorylation of Tau and production of β-amyloids may be the result of aberrant GSK-3β signaling[122,211]. GSK-3β is inhibited via phosphorylation by Akt. As Akt is a central mediator of the insulin pathway, any disruption in normal insulin signaling can lead to dysfunction of Akt, and thus GSK-3β mal-activity[122,123,211].

Chronic inflammation may also contribute to the accumulation of amyloid-β (Aβ) plaques through modulation of amyloid precursor protein (APP) and inducible nitric oxide synthase (iNOS) expression. Increased iNOS activity leads to enhanced production of nitric oxide (NO) and associated nitrosative activity known to potentiate β-secretase[157]- and γ-secretase[158]-mediated formation of Aβ peptides. Given this link to β-amyloidogenesis, it is clear that ordinarily beneficial acute inflammatory responses can become neurotoxic when active for exaggerated periods of time. Interestingly, high sugar diets may instigate process that allow for the transition from acute inflammation to chronic. First, caloric excess contributes to inflammation directly, highlighting potential synergy between insulin resistant conditions and inflammatory events[212–214]. Second, caloric excess may be able to enhance the propagation of inflammatory mediators into the brain through disruption of the blood-brain-barrier (BBB)[215,216] and glucocorticoid-mediated alteration of anti-inflammatory and antioxidant capacities[86,87].

To test the hypothesis that mild inflammatory events will accelerate AD-associated pathological processes in an established high-sucrose model of neurodegeneration, I combined a high-sucrose diet (20% of the drinking water) with repeated systemic injections of lipopolysaccharide (LPS; 0.1 mg/kg) in reproductively normal female wild-type mice. While the dose of LPS chosen is likely insufficient to induce significant neuroinflammation on its own, I propose that a high sucrose diet will enable establishment of a chronic inflammatory state.

3.2 EXPERIMENTAL MICE AND STUDY GROUPS

Three-month-old female C57/Bl6 (Charles River, Canada) mice were randomized into four groups of ten animals constituting a 2x2 design. The control group was provided normal drinking water and given three intraperitoneal (IP) saline injections delivered once per month for three months beginning after the 4th week of treatment. A lipopolysaccharide (LPS; Sigma, 0.1 mg/kg IP) group was administered LPS in place of saline. A high-sucrose group was provided 20% sucrose in drinking water with three IP saline injections. A high-sucrose-LPS (hSL) combined treatment group followed the regimens of hS and LPS treated mice. See figure 3.1 for a summary of experimental groups and treatments. Mice were housed in pairs and kept on a 12-hr light/dark cycle. Behavioral testing began after six months of treatment with animals being sacrificed for tissues after seven months. Mice aged 3 months and 10 months can be roughly equated to humans aged 20 years and 40 years. Thus, the animals in this study approximate the period between human maturity and middle age[217,218]. All experiments were approved by the University of Saskatchewan Animal Research Ethics Board and done according to the Canadian Council on Animal Care.

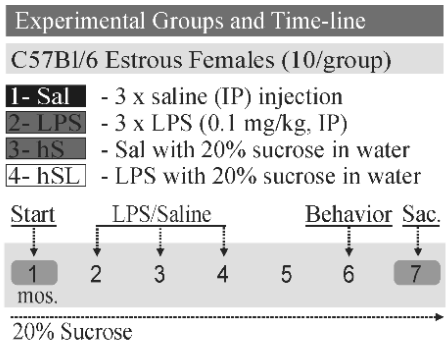

Fig. 3.1. Schematic outline of experimental groups and time-line. Two-by-two design comparing LPS injections and a high-sucrose diet.

3.3 A HIGH-SUCROSE DIET AS A MODEL OF CHRONIC STRESS AND LIVER STEATOSIS

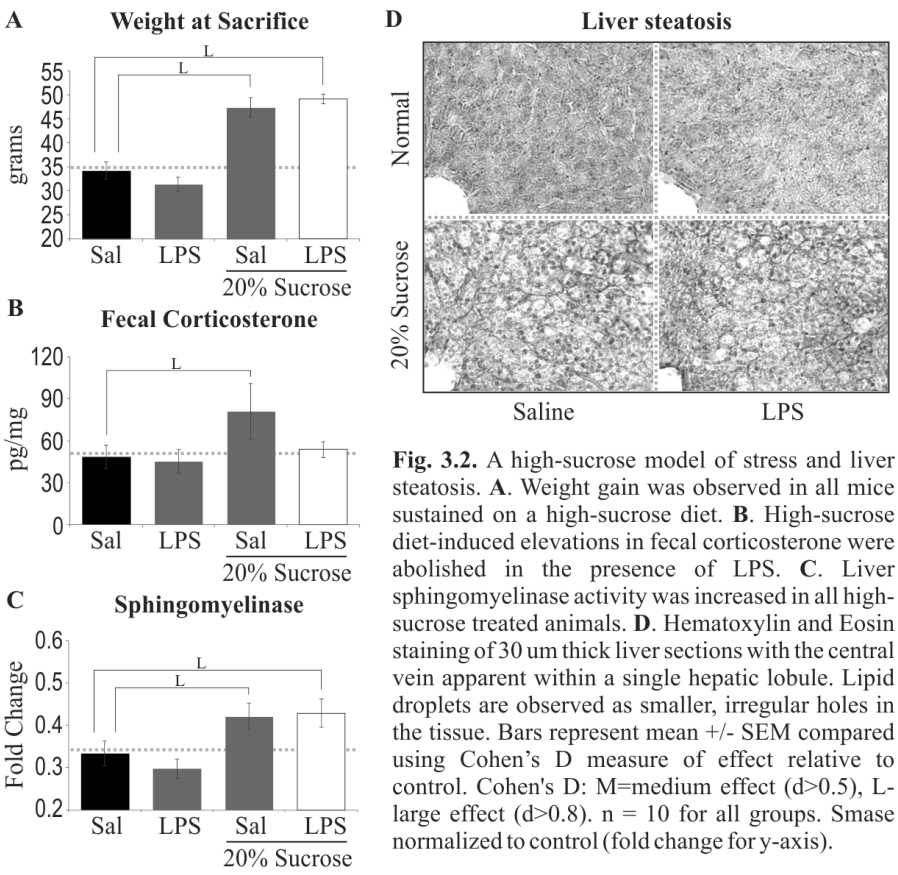

Fig. 3.2. A high-sucrose model of stress and liver steatosis. **A**. Weight gain was observed in all mice sustained on a high-sucrose diet. **B**. High-sucrose diet-induced elevations in fecal corticosterone were abolished in the presence of LPS. **C**. Liver sphingomyelinase activity was increased in all high-sucrose treated animals. **D**. Hematoxylin and Eosin staining of 30 um thick liver sections with the central vein apparent within a single hepatic lobule. Lipid droplets are observed as smaller, irregular holes in the tissue. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups. Smase normalized to control (fold change for y-axis).

Animals maintained on 20% sucrose in their drinking water (hS) demonstrated significant weight gain (sucrose effect P<0.001, two-way ANOVA) at the time of sacrifice (7 months) concomitant with an increasing effect on fecal corticosterone expression, as reflected by Cohen's D measure of effect size[201] and two-way ANOVA analysis of treatment effect (D=0.739 for hS) (sucrose effect P=0.023, two-way ANOVA)(Fig. 3.2a,b). Interestingly, co-administration of LPS prevented/reset the observed hS-induced increase in glucocorticoids. Increased liver neutral sphingomyelinase activity (sucrose effect P=0.024, two-way ANOVA) (Fig. 3.2c) and hepatic steatosis (Fig. 3.2d) were similarly observed in high sucrose fed animals. Given that corticosterone injections are known to affect ceramide production and steatosis[219], it is interesting to find that LPS did not show any antagonistic effects on sphingomyelinase activity or steatosis in the combined group, despite normalizing fecal corticosterone levels, supporting the notion that a high sucrose diet will increase sphingomyelinase activity independent of baseline corticosterone levels

3.4 SEVEN MONTHS ON A HIGH-SUCROSE DIET ALTERS INSULIN-RELATED SIGNALING

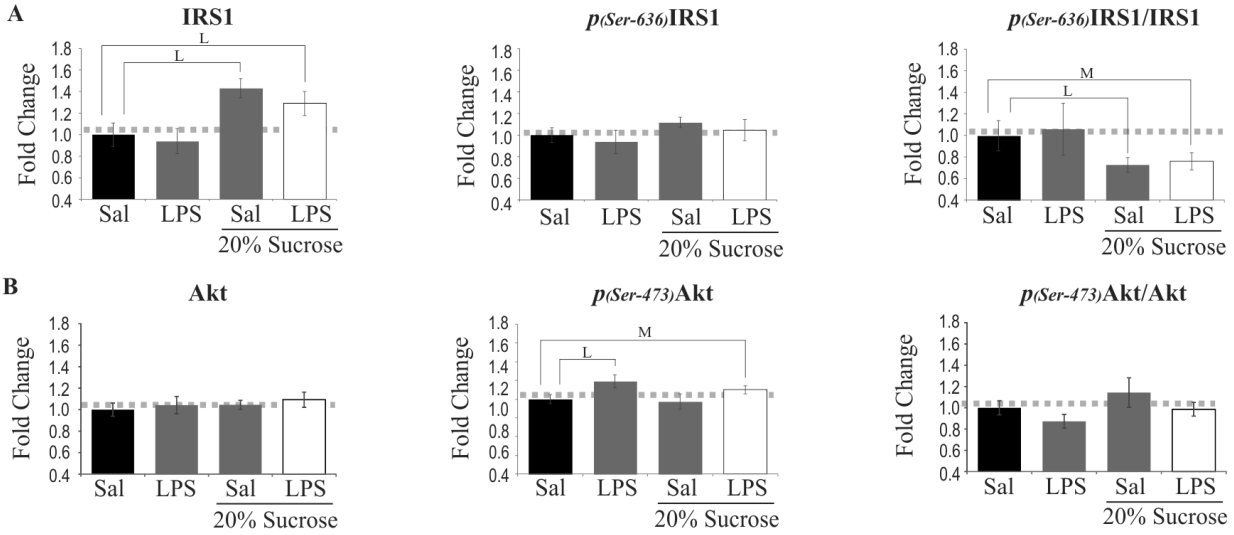

Fig. 3.3. Seven months on a high-sucrose diet promotes early irregularities in brain insulin pathway activity: Part A. **A**. High-sucrose treatment increased total IRS1 protein levels without affecting the state of phosphorylation at Ser-612. **B**. Akt phosphorylation at Ser-473 was elevated in LPS treated animals, regardless of the presence/absence of sucrose. No changes in total Akt expression were noted. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 9-10 for all groups. All values are normalized to control (fold change for y-axis).

Total insulin-receptor substrate 1 (IRS1) protein levels were increased after seven months on a 20% sucrose diet (sucrose effect P = 0.003, two-way ANOVA) (Fig. 3.3a, left), while phosphorylated levels remained consistent. Although the ratio of phosphorylated to total IRS1 protein did not change significantly (sucrose effect P=0.107, two-way ANOVA), it demonstrated a decreasing effect (reduced expression) in hS-treated groups (D = 0.554) (Fig. 3.3a, right). Neither protein expression nor phosphorylation state of Akt were altered in hS mice. Conversely, activation of the downstream mediator Akt was increased following LPS treatment, regardless of the presence/absence of a high sucrose diet (LPS effect P=0.011, two-way ANOVA)(Fig. 3.3b, middle). This increase in *p*Akt coincided with an enhanced degree of mTOR phosphorylation (D=0.585), which is regulated in part by Akt activity (Fig. 3.4b, bottom). Total and phosphorylated protein levels for GSK-3β and p70S6K (Fig. 3.4a,c), downstream effectors of the insulin pathway, were unaffected by hS or LPS treatment. This lack of second messenger activity downstream of IRS1 despite increased availability of the protein may suggest an impairment or dysregulation induced by hS within the brain insulin pathway (Fig. 3.3). Decreased phosphorylation of mTOR (phosphorylation implies activation) further supports this notion (hS effect P=0.006, two-way ANOVA) (Fig. 3.4b, middle).

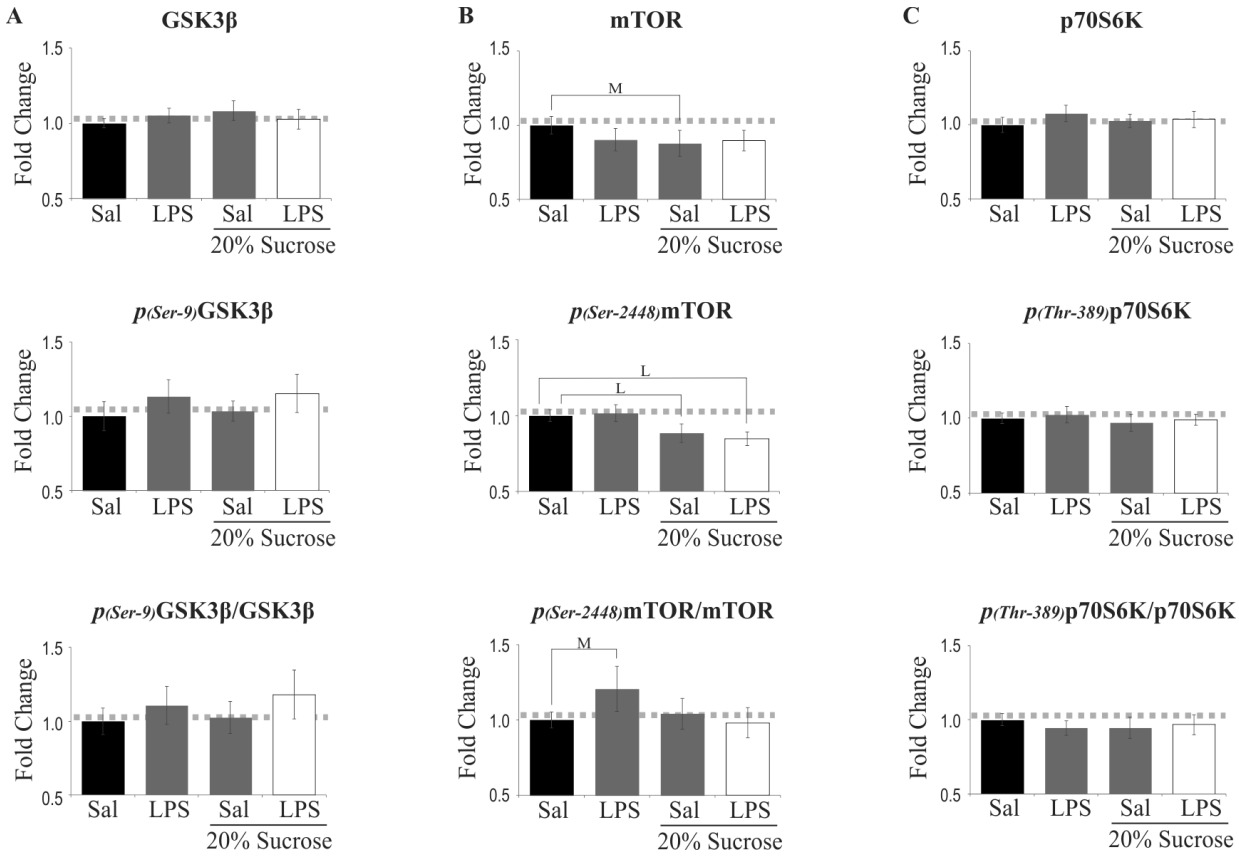

Fig. 3.4. Seven months on a high-sucrose diet promotes early irregularities in brain insulin pathway activity: Part B. **A**. No effects on GSK3β total or phosphorylated protein levels were observed in any treatment group. **B**. Levels of phosphorylated mTOR were reduced in all animals sustained on a high sugar diet. While a trend toward decreased *p*mTOR expression was observed in animals following the hS regimen, no significant differences in total protein levels or the ratio of total to phosphorylated proteins were noted. **C**. No changes in total or phosphorylated p70S6K protein levels were noted. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 9-10 for all groups. All values are normalized to control (fold change for y-axis).

3.5 HIGH-SUCROSE TREATMENT CONTRIBUTES TO NEURODEGENERATIVE PROCESSES

Considering the well-established role of neuroinflammation in the etiology and progression of neurodegenerative conditions, I examined nitric oxide activity and cytokine expression to assess any potential inflammatory phenotype. Further, given the importance of acetylcholine in memory and cognition, and the loss of this system in neurodegenerative conditions, I evaluated acetylcholinesterase activity. Nitrate levels, indicative of NO activity, were elevated in all groups receiving high-sucrose in the drinking water (high-sucrose effect P=0.002, two-way ANOVA) (Fig. 3.5a). No changes in acetylcholinesterase activity were observed in any group (Fig. 3.5b).

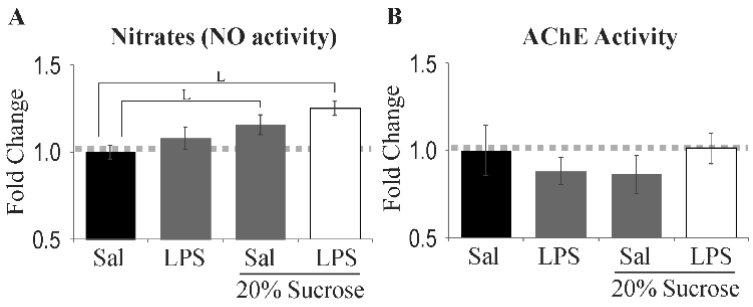

Fig. 3.5. A high-sucrose diet and repeated lipopolysaccharide challenge exert differing effects on general inflammation and cytokine expression. **A**. A high-sucrose diet increased nitrate expression regardless of LPS exposure. **B**. No notable effect on acetylcholinesterase activity was observed. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M = medium effect (d>0.5), L = large effect (d>0.8). n = 10 for all groups. All groups normalized to control (fold change for y-axis).

Interestingly, only hSL groups demonstrated a decreasing effect (reduced expression) on levels of the proinflammatory cytokines IL-5 (D=0.987), IL-1β (D=0.952) and IL-6 (D=0.768) (Table 3.1). In contrast to its effects on nitrate/nitrite expression, the high sugar diet appeared to be the primary mediator of this observed attenuation of pro-inflammatory mediator release/production (high-sucrose effect P=0.017 for IL-5, P=0.091 for IL-1β).

	IL-10	TNFα	IL-6	IL-1β	IL-5
Control	2.704 ± 0.147	0.118 ± 0.008	2.633 ± 0.146	0.314 ± 0.023	0.126 ± 0.008
LPS	2.786 ± 0.187	0.124 ± 0.013	2.360 ± 0.155	0.313 ± 0.019	0.130 ± 0.009
hS	2.812 ± 0.219	0.108 ± 0.010	2.428 ± 0.189	0.303 ± 0.034	0.120 ± 0.008
hSL	2.900 ± 0.171	0.122 ± 0.029	**2.235 ± 0.148^{M}**	**0.244 ± 0.016^{L}**	**0.098 ± 0.008^{L}**

Table 3.1. A high-sucrose diet and repeated lipopolysaccharide challenge intersect to suppress proinflammatory cytokine expression. All pro-inflammatory cytokines are normalized to interleukin-10. The addition of lipopolysaccharide to mice sustained on a high-sucrose diet suppressed brain interleukin-5, interleukin-1β, and interleukin-6 expression. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups.

As hypo-activation of the insulin pathway is known to contribute to hyperphosphorylation of the microtubule associated protein Tau and increased genesis of the amyloid-β_{42} protein[77,92,114,117] (hallmarks of AD-associated neurodegeneration), I assessed treatment effects on hemibrain Tau and Aβ_{42} levels. Only hS treated mice displayed an increase in effect for Aβ_{42} production (D = 0.619) (Fig. 3.6a, top). No changes in Aβ_{40} levels were observed (Fig. 3.6a, middle). Interestingly, while both hS and LPS groups demonstrated increased effect on the ratio

of $A\beta_{42}$ to $A\beta_{40}$ (Cohen's D=0.570 and D=0.580, respectively), this was lost in combination, suggesting antagonistic interaction (P=0.017, two-way ANOVA *interaction*) (Fig. 3.6a, bottom). No differences between groups were observed for total Tau expression, while phosphorylated Tau (Thr^{231}) demonstrated increased effect in hS mice (D = 0.517) that was blocked with concurrent addition of LPS (Fig. 3.6b, top and middle). Of note, treatment with LPS suppressed Tau phosphorylation regardless of the presence or absence of high sucrose (LPS effect P=0.036, two-way ANOVA) (Fig. 3.6b), though this was lost when normalized to total Tau protein (total and phosphorylated protein levels were similarly reduced). The modest effects of hS treatment on Tau phosphorylation and β-amyloidogenesis could have been due to the use of hemi-brain homogenates (may have diluted region-specific differences) and early time point of assessment. It is also possible that increased phosphorylation of Tau occurred on sites other than Thr^{231}. Nonetheless, when paired with corticosterone, sphingomyelinase, and NO data, the results obtained in a non-transgenic wild-type model at this early time-point support a high-sucrose diet-mediated upregulation of neurodegenerative processes antagonized by acute inflammatory events.

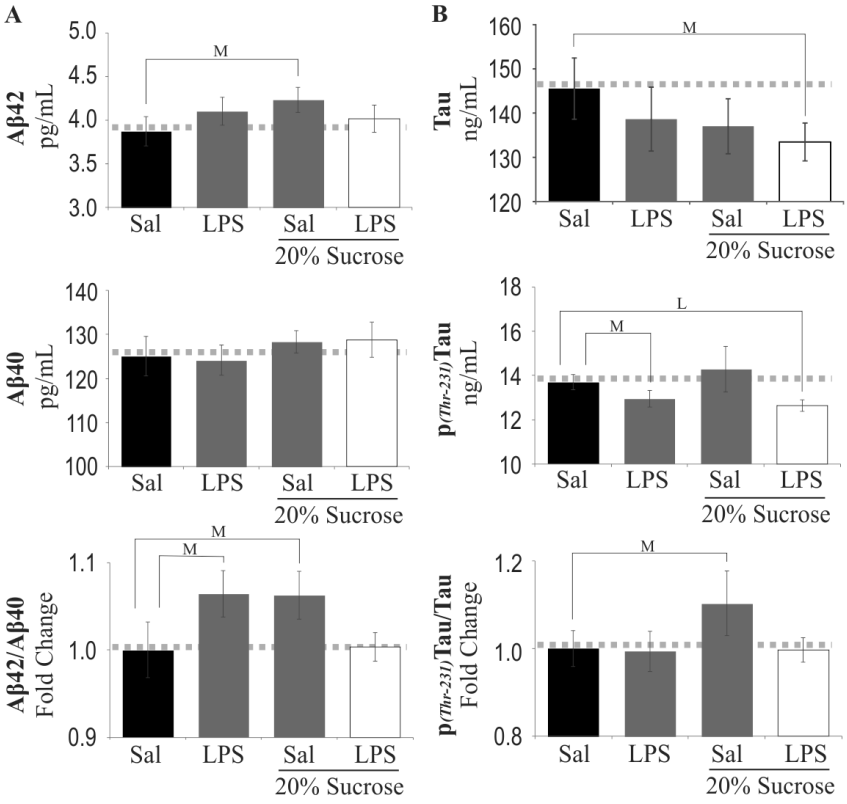

Fig. 3.6. Effects of a high-sucrose diet and/or lipopolysaccharide on β-amyloidogenesis and Tau phosphorylation in estrous female mice. **A**. Aβ42 expression demonstrated a trend toward increase in mice sustained on a high-sucrose diet. Aβ40 was unaffected. An interaction between treatments concerning the ratio of Aβ42 to Aβ40 was observed using 2-way ANOVA. **B**. Mice sustained on a high-sucrose diet demonstrated a trend toward increased Tau phosphorylation that was blocked with concomitant addition of LPS; LPS treatment suppressed Tau phosphorylation regardless of the presence/absence of high-sucrose. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L=large effect (d>0.8). n = 8-10 for all groups. Aβ42/Aβ40 and pTau/Tau ratios were normalized to control (fold change for y-axis).

3.6 A HIGH SUGAR DIET ALTERS BEHAVIOR DIFFERENTLY IN THE PRESENCE/ABSENCE OF LIPOPOLYSACCHARIDE

Behavioral studies performed during the 6th month of treatment demonstrated differing high-sucrose-associated behavioral phenotypes in the presence and absence of lipopolysaccharide. Animals sustained on hS alone displayed worsened spatial learning performance in the Barnes maze (D=0.933), characterized by increased latency to escape and frequency of errors. This impairment aligned with elevated fecal corticosterone expression (Fig. 3.7a, left; Fig. 3.2a, middle). Such results are consistent with the effects of chronic corticosterone on spatial learning observed elsewhere[77,220,221], suggesting a glucocorticoid-mediated impairment in our hS mice. In contradiction to this notion, hSL mice demonstrated an impairment in spatial learning (D=0.761) that did not coincide with increased fecal corticosterone levels. No effects on long-term spatial recall were observed for any group (Fig. 3.7a, right).

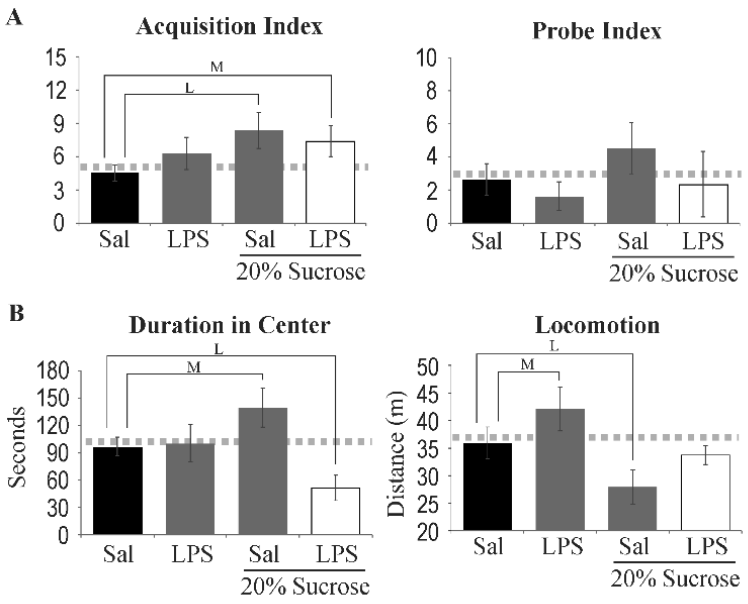

Fig. 3.7. Differential alteration of Barnes maze-associated spatial learning performance and Open Field duration in center in response to high-sucrose feeding and/or systemic LPS injection. **A**. **Barnes maze**. Acquisition index is defined as (time to escape) x (# of errors) averaged over 7 days for each animal. The probe index is similarly derived from a single trial 48 hours after the final training period. Rate of spatial memory acquisition was impaired in animals sustained on a high-sucrose diet. The addition of LPS attenuated - though did not abolish - these deficits. No differences in 48 hour recall of the escape location were observed. **B**. **Open Field**. The addition of systemic LPS to high-sucrose fed mice appeared to decrease duration in center; significant interaction between treatments was noted. Animals fed a high sugar diet demonstrated a trend toward increased duration in center. Locomotion did not differ significantly between groups, though a trend toward increased and reduced distance traveled was noted for LPS and hS animals, respectively. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups.

During the Open Field test, only the combination of LPS- and hS-treatment precipitated a potentially anxiogenic phenotype, characterized by increased thigmotaxis (D=1.080) (Fig. 3.7b, left). Avoidance of the center of the field is often associated with anxiety-like behavior[222–224], suggesting that some anxiogenic interaction (P=0.076, two-way ANOVA *interaction*) between a high-sucrose diet and LPS exists that is absent following the individual treatments. Furthermore, hS alone induced increased entry into the center (D=0.784), highlighting a significant difference in the phenotypes observed in the presence/absence of concurrent LPS treatment. Weight gain

resulting from the hS diet is unlikely to account for these differences in thigmotaxis, as overall locomotion in hSL mice did not differ from control (Fig. 3.7b, right).

3.7 DISCUSSION

3.7.1 Summary

Given that trends in AD incidence coincide with similar trends in obesity, insulin resistant conditions, and chronic inflammation - all known risk factors for AD[12,75,225] – the present study combined a high-sucrose drinking water regimen with repeated monthly intraperitoneal lipopolysaccharide injections to accelerate AD-related pathology in reproductively normal-female wild-type mice in a manner that better captures sporadic late-onset neurodegeneration. Female mice were used as the bulk of AD literature concerns male animal models despite nearly two-thirds of all AD patients being women. I demonstrated that high sugar consumption promotes mild upregulation of AD-related processes after just 6-7 months, as evidenced by elevated fecal corticosterone (Fig. 3.2), increased liver sphingomyelinase activity (Fig. 3.2), brain insulin pathway dysregulation (Fig. 3.3; Fig. 3.4), increased β-amyloidogenesis (Fig. 3.6) and Tau protein phosphorylation (Fig. 3.6), an altered brain inflammation state (Fig 3.5), and worsened spatial learning in the Barnes maze (Fig.3.7). Interestingly, the addition of LPS blocked many of these effects, as fecal corticosterone (Fig. 3.2), insulin pathway activity (Fig. 3.3), β-amyloidogenesis (Fig. 3.6) and Tau phosphorylation (Fig. 3.6) were rescued.

3.7.2 A high-sucrose diet as a model of mild neurodegeneration

Previous studies have shown that caloric excess promotes lipogenesis and triglyceride storage in both the liver and adipose tissue[226]. In cases of chronic excess, exacerbated intake leads to liver insulin resistance and steatohepatitis (inflammation of fatty liver)[2,206,207]. This inflammatory state promotes lipolysis and degeneration of the liver, culminating in mitochondrial- and/or apoptotic-mediated cell-death and ceramide synthesis[104,105]. The resultant free fatty acids, proinflammatory cytokines, and ceramides can induced both systemic and central dysregulation of the insulin pathway, leading to eventual neurotoxicity[92,207]. In this study, animals sustained on 20% sucrose in their drinking water displayed weight gain, hepatic steatosis, and elevated fecal corticosterone expression and neutral sphingomyelinase activity (ceramides) after 7 months. As noted prior, these conditions have been linked with insulin resistance, neurodegeneration and cognitive impairment[74,207,219,227]. A recent study by Chen *et al* (2017) demonstrated that liver ceramide synthesis is upregulated by glucocorticoid signaling[219], thus opening the possibility that

glucocorticoids are partly responsible for the high-sucrose-induced liver pathologies in this study. While I did not assess systemic insulin sensitivity directly, observed increases in corticosterone, sphingomyelinase activity and liver steatosis suggest that our high-sucrose animals were likely insulin resistant. Furthermore, ceramides have been shown to inhibit brain insulin signaling[92,219,227] and promote both neuroinflammation[228] and oxidative stress in the CNS[74,207,227]. Given that glucocorticoids are known to induce brain insulin resistance[88], it seems possible that high-sucrose diet-induced effects on brain insulin signaling are mediated through chronic corticosterone- and ceramide-associated activity.

A high-sucrose diet induced irregular alterations in the brain insulin pathway. Total IRS1 protein expression was found to be increased in whole hemi-brain homogenates without concomitant activation of downstream mediators such as Akt and p70S6K, suggesting a possible compensatory increase in IRS1 proteins in response to reduced insulin signaling. Phosphorylated mTOR, a target of Akt, was also decreased. Elevations in total IRS1 proteins without concurrent increase in Akt phosphorylation are consistent with results observed following injection with ceramides, as demonstrated by de la Monte, *et al*[227], suggesting that a high sugar diet influences the insulin pathway through pathways similar to those implicating ceramide expression. Dysregulation of brain insulin signaling has been proposed to set the stage for β-amyloidogenesis and Tau protein hyperphosphorylation, the hallmark processes of AD pathogenesis[92,114,117]. In fact, I previously demonstrated that a 20% sucrose diet induces irregularities in the insulin pathway and increases Tau phosphorylation after just 4-months in male mice[77]. Both total $A\beta_{42}$ and the ratio of $A\beta_{42}$ to $A\beta_{40}$ were mildly increased by the high sugar diet, while the proportion of phosphorylated Tau proteins was similarly elevated. Furthermore, high-sucrose animals displayed increased nitrate/nitrite expression, suggesting enhanced NO production (perhaps through upregulated iNOS). Exaggerated NO has been linked to neuroinflammation and nitrosative activity, both of which are known to enhance the processing of APP to Aβ[157,158]. Surprisingly, cytokine levels were - in apparent contradiction to NO expression - decreased in animals on a high sugar diet. Clearly, the role of microglia in neurodegeneration remains controversial. Regardless, increased β-amyloidogenesis, Tau phosphorylation and nitrate

expression were associated with worsened spatial learning performance, highlighting a potential decline in cognition. Given the data presented, it seems possible that a high-sugar diet upregulated neurodegenerative processes (*i.e.* β-amyloidogenesis, nitrosative activity, etc.) through glucocorticoid[88]- and hepatic ceramide-mediated mechanisms[74,74,207] to influence spatial memory. It should be noted that the mild phenotype observed may have been due to the sex of the animals and could represent an early AD-related phenotype (fig. 3.8). Estrogen has been shown to exert neuroprotection in a variety of models (cell culture as well as animal) and can diminish any pathological processes associated with AD, such as β-amyloidopathy, glucocorticoid over-expression, mitochondrial dysfunction, and oxidative stress[33,205].

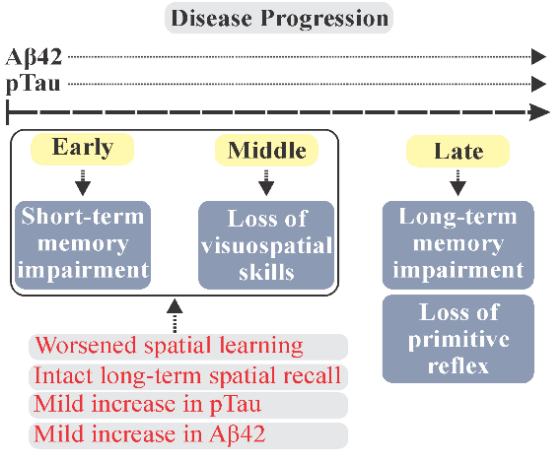

Fig 3.8: Seven months on a high-sucrose diet induces behavioral abnormalities and upregulation of neurodegenerative processes suggestive of an early AD-like phenotype. Early AD is characterized, in part, by impairments in short-term memory formation. As the disease progresses, long-term recall begins to diminish. This coincides with increased Aβ and pTau burden. Our mice demonstrate a mild though present Aβ and pTau phenotype that coincides with worsened spatial acquisition (delayed learning of a visual escape location in the Barnes maze) and spared long-term recall. This may suggest that our mice display an early AD-like phenotype.

In contrast to the high sugar diet, individual treatment with LPS did almost nothing. No effects on ceramides, sphingomyelinase, steatosis, brain insulin dysregulation or behavior were noted. However, LPS did appear to have two notable effects: elevated phosphorylation of Akt (increased activity) and enhanced production of Amyloid-β_{42} relative to Amyloid-β_{40}. Given the lack of associated pathology or behavioral abnormalities, it is possible that the observed increase in β-amyloidogenesis may have been the result of an evolutionarily conserved antimicrobial defense mechanism[229,230]. Separate studies by Miklossy and Ishida have shown that rat and mouse neurons exposed to LPS (via bacterial infection) upregulate APP[231] and Amyloid-β_{42}[232] expression. Clearly, a relationship between LPS and β-amyloidogenesis exists. Interestingly, this association may be beneficial. Soscia and colleagues (2010) demonstrated that Aβ displayed antimicrobial activity against eight common human pathogens, many of which were gram-negative (thus containing LPS)[229]. For seven of these pathogens, Aβ displayed an antimicrobial potency equal to that of a known antimicrobial peptide, LL-37[229]. It should be noted that LPS has difficulty reaching the CNS, which casts doubt on the mechanism proposed above[233]. Alternatively, is also possible that the increased activity of Akt played a role, as hyperactivity of Akt has been linked to increased β-amyloidogenesis; however, this seems unlikely given the lack of a behavioral phenotype displayed by our LPS animals (Akt hyperactivation is associated with cognitive decline[234]).

The modest effects of LPS in isolation may have been related to the administered dose. LPS interacts with toll-like receptor 4 (TLR4) to initiate the inflammatory response[235]. High concentrations of LPS (greater than 1 mg/kg) are known to initiate endotoxemia and associated septic shock-like events linked to increased mortality and neuorinflammation[236]. Conversely, lower doses of LPS have been shown to reduce inflammation and mortality in response to subsequent exposure to LPS and other TLR4 ligands[233,237]. Considering the lack of increased nitrate/nitrite and cytokine expression, it is likely that the low dose used in this study (0.1 mg/kg) failed to initiate a chronic inflammatory response. In addition, the sex of the animals may have also contributed to the lack of phenotype. Everhardt and associates (2016) demonstrated that female mice injected with LPS displayed subdued proinflammatory responses relative to their male counterparts[38]. This attenuated inflammatory response may be due to the protective influence of estrogen. In a 2001 study, Merkel demonstrated that gonadectomy increased LPS-associated mortality in female rats and that estrogen replacement therapy protected against this LPS-induced endotoxic shock[238].

3.7.3 Acute inflammatory events protect against high sugar diet-induced pathology

Counter to our expectations that a high sugar diet would exacerbate the effects of LPS, its addition to a high sugar diet failed to accelerate development of a neurodegenerative phenotype. Instead, the combination appeared to be protective, as antagonistic interactions were noted at several levels. First, and perhaps most notably, animals on the combined high-sucrose and LPS regimen did not display an elevation in fecal corticosterone. Given the proposed central nature of glucocorticoids to high-sucrose diet-mediated pathology[88], attenuation of chronic corticosterone expression may have proved to be quite beneficial. In fact, LPS was found to both suppress Tau phosphorylation (with or without sucrose) and rescue $A\beta_{42}$ levels in animals sustained on a high sugar diet.

Interestingly, the only pathology that arose due to the combination was observed in the open field behavioral test, where the addition of LPS to high-sucrose animals elevated thigmotaxis. Avoidance of the center has been linked to anxiety-like behavior, suggesting the combination treatment to be anxiogenic. This anxiety-like phenotype was coincident with reduced expression of IL-5, IL-6 and IL-1β. Aberrant IL-6 and IL-1β activity have been linked to anxiety-like behavior in both humans[239] and animal models[240], though expression of the cytokines are typically found to be increased in such conditions[239–242]. IL-5 is associated with neuroinflammation, though I am not aware of any role in anxiety-related behavior. Highlighting the complicated relationship between cytokine expression and behavior, contrasting studies have shown either no change[243], an increase[244], or a decrease in the levels of proinflammatory cytokines in patients with anxiety

disorders[245]. Furthermore, the expression of anti-inflammatory cytokines has been found to be either increased[244] or decreased in anxiety-related conditions[246]. Given that much work remains to be done to untangle the role of cytokines in anxiety-like behavior, it remains possible that reduced proinflammatory cytokine levels could be related to mal-adaptive behavioral phenotypes. The increased NO activity coupled with decreased proinflammatory cytokines observed in our hSL mice represents a 3rd inflammatory phenotype distinct from those observed in the LPS (no change relative to control) and hS (increased NO and with no change in proinflammatory cytokine expression) groups. As only our hSL mice demonstrated increased thigmotaxis in the open field, it is possible that increased NO expression concurrent with suppressed proinflammatory cytokine levels may promote anxiety-like behavior.

The protective effects of LPS may have been due to the acute nature of the inflammation induced. Glucocorticoids engage in a form of negative feedback through a hippocampal-HPA axis circuit. Once a temporal and spatial threshold of interaction between glucocorticoids and their receptors in the hippocampus is reached, activation of the HPA is suppressed. This process allows for robust yet transient corticosterone/cortisol responses to stressful stimuli[84]. Acute inflammation has been shown to promote a robust increase in circulating glucocorticoids[151] capable of triggering negative feedback. Thus, it is possible that this spike in corticosterone activity may have 'reset' the high-sucrose diet-induced chronic low-level elevation in glucocorticoids through activation of the aforementioned feedback loop. Also of note, the addition of low-dose LPS suppressed proinflammatory cytokine expression (IL-5, IL-6 and IL-1β), as noted previously. Proinflammatory cytokines are heavily implicated in the progression of neurodegeneration.

If acute inflammation did exert its protective influence through 're-setting' the corticosterone response, it reinforces both the notion that high sugar diet-induced pathology is mediated by glucocorticoids, and that transient acute inflammatory events are beneficial in the long-term management of chronic stress.

3.7.4 Conclusions

In conclusion, the present work reinforces the idea that high sugar diets contribute to the pathologic processes involved in neurodegeneration. While the impairment of spatial learning and increases in $A\beta_{42}$ and pTau expression are mild, they appear to suggest an early neurodegenerative phenotype (fig. 3.8). As some have proposed that brain insulin resistance is a late event in transgenic animal models[247], the presence of any phenotype at seven months in wild-type mice is thus significant. These studies also demonstrate that acute inflammatory events may antagonize the neurodegenerative processes associated with these diets, at least in

reproductively normal females. While it is still possible that modern lifestyle changes (*i.e.* high sugar diets) and medical complications (*i.e.* inflammation, infections, stress) can intersect to accelerate development of an AD phenotype, it remains clear that much work is to be done to untangle the relationships between diet, stress and neurodegeneration. Furthermore, this study may highlight a potential reason as to why AD occurrence is so variable among individuals. If acute stress/inflammation is protective, then perhaps differences in lifestyle, diet, exercise and work/home environments contribute to long-term cognitive health; *i.e.* some stress is needed to 're-set' cortisol homeostasis, at least in females. It also remains likely that males and females respond differently to inflammatory events due to differences in sex hormones, thus raising the possibility that the combination of a high sugar diet and LPS injection would be neurotoxic in males and post-menopausal females. Studies comparing the effects of high sugar and/or LPS in reproductively normal male and female mice to ovariectomized female mice would thus be useful for further characterizing the role of sex hormones in neurodegeneration.

CHAPTER 4: INTERACTIONS BETWEEN LITHIUM AND DIETARY/INFLAMMATORY STRESS

4.1 INTRODUCTION

AD is characterized by the presence of senile plaques and neurofibrillary tangles composed of insoluble β-amyloid fibrils and aggregates of paired helical filaments of hyperphosphorylated Tau proteins, respectively. Exaggerated Tau phosphorylation and β-amyloidogenesis are known to have many origins. One such origin is proposed in the 'GSK-3 hypothesis of AD' which presents GSK-3β mal-activity as a central mediator of AD-associated neurodegeneration[118–122,211]. GSK-3β is inhibited via phosphorylation by Akt, a central downstream effector kinase of the insulin pathway. Disruptions in normal insulin signaling, such as those observed in animals sustained on a high-sucrose diet[75,77,197], can lead to dysfunction of Akt and aberrant GSK-3β signaling[122,123,211]. GSK-3β has been linked directly to Tau phosphorylation and upregulation of β-secretase activity, leading to increased concentrations of paired helical filament and enhanced production of Aβ[118,120,122]. Inhibitors of GSK-3β have thus garnered considerable interest for their putative role in delaying pathogenesis of neurodegeneration[248,249].

Interestingly, a means for counteracting the deleterious effects of aberrant GSK-3β may already exist in the form of a common treatment for bipolar disorder - lithium. Although lithium has demonstrated little efficacy as a primary treatment for AD[165,166], it may have promise as a prophylactic treatment against processes involved in disease pathogenesis through mechanisms involving GSK-3β inhibition. Owing to its similar ionic radii to magnesium, lithium attenuates GSK-3β activity via competition with magnesium for binding at its catalytic core[169]. As GSK-3β has been proposed as central to neurodegenerative processes associated with brain insulin resistance (*i.e.* the 'GSK-3β hypothesis' of AD[122]), lithium may prevent the AD-like phenotype observed in mice sustained on a high-sucrose diet. In addition, lithium may counteract the neurodegenerative effects of inflammatory insult through a reported ability to attenuate microglial-mediated inflammatory responses[200,250,251]. As inflammation is known to contribute to insulin resistance, GSK-3β inhibition may represent a means by which lithium can exert two-front antagonism against the effects of chronic inflammation: 1) direct blockade of the inflammatory response via attenuation of microglial-activation, and 2) suppression of inflammation-induced insulin pathway dysregulation via prevention of GSK-3β over-activity[169].

To assess the prophylactic potential of lithium against dietary- and inflammatory-mediated neurodegenerative insult, lithium orotate was added to the drinking water of female wild-type mice sustained on a high sugar diet and concurrent intraperitoneal LPS injections.

4.2 STUDY-SPECIFIC METHODS

4.2.1 Experimental mice and study groups

Three-month-old female C57/Bl6 (Charles River, Canada) mice were randomized into four groups of ten animals constituting a 2x2 design. The control group was provided normal drinking water and given three intraperitoneal (IP) saline injections delivered once per month for three months beginning after 1 month. A high-sucrose-LPS (hSL) group was provided high-sucrose (20% of the drinking water) and IP LPS injections (0.1 mg/kg, once per month for three months). A high-sucrose-LPS-lithium group was provided with lithium (1 mg/L in the drinking water) in addition to the hSL treatment. See figure 4.1 for a summary of experimental groups and treatments. Mice were housed in pairs and kept on a 12-hr light/dark cycle. Behavioral testing began after six months of treatment with animals being sacrificed for tissues after seven months. Mice aged 3 months and 10 months can be roughly equated to humans aged 20 and 40 years[217,218]. All experiments were approved by the University of Saskatchewan Animal Research Ethics Board and done according to the Canadian Council on Animal Care.

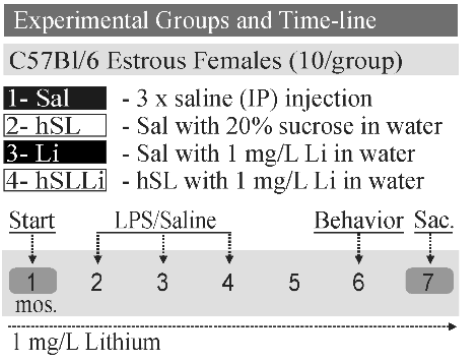

Fig. 4.1. Schematic outline of experimental groups and time-line. Two-by-two design comparing combined high-sucrose and LPS treatments to lithium orotate supplementation.

4.2.2 Lithium solution preparation

A 14.4 mM (100x) lithium orotate solution was prepared using lithium hydroxide and orotic acid. This solution was diluted 100-fold prior to use. To make 1 L of 100x solution, 0.345 g of lithium hydroxide were combined with 2.509 g orotic acid in double distilled water (ddH_2O). 16 mL of this stock were diluted with ddH_2O to a final volume of 1.6 L (1 mg Li^+/L water). Average daily water consumption was between 10-11 for mice receiving high-sucrose water and 4-5 for mice without. Average daily lithium intake was thus 0.0105 mg for hSLLi mice and 0.005 mg for Li mice. The recommended human dosage of lithium carbonate is 900-1200 mg/day[252], which translates to roughly 224 mg elemental lithium (in a 1200 mg dose). The average female bipolar disorder patient will thus consume roughly 2.89 mg elemental Li^+/kg of body weight (based on 77.4 kg being the rough average weight of a 20 year old American woman[253]). In contrast, our mice received 0.214 mg/kg (hSLLi) or 0.158 mg/kg (Li). The doses administered can thus be classified as trace, as they represent just 7.4% (hSLLi) and 5.5% (Li) of a therapeutic dose (1200 mg).

4.3 LITHIUM AFFECTS TOTAL AND PHOSPHORYLATED PROTEIN EXPRESSION OF BRAIN INSULIN PATHWAY SECOND MESSENGERS

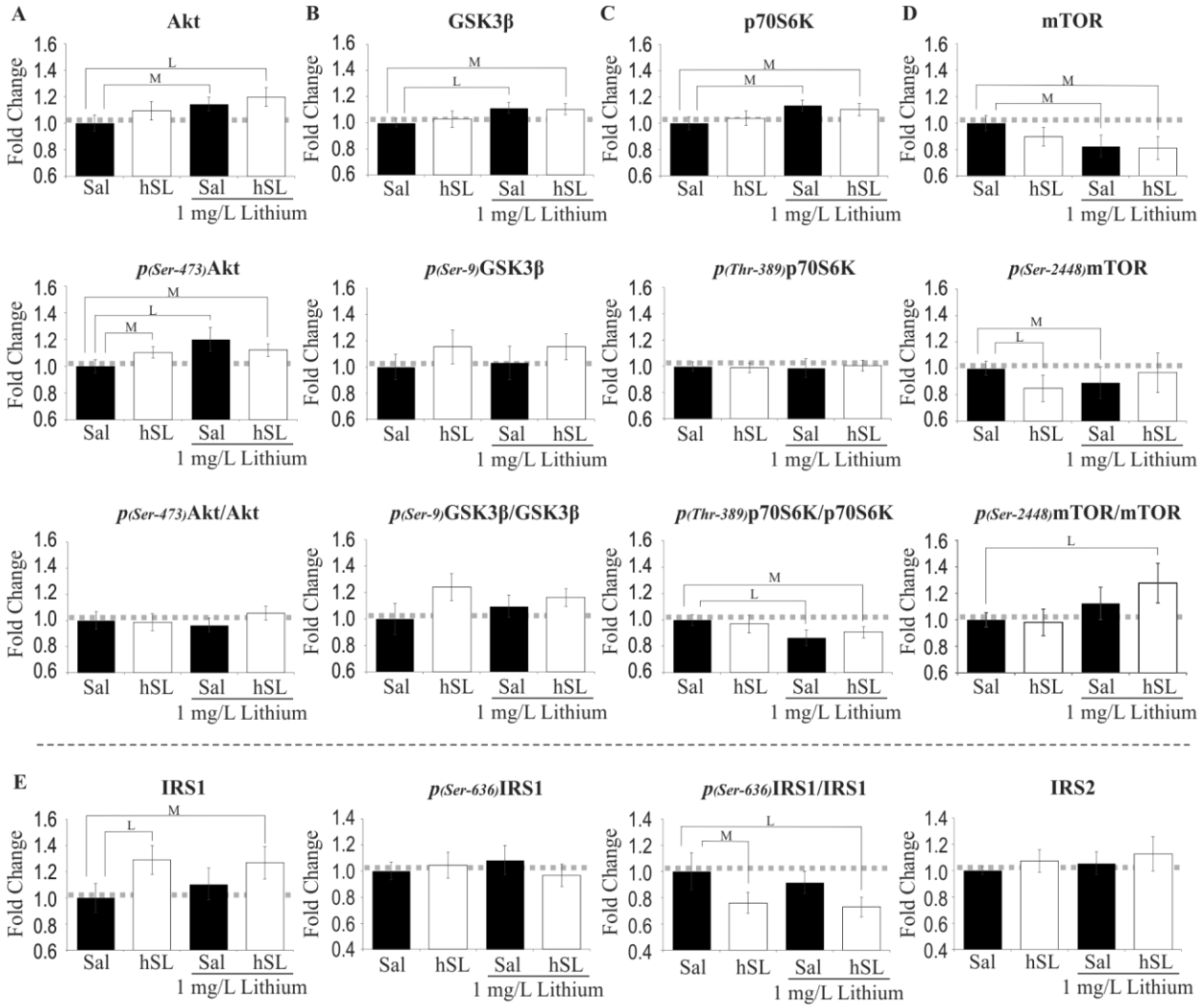

Fig. 4.2. Effects of lithium supplementation and/or hSL treatment on insulin pathway second messenger expression and activity. **A**, B, **C**. Lithium increased total expression of GSK3β, and p70S6K without altering levels of phosphorylated protein. Lithium increased both total and phosphorylated levels of the upstream mediator Akt. However, the phosphoprotein to total protein ratios were unchanged for Akt (phospho = active) and GSK3β (phospho = inactive), while p70S6K demonstrated a trend toward decreased degree of phosphorylation (phospho = active) both alone and in combination with hSL. **D**. Animals given lithium orotate in the drinking water demonstrated a trend toward decreased total mTOR protein expression, regardless of the presence/absence of hSL. Addition of LPS to a high sucrose diet reduced levels of phoshporylated mTOR; lithium blocked this effect, suggesting an interaction. The ratio of phosphorylated to total mTOR was increased only in animals receiving all 3 treatments. **E**. Supplementation with lithium had no effect on IRS1 expression, as levels were increased in all animals sustained on the combined experimental regimen. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L=large effect (d>0.8). n = 10 for all groups. All values normalized to control (fold change for y-axis).

Supplementation with lithium orotate (1 mg elemental lithium/L of drinking water) increased total protein expression for Akt (lithium effect P=0.052, two-way ANOVA) (Fig. 4.2a), GSK-3β (lithium effect P=0.040, two-way ANOVA) (Fig. 4.2b) and p70S6K (lithium effect P=0.038, two-way ANOVA) (Fig. 4.2c). While phosphorylated protein levels for GSK-3β (Fig. 4.2b) and p70S6K

(Fig 4.2c) were unaffected by lithium treatment, phosphoprotein expression was increased for Akt (lithium effect P=0.053, two-way ANOVA), which may indicate elevated activity. (Fig. 4.2a). However, given that the degree of phosphorylation (ratio of total to phosphorylated proteins) was unaffected for GSK-3β and reduced for p70S6K (D=0.802 for Li and D=0.612 for hSLLi), both downstream targets of Akt, it seems unlikely that lithium promoted Akt activity despite increased expression of *p*Akt. Lithium had no notable effects on IRS1 or IRS2 expression (Fig. 4.2e), as the increase in total IRS1 was the result of hSL treatment (hSL effect P=0.049, two-way ANOVA) (Fig. 4.2e). In contrast to second messengers discussed thus far, total mTOR expression was decreased (D=0.747 for Li and D=0.798 for hSLLi) in mice provided with lithium in the drinking water. Interestingly, lithium exerted a mild suppression of *p*mTOR levels (D=0.639) while reversing an hSL-induced decrease (D= 1.04) in hSLLi mice (Fig. 4.2d), supporting an antagonistic interaction between treatments (P=0.017, two-way ANOVA *interaction*).

4.4 LITHIUM ALTERS BEHAVIORAL DIFFERENTLY IN THE PRESENCE/ABSENCE OF HSL

Behavioral studies performed during the 6th month of treatment revealed differing behavioral phenotypes in hSL mice in the presence/absence of concurrent lithium supplementation. Mice following the hSL regimen displayed impaired spatial learning during the acquisition phase of the Barnes maze (D=0.761) that was completely abolished by the addition of lithium, suggesting a potential protective effect (Fig. 4.3a, top). Interestingly, lithium worsened spatial learning performance when administered alone (D=0.924) (Fig. 4.3a, top). No effect on long-term recall of the escape location was observed for any treatment (Fig. 4.3a, bottom). Mice in the hSL group demonstrated anxiety-like behavior in the Open Field characterized by increased thigmotaxis (D=1.080). This phenotype was blocked

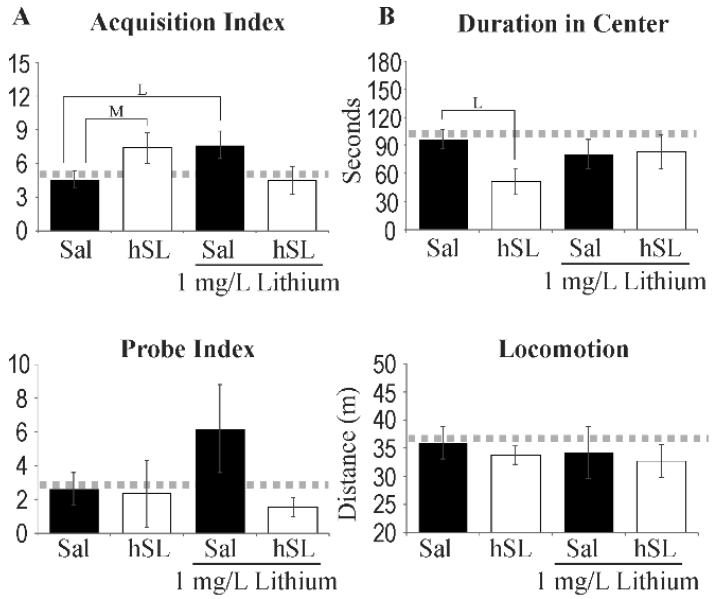

Fig. 4.3. Effects of lithium supplementation on hSL-mediated behavioral abnormalities. **A**. While learning was impaired in animals treated with lithium alone, deficits during Barnes maze acquisition trials were attenuated following addition of the element to hSL mice. No effect on long-term spatial memory was observed. **B**. Anxiety like behavior observed during the Open Field in hSL mice was abolished by concurrent lithium supplementation. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups.

following addition of lithium, suggesting an anxiolytic effect (Fig. 4.3b, top). As locomotion was unaffected in hSL mice, weight is unlikely to have contributed to differences in thigmotaxis (Fig. 4.3b, bottom).

4.5 LITHIUM ALTERS GLUCOCORTICOID HOMEOSTASIS IN RESPONSE TO HSL TREATMENT

While hSL alone did not affect fecal corticosterone levels, the addition of lithium to hSL mice increased expression (D=0.827) (Fig. 4.4b), suggesting an interaction between the treatments (P=0.040, two-way ANOVA *interaction*). Mice in the hSL group displayed increased weight gain (D=2.638 for hSL and D=1.177 for hSLLi) and sphingomyelinase activity (D=1.002 for hSL and D=0.970 for hSLLi), regardless of the presence/absence of lithium (Fig. 4.4a,c). Lithium had no notable individual effect on weight gain or sphingomyelinase activity at the time of sacrifice (Fig. 4.4a,c). Steatosis was observed in all mice following the hSL regimen, with or without concurrent addition of lithium. Lithium alone had no effect. (Fig. 4.4d).

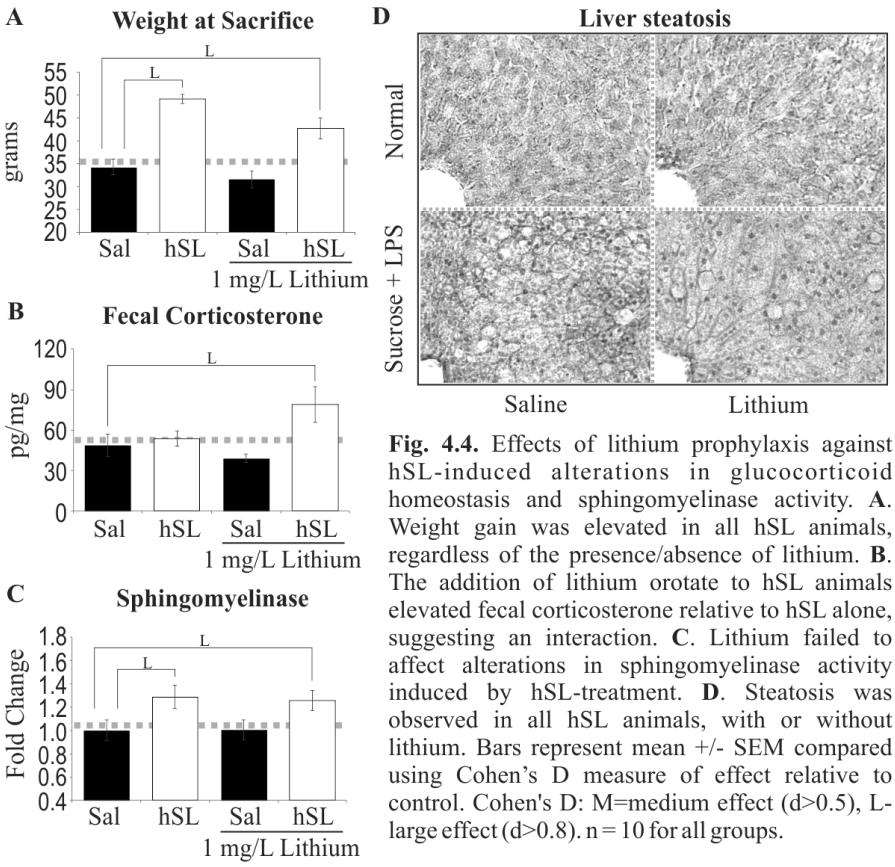

Fig. 4.4. Effects of lithium prophylaxis against hSL-induced alterations in glucocorticoid homeostasis and sphingomyelinase activity. **A**. Weight gain was elevated in all hSL animals, regardless of the presence/absence of lithium. **B**. The addition of lithium orotate to hSL animals elevated fecal corticosterone relative to hSL alone, suggesting an interaction. **C**. Lithium failed to affect alterations in sphingomyelinase activity induced by hSL-treatment. **D**. Steatosis was observed in all hSL animals, with or without lithium. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups.

4.6 LITHIUM AFFECTS INFLAMMATORY AND NEURODEGENERATIVE PHENOTYPES IN HSL MICE

As neuroinflammation is known to play a central role in the etiology and progression of neurodegeneration, I examined several markers of inflammation, including expression of nitric oxide and various pro- and anti-inflammatory cytokines. I also assessed acetylcholinesterase activity for its importance to cognition and its frequently altered state in neurodegenerative conditions. Mice following the hSL regimen demonstrated reduced IL-5 (D=0.987), IL-1β (D=0.952), and IL-6 (D=0.768) expression. Lithium blocked this effect for IL-5 and IL-1β, but not IL-6 (D=0.595 for hSLLi) (Table 4.1). Similar changes to NO levels (represented by nitrate/nitrite metabolites) were not observed, with lithium (D= 1.016) and hSL groups (D=1.742 for hSL and D=1.822 for hSLLi) displaying an increase in effect (Fig. 4.5a). No changes in acetylcholinesterase activity were noted (Fig. 4.5b).

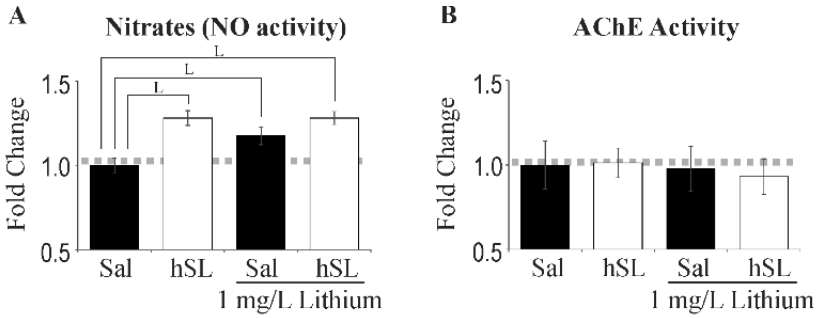

Fig. 4.5. Lithium alters neuorinflammatory processes associated with hSL treatment. **A**. Lithium increased nitrite expression, both alone and in combination with hSL. **B**. Lithium had no effect on acetylcholinesterase activity. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups. All groups normalized to control (fold change for y-axis).

	IL-10	TNFα	IL-6	IL-1β	IL-5
Control	2.704 ± 0.147	0.118 ± 0.008	2.633 ± 0.146	0.314 ± 0.023	0.126 ± 0.008
hSL	2.900 ± 0.171	0.122 ± 0.029	**2.235 ± 0.148^{M}**	**0.244 ± 0.016^{L}**	**0.098 ± 0.008^{L}**
Li	2.712 ± 0.140	0.192 ± 0.082	2.309 ± 0.126	0.349 ± 0.044	0.104 ± 0.009
hSLLi	2.672 ± 0.155	0.115 ± 0.012	**2.319 ± 0.156^{M}**	0.306 ± 0.031	0.115 ± 0.008

Table 4.1. All pro-inflammatory cytokines are normalized to interleukin-10. The addition of lithium to hSL-treated mice restored brain interleukin-5 and interleukin-1β expression to control. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L-large effect (d>0.8). n = 10 for all groups.

Given the importance of β-amyloidogenesis, and Tau hyperphosphorylation to AD-like neurodegeneration, I assessed treatment effects on hemibrain Tau and $A\beta_{42}$ levels. While hSL mice did not demonstrate a significant change in $A\beta_{42}$ production, concurrent treatment with lithium resulted in an increase in effect on expression of $A\beta_{42}$ (D=0.540) (Fig. 4.6a, left). No effects on $A\beta_{40}$ (Fig. 4.6a, middle) or the $A\beta_{42}/A\beta_{40}$ ratio were observed (Fig. 4.6a, right). Expression of pTau was reduced in all mice treated with hSL, with or without lithium (hSL effect P = 0.024, two-way ANOVA) (Fig. 4.6b, middle). Lithium alone had no effect on pTau (Fig. 6b, middle). A reduced effect on total Tau was noted in hSL mice (D=0.532) (Fig. 4.6b, left), while no changes in the pTau/Tau ratio were observed for any group (fig. 4.6b, right).

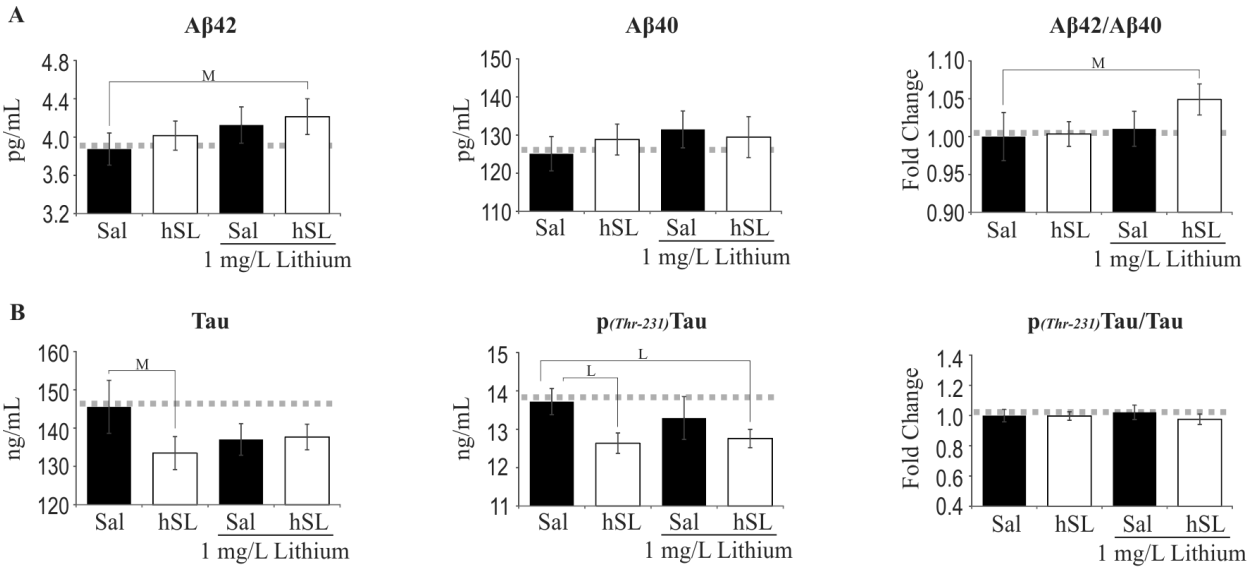

Fig. 4.6. Lithium alters neurodegenerative processes associated with hSL treatment. **A**. The addition of lithium to hSL mice increased Aβ42 levels relative to those following the hSL regimen alone. Neither total Aβ40 levels nor the ratio of Aβ42 to Aβ40 were influenced by lithium. B. Lithium supplementation had no effect on Tau phosphorylation, as all mice receiving the hSL-treatment demonstrated reduced pTau expression. Bars represent mean +/- SEM compared using Cohen's D measure of effect relative to control. Cohen's D: M=medium effect (d>0.5), L=large effect (d>0.8). n = 10 for all groups. Aβ42/Aβ40 and pTau/Tau ratios normalized to control (fold change for y-axis).

4.7 DISCUSSION

4.7.1 Summary

Modern society is rife with stressors and dietary patterns that contribute to obesity, insulin resistance, and chronic inflammation - all known risk factors for AD[12,75,225]. Given that lithium is a potent inhibitor of GSK-3β, which is believed to be central to both insulin resistant- and inflammatory condition-mediated neurodegeneration, the presented work added lithium orotate to the drinking water of female mice to assess its prophylactic potential against neurodegenerative insults resultant of combined dietary and inflammatory stressors. Female mice were used in response to the lack of literature surrounding AD pathogenesis in female models. Lithium increased insulin pathway second messenger protein expression (Fig. 4.2) and impaired spatial learning performance in the Barnes maze when administered individually. Counter to these effects in isolation, lithium completely abolished hSL-induced spatial learning deficits (Fig. 4.3), suggesting a beneficial role for the element in preserving cognition in the presence of insult. Elevations in protein expression were observed regardless of the presence/absence of combined hSL treatment (Fig. 4.2). When added to mice following the hSL regimen, lithium increased levels of proinflammatory cytokines (Table 4.1), fecal corticosterone (Fig. 4.4) and $A\beta_{42}$ peptides (Fig. 4.5), supporting a significant interaction between the treatments. Finally, lithium demonstrated anxiolytic potential in the Open Field. The addition of

lithium to hSL mice blocked the increase in thigmotaxis observed as a result of the combined treatment alone (Fig. 4.3).

4.7.2 Lithium supplementation demonstrates significant interaction with the hsl treatment

Lithium is known to attenuate LPS-induced inflammation through suppression of macrophagic and microglial activation in response to LPS exposure[200,250]. LPS, among other mechanisms, interacts with TLR4 to induce activation of macrophagic and microglial cells in the periphery and brain, respectively. One of the downstream effects of TLR4 signaling is induction of the NFκβ pathway, which has been associated with release of proinflammatory mediators, production of NO, and formation of ROS. Given that neuroinflammation, nitrosative activity, and oxidative damage have been linked to several processes implicated in neurodegeneration, it is of no surprise that LPS can contribute to neurodegenerative pathologies in murine models[12].

In contrast to potent concentrations of LPS[236], low doses have been shown to attenuate macrophagic[233,237,254] and microglial[255] responses to future inflammatory events. In the present work, I confirmed a seemingly anti-inflammatory phenotype resultant of treatment with low-dose LPS. Supplementation with lithium blocked this effect, as evidenced by a return of proinflammatory cytokine expression to control. As noted above, low doses of LPS have been shown to decrease the magnitude of macrophagic[237,254]- and microglial-mediated[255] inflammation in response to future inflammatory challenge. If lithium blocked the effects of LPS, it could explain why proinflammatory cytokine levels were returned to control in hSLLi mice.

Alternatively, LPS is known to induce an acute inflammatory response associated with a robust increase in circulating glucocorticoids[151] that, in contrast to their chronic effects[87,256], act to resolve inflammation[257–259]. It is therefore possible that an LPS-induced acute glucocorticoid response could have resulted in the suppression of inflammatory cytokine production displayed by members of the hSL group. Furthermore, this acute glucocorticoid response may have also 're-set' the chronic elevations in corticosterone associated with a high-sucrose diet[77,85]. Sufficient interaction between glucocorticoids and their receptors in the hippocampus triggers a hippocampal-HPA axis-mediated negative feedback loop that quenches glucocorticoid release from the adrenal cortex[84], as discussed previously. By antagonizing LPS-mediated signaling[200,250], lithium may have thus 1) prevented an acute LPS-induced anti-inflammatory glucocorticoid response, and 2) allowed for high-sucrose diet-induced chronic elevations in glucocorticoid expression (proinflammatory[256]) to progress unchecked (due to lack of HPA-dependent negative feedback[84]).

Intriguingly, lithium increased NO production in isolation (indicative of inflammation) but did not promote an additive increase in NO expression when introduced to hSL mice. These seemingly inconsistent effects may be due to an interesting duality in the relationship between lithium and the NO-NOS pathway. In the absence of additional treatment, lithium has repeatedly demonstrated an ability to increase NO expression in the brain[260,261]. However, lithium was found to attenuate microglial-mediated production of NO in response to LPS[251]. It is thus possible that the high levels of NO observed in our hSLLi group were the result of lithium increasing NO expression while simultaneously inhibiting hS- or LPS-induced NO production; *i.e.* hSLLi NO levels do not differ from those observed for lithium or hSL treatment alone.

Finally, lithium increased $A\beta_{42}$ levels relative to control when added to mice following the hSL regimen, despite having no effect in isolation. This effect may have been due to elevated proinflammatory cytokines and/or glucocorticoids observed in hSLLi mice relative to those in the hSL group, as proinflammatory cytokines and glucocorticoids are associated with β-amyloidogenic processing of APP[11,17,157,158,160]. Although lithium appeared to 'un-mask' hS-induced Aβ production in hSL mice, it may have prevented toxicity resultant of increased Aβ levels. Alvarez *et al* demonstrated that pre-treatment of cultured neurons with lithium prevented Aβ-induced hyperphosphorylation of Tau proteins[262,263]. The lack of elevated Tau phosphorylation displayed in the hSLLi group may thus support the notion of a lithium-mediated protective effect against $A\beta_{42}$-associated toxicity. Taking into summation the antagonistic interactions between LPS and lithium reported elsewhere[200,250], and the observed interactions between hSL and lithium on levels of fecal corticosterone, proinflammatory cytokines, NO and $A\beta_{42}$ demonstrated here, it seems reasonable to propose that lithium interfered with the activity of LPS.

4.7.3 Lithium as an anxiolytic agent

Lithium is an efficacious adjunctive treatment in the management of mood disorders[264–266] that has shown promise as an anti-depressant in both humans[267] and animal models[268,269]. A role for lithium in the management of anxiety-related conditions is far less characterized. I found that supplementation with a sub-therapeutic dose of lithium completely rescued elevations in thigmotaxis observed in hSL-treated mice. Given that avoidance of the center during the Open Field test is believed to be characteristic of anxiety-like behavior in rodents[222–224], attenuation of thigmotaxis may suggest anxiolytic properties of lithium. In fact, work by Yu *et al* supports this notion, as lithium was found to attenuate anxiety-like behavior in the Open Field in male mice following traumatic brain injury[270].

Interestingly, only a reduction in IL-5 and IL-1β coincided with the anxiogenic effect of the hSL treatment. The addition of lithium to hSL mice restored IL-5 and IL-1β levels to control while abolishing anxiety-like behavior in the Open Field, suggesting a possible role for IL-5 and IL-1β in anxiety-associated behaviors. As repeated injection with low-dose LPS has been shown to skew activated microglia toward an anti-inflammatory M2 phenotype[255], the inhibitory effect of lithium on LPS-induced inflammation may have prevented such a shift[200], resulting in increased levels of IL-5 and IL-1β in hSLLi mice. In addition, lithium may have attenuated the acute glucocorticoid response induced by LPS administration[151]. Acute glucocorticoid responses are associated with anti-inflammatory macrophage/microglia[271] phenotypes. Conversely, persistent glucocorticoid receptor agonism leads to reduced expression of M2 microglial polarization[272] and exacerbated pro-inflammatory responses[87,273]; hSLLi mice displayed chronic glucocorticoid activity. Clearly, numerous avenues exist by which lithium could have attenuated LPS-induced suppression of proinflammatory cytokines.

Regardless of mechanism, hSL mice demonstrated increased anxiety-like behavior in the Open Field that was abolished by concurrent treatment with lithium, supporting the notion of an antagonistic interaction between lithium and LPS proposed earlier. Whether or not mechanisms involving IL-5 and IL-1β were involved can be neither deduced nor inferred at this time.

4.7.4 A role for lithium in the preservation of spatial memory

AD-like neurodegeneration is characterized by a progressive decline in memory and cognition[13–16]. Behavioral tests on animal models of AD have been widely performed in an attempt to create behavioral analogues of the human condition. To this end, the Barnes maze is believed to capture certain elements of spatial cognition that in part represent the cognitive decline associated with neurodegeneration. Impaired spatial learning and spatial memory during the Barnes maze paradigm is often used as an indicator of disrupted memory formation/recall. Mice sustained on a high sugar diet with concurrent injections of LPS demonstrate worsened spatial learning performance in the Barnes maze relative to control animals.

Elevated GSK-3β activity has been linked to impaired memory consolidation[274]. As lithium inhibits GSK-3β function[198,199,275], it is possible that spatial learning deficits in hSL mice could have been the result of GSK-3β mal-signaling. GSK-3β promotes long-term depression (LTD) over long-term potentiation (LTP), and is associated with increased hippocampal LTD[276] and impaired spatial learning[276,277]. These spatial learning deficits are rescued through use of GSK-3β inhibitors[276]. While hSL mice demonstrated an apparent lack of aberrant GSK-3β activity, our method of homogenate preparation could have been to blame. As abnormal GSK-3β phosphorylation states are most commonly reported in the hippocampus, our use of full hemi-

brain homogenates may have diluted our samples and washed out region-specific differences in GSK-3β and *p*GSK-3β expression. Furthermore, as lithium primarily exerts its inhibition of GSK-3β through competition with magnesium for binding at the enzyme's catalytic core[198,199], the effects of the element on GSK-3β activity cannot be fully captured through analysis of total and phosphorylated protein levels. Thus, it remains possible that lithium could have preserved spatial learning via inhibition of hyperactive GSK-3β.

Interestingly, suppression of GSK-3β in the absence of aberrant activity may impair spatial memory. Due to its central role in the balance of LTD and LTP, GSK-3β signaling is required for proper regulation of spatial memory consolidation[278]. Results from our lithium group support this notion, as spatial learning performance was worsened in the absence of hSL. Alternatively, a reduced sensitivity of mice treated with lithium to aversive stimuli used to increase escape motivation could be responsible. A series of studies conducted by Hines *et al* found that rodents treated with lithium chloride display attenuated responses to low-intensity aversive stimuli[279–281]. Thus, worsened spatial learning performance observed in our lithium treated mice may have been the result of reduced escape motivation in response to the aversive sound-based stimulus employed.

Future work exploring treatment effects on hippocampal BDNF, neurogenesis and GSK-3β phosphorylation would shed light on the underlying mechanism for hSL-induced spatial learning impairment and lithium-mediated rescue.

4.7.5 Conclusions

The present work reinforces the notion of an antagonistic interaction between lithium and LPS[200,282], perhaps through attenuation of LPS-induced acute glucocorticoid responses. As the low dose of LPS chosen proved to be protective in the face of challenges introduced by a high sugar diet observed in Chapter 3, this antagonism may not have been beneficial. However, it should be noted that lithium-induced attenuation of LPS activity has demonstrated to be overwhelmingly neuroprotective when more potent doses of LPS are administered[200,250,282]. Lithium-induced sparing of spatial learning and a stabilizing effect on anxiety-like behavior were also supported[270], though the mechanisms responsible remain unclear. In conclusion, while lithium reversed many the protective effects of low-dose LPS against hS-induced pathology, it appeared to do so through a similar mechanism as is observed in the attenuation of endotoxemia at higher doses of LPS[200,282]. Thus, lithium may still have use as a prophylactic agent against neurodegeneration. Higher concentrations of LPS in future studies would address this question.

CHAPTER 5: GENERAL DISCUSSION

5.1 HIGH SUGAR DIETS MAY BE A FACTOR IN THE AD CRISIS

Brain insulin resistance is believed to be involved, in part, in the pathogenesis of AD[68,76]. While this insulin resistant state can arise from a number of conditions, glucocorticoid- and ceramide-mediated mechanisms have proven to be particularly robust[90,207,219,227]. High-sugar diets, as demonstrated both within this work (chapter 3) and eslewhere[77,78,197], are capable of inducing brain insulin resistance and increasing ceramide and glucocorticoid expression. Given the relationship between high-sucrose diets and neurodegeneration, the observation that trends in average sucrose consumption[44,45] coincide with increased incidence of AD[1] suggests that excess sucrose consumption is contributing to the burgeoning AD crisis. Irregular insulin pathway activity, increased glucocorticoid, ceramide, $A\beta_{42}$ and pTau expression, and spatial learning deficits displayed in our mice sustained on a high-sucrose diet support this possibility.

It has been proposed that an insulin resistant brain state precipitates exaggerated activity of GSK-3β, which has been demonstrated to contribute directly to the hallmark pathological processes associated with AD[77,118,120,122,211]. While our high-sucrose mice did not demonstrate an increase in GSK-3β signaling, spatial learning deficits and increased Aβ and pTau expression - pathologies associated with aberrant GSK-3β activity[118,120,122,211,283] - were noted. It is thus possible that our lack of an observed effect on GSK-3β was due to the use of whole hemi-brain homogenates. Use of reproductively normal females and unaccounted for interactions of estrogen with the pathways explored could also have contributed. As AD-related changes in GSK-3β expression/phosphorylation are typically observed in the hippocampus[284–286], I may have 'washed-out' region-specific alterations in enzyme activity by diluting our samples with the rest of the hemi-brain. Thus, it is possible that our high-sucrose mice demonstrated a neurodegenerative phenotype associated with brain insulin dysregulation despite lack of an observed increase in GSK-3β activity.

5.2 INFLAMMATORY EVENTS MAY PROTECT AGAINST DIET-INDUCED NEURODEGENERATION THROUGH MECHANISMS INVOLVING GLUCOCORTICOID HOMEOSTASIS

Neuroinflammation has been proposed as a central player in the development and progression of neurodegenerative conditions, including AD[6–12]. It is thus of great interest that the introduction of LPS - a known inducer of neuroinflammation[12] - to mice sustained on a high-sucrose diet attenuated hS-induced neurodegenerative processes. As discussed in chapter 3, LPS

could have 're-set' glucocorticoid levels by triggering hippocampal-HPA axis-mediated feedback (Fig. 5). Glucocorticoids interact with their receptors in the hippocampus as part of a negative feedback loop with the HPA that regulates cortisol/corticosterone activity. Once a spatial and temporal threshold is reached, HPA-mediated release of glucocorticoids from the adrenal cortex is suppressed[84]. LPS produces a robust and transient increase in corticosterone expression[151] that could possibly trigger this response. LPS may also have exerted its protective effects either through quenching overall inflammation in the CNS, as others have demonstrated following introduction of low-dose LPS[254], or instigating[151] acute glucocorticoid-mediated immune suppression[257–259]. I demonstrated that systemic LPS reduced expression of the proinflammatory mediators IL-1β, IL-6 and IL-5 in the brain when added to mice sustained on a high-sucrose diet. As IL-1β and IL-5 are associated with activated M1 microglia, which have been linked to neurodegeneration[131,287], repeated low-dose LPS injections may have increased M2 microglial polarization over the long-term[255]. M2 microglial polarization is believed to be protective in most circumstances[131]. Future immunohistochemical work (*i.e.* Iba-1 and/or arginase-1) examining the activation state of microglia in hS, LPS and hSL mice would shed light on this proposed mechanism.

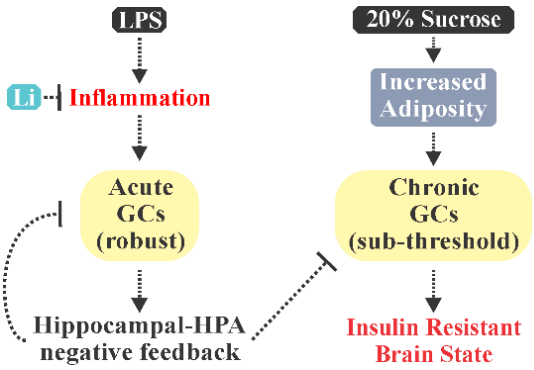

Fig 5. LPS may have conferred protection via attenuation of chronic glucocorticoid signaling. LPS is known to induce a robust inflammatory response associated with elevated glucocorticoid release via activation of the HPA axis. Glucocorticoids self-regulate their activity by induction of negative feedback through a hippocampal-HPA axis-dependent loop. High-sucrose diets could potentially promote chronic increases in glucocorticoid expression without triggering this negative feedback (sub-threshold elevations). Transient and robust glucocorticoid responses following LPS administration may have 're-set' this chronic increase. Lithium pre-treatment could have unmasked the high-sucrose induced effect on glucocorticoids by blocking the activity of LPS.

5.3 LITHIUM MAY HAVE 'UNMASKED' HIGH-SUCROSE EFFECTS ON GLUCOCORTICOID HOMEOSTASIS IN HSL MICE THROUGH ANTAGONISM OF LPS ACTIVITY

Lithium appeared to block the protective effects of low-dose LPS in mice sustained on a high-sucrose diet. In chapter 3, I demonstrated that the addition of LPS to mice sustained on high-sucrose diet attenuated fecal corticosterone. Concurrent supplementation with lithium increased corticosterone levels relative to control, as shown in chapter 4. In fact, corticosterone expression in hSLLi mice was similar to those observed in animals receiving a high-sucrose diet alone. Given the known effects of high-sucrose on glucocorticoid expression[77,85], it is thus reasonable to propose that lithium antagonized the actions of LPS to 'unmask' the high-sucrose

diet-dependent glucocorticoid phenotype reported both here in chapter 3 and elswehere[77,85]. As lithium is known to be anti-inflammatory in nature[200,250,288,289], it is possible that lithium prevented induction of the hippocampal-HPA negative feedback loop by attenuating the initial LPS-mediated systemic inflammatory response; no inflammation -> no robust glucocorticoid response -> no feedback inhibition. Supporting this idea, some studies have suggested that LPS-induced activation of macrophage/microglia is dependent on signaling through GSK-3β, as evidenced by attenuation of macrophage[290] and microglial[251] responses to inflammatory stimuli following administration of GSK-3β inhibitors. As lithium exerts potent inhibition of GSK-3β, it may suppress LPS-induced signaling in both the brain and periphery by blocking activity of this required downstream mediator. Regardless of mechanism, it appears clear that lithium partially unmasked the hS-phenotype when added to hSL mice, supporting the idea that an interaction between lithium and LPS took place. Future work directly comparing LPS and lithium and a high-sucrose diet and lithium would assist in understanding the nature of the interaction between these three treatments.

CHAPTER 6: LIMITATIONS AND FUTURE DIRECTIONS

6.1 MAJOR THESIS FINDINGS

1. A high-sucrose diet precipitated behavioral and biochemical phenotypes characteristic of AD-related neurodegeneration as evidenced by impaired spatial learning, elevated glucocorticoids, insulin pathway irregularities, and increased NO, $A\beta_{42}$, and pTau expression.

2. The addition of LPS to mice sustained on a high-sucrose diet diminished development of a neurodegenerative phenotype. Spatial learning performance was improved (though was not rescued), glucocorticoid levels were reset, irregularities in insulin pathway second messenger expression and phosphorylation were abolished, and $A\beta_{42}$ and pTau levels were returned to those observed in control mice.

3. Supplementation with lithium antagonized phenotypes resultant of the combined hSL treatment. Barnes maze-related spatial learning deficits and Open Field anxiety-like behavior were abolished. Glucocorticoid and $A\beta_{42}$ levels, though not NO or pTau, were returned to those observed in mice sustained on a high-sucrose diet alone.

6.2 LIMITATIONS

6.2.1 Use of hemi-brain homogenates and choice of phosphoprotein analytes

Pathological changes in Tau phosphorylation or insulin pathway second messenger expression and phosphorylation are most frequently observed in the hippocampus and temporal cortex[284–286]. As such, our use of hemi-brain homogenates for biochemical tests may have diluted our samples, resulting in reduced protein expression relative to what would be observed in hippocampal isolations. Also, it is possible that Tau proteins were phosphorylated at sites other than the Threonine 231 assessed.

6.2.2 Timing of the LPS injections

The relatively early time of injection (1st, 2nd and 3rd months) may have ‘re-set’ hS-induced elevations in corticosterone by triggering negative feedback within the hippocampal-HPA pathway. Given the proposed central role of chronic glucocorticoid activity in sensitizing the CNS to inflammation, it is possible that not enough time was allowed for the necessary glucocorticoid-

mediated processes to exert an effect. Delaying the introduction of the first LPS injection to the 2nd or 3rd month may allow for development of a state more conducive to LPS-induced instigation of neuroinflammation.

6.3 FUTURE DIRECTIONS

There are several future directions which could expand upon the work presented, including elucidating the relationship between hSL and lithium and the role of sex hormones in diet- and inflammation-induced neurodegeneration

6.3.1 Treatment effects in male wild-type mice

While an identical male cohort was run, the data obtained could not be used on account of a compromised control group. Male control animals displayed similar weight gain, liver steatosis, and biochemical phenotypes to high sucrose fed mice. These findings were in complete contradiction to results demonstrated elsewhere[75,77,197]. Prior to assessing behavior, male mice were transferred to a new room for housing during the 6th month of the study (for ease of access during behavioral testing). This room change may have acted as a chronic stressor (the room change was maintained for a full month). In addition, frequent building construction and the scent of other animals (mice were moved from the mouse suit to the multi-species suite) may have contributed to this stress. This does, however, support the idea that chronic stress is the major contributing factor to behavioral effects as a result of caloric excess.

As female mice demonstrate increased LPS tolerance relative to their male counterparts[38], it is likely that the combination of a high-sucrose diet and LPS would have netted a more aggressive neurodegenerative phenotype in male mice. Running the male cohort a second time, free from room changes or construction issues, would allow for direct comparison of the effects of high-sucrose and/or LPS in males and females. Furthermore, should the hSL-phenotype prove to be stronger in males, more robust data regarding the prophylactic potential of lithium against neurodegeneration would become available.

6.3.2 The role of sex hormones in diet-induced neurodegeneration

Post-menopausal women represent nearly two-thirds of all current and expected AD patients, strongly implicating the age-related loss of estrogen in the pathogenesis of AD in females[1]. Estrogen has been shown to antagonize several processes involved in AD[33], such as β-amyloidogenesis[34], Tau hyperphosphorylation[35], and inflammation[38]. In addition, estrogen counteracts glucocorticoid activity, a central mediator of high-sucrose diet-associated

neurodegeneration. Post-menopausal females may therefore demonstrate a heightened sensitivity to dietary stressors.

To explore the role of estrogen in diet-induced neurodegeneration, ovariectomized female mice would be compared to reproductively normal males and females. Ovariectomized females would serve as a post-menopausal model to examine the expression of neurodegenerative processes relative to normal female mice when challenged with a high-sucrose diet. Reproductively normal males would serve to elucidate potential differences between predominant expression of testosterone or estrogen in the pathogenesis of diet-induced neurodegeneration. Such a study could utilize the following two-by-two structure. Three cohorts of male, female, and ovariectomized females, with each cohort containing two groups, control and high-sucrose (20% sucrose in the drinking water). Lithium prophylaxis could also be examined by running four groups per cohort instead of two: control, high-sucrose, lithium, and lithium plus high-sucrose.

6.3.3 The interaction between hSL and lithium

Whether lithium primarily antagonized LPS- or hS-induced processes when added to mice following the hSL combined regimen is difficult to deduce in the absence of groups comparing hS and lithium and LPS and lithium directly. Furthermore, the prophylactic potential of lithium against neurodegeneration could not be assessed in the work presented due to the absence of a prominent neurodegenerative phenotype in hSL mice. An additional group comparing hS and lithum (hSLi) would address this concern.

To sum, groups comparing hS and lithium (hSLi) and LPS and lithium (LPSLi) are needed to characterize the nature of the interaction between the hSL treatment and lithium, and to assess the prophylactic potential of lithium against hS-induced neurodegeneration.

6.3.4 Differing effects of low-dose vs moderate-to-high-dose LPS (Dose versus timing of LPS)

Clear differences in effect between high and low doses of LPS have been reported. Acute concentrations contribute to neuroinflammation and neurodegeneration[12], while low doses appear to confer beneficial effects[254]. I proposed that a high-sucrose diet would sensitize the brain to inflammatory insult, allowing low concentrations of LPS to instigate central inflammation. This hypothesis proved incorrect, as not only did the low-dose of LPS not aggravate neurodegeneration, it countered the pathological effects of the high-sucrose diet. However, it is possible that a higher dose of LPS would result in accelerated neurodegeneration when paired with high-sucrose. A comparison of the effects of low vs high concentrations of LPS in conjunction

with a high-sucrose diet would shed light on the nature of the interaction between the two treatments (*i.e* is LPS always protective against hS-induced pathology, or is the interaction dose-dependent? Would an increased concentration of LPS aggravate hS-induced neurodegeneration?). Using LPS concentrations of 0.1, 1 and 5 mg/kg would allow for comparison of a protective low dose (as demonstrated in this thesis), a moderate-to-high dose, and a dose capable of mimicking a septic shock-like event.

6.3.5 Use of APOE4 Knock-in transgenic animal models

The apolipoprotein ε-4 (APO ε4) allele exhibits an estimated frequency of 40% in AD patients[291]. As apolipoprotein E4 (resultant of APO ε4) is known to impair clearance of Aβ peptides[292], carriers of the APO ε4 allele may demonstrate increased susceptibility to β-amyloidopathy in the face of β-amyloidogenic challenge. Given that high-sucrose diets promote Aβ production, contrasting the effects of high-sucrose consumption in normal and APO ε4 knock-in transgenic mice (*i.e.* B6(SJL)-APOE4 KI from Jackson labs) would provide insight into whether APO ε4 carriers are more susceptible to diet-induced neurodegeneration.

6.3.6 Immunohistochemical analysis of samples

6.3.6.1 Effects of high-sucrose diet-associated pathology on neurogenesis

Hippocampal neurogenesis is characterized by the proliferation and differentiation of multipotent stem cells in the subgranular zone of the dentate gyrus[293,294]. This process of generating new dentate gyrus granule neurons is believed to play a key role in learning and memory[295–298], highlighting its importance to cognitive function. An early disruption of hippocampal neurogenesis has been proposed as a precursor to gross neurodegenerative alterations in AD, with several animal models of the disease demonstrating a reduction in neurogenesis as pTau and $A\beta_{42}$ levels increase[299–302]. Given that a high-sucrose diet can increase expression of $A\beta_{42}$ and pTau, as demonstrated both within this thesis and elsewhere[75,77,197], it is reasonable to propose that mice sustained on a high-sucrose diet may display reduced hippocampal neurogenesis. Furthermore, the addition of a sub-therapeutic dose of lithium to mice following the hSL regimen completely rescued spatial learning performance in Barnes maze acquisition trials, as demonstrated in chapter 4. Through inhibition of GSK-3β, lithium is known to upregulate expression of several neurotrophic factors involved in memory, cognition, and neurogenesis, such as brain-derived neurotrophic factor (BDNF) and β-catenin[181,198,303–305]. Supporting this GSK-3β-inhibition-mediated mechanism of lithium, GSK-3β antagonists are able to increase hippocampal neurogenesis[283]. As loss of β-catenin, increased apoptosis, and reduced

neurogenesis are associated with cognitive decline, it is possible that lithium may have rescued spatial learning through increased neurotrophic factor production and restoration of neurogenesis[289,306]. Neurogenesis in response to a high-sucrose diet and/or inflammation could be assessed via simple doublecortin staining for immature neurons within the dentate gyrus. Although not included in this thesis, brains are currently being processed for this purpose.

6.3.6.2 Analysis of microglial morphology

Microglia are known to mount an immune response when exposed to harmful stimuli such as misfolded proteins (*i.e.* Aβ) and foreign pathogens (*i.e.* LPS). While ordinarily beneficial, failure to resolve this response leads to chronic over-activation of microglia and a diversion of their typical physiological functions. Activated microglia have been found to associate with Aβ deposits in the brain of both human AD patients[307] and animal models[308,309]. As previously discussed, inflammation contributes to β-amyloidogenesis[11,157,158,287], while Aβ leads to continued inflammatory activation of microglia[161,287]. Thus, the Aβ-microglia interaction may represent a means by which central inflammation becomes self-perpetuating[287]. Assessment of the activation state of microglia would thus assist in the characterization of the phenotypes presented in this thesis.

Ionized calcium-binding adaptor protein-1 (Iba-1) is an actin-binding protein ubiquitously expressed in microglia[310]. Iba-1 is involved in the membrane ruffling of microglia during the morphological changes associated with activation[311]. As its expression is increased during microglial activation, Iba-1 is an effective marker of activated microglia in immunohistochemical analysis[310]. Iba-1 staining of 40 μm thick brain sections obtained during completion of this thesis would allow for initial characterization of microglial activation in our mice. Given the increase in Aβ observed, increased microglial activation in hS mice, particularly within the hippocampus, would strengthen the neurodegenerative phenotype displayed by those animals.

REFERENCES

1. Alzheimer's Association. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement. J. Alzheimers Assoc.* **12**, 459–509 (2016).
2. de la Monte, S. M. & Wands, J. R. Alzheimer's Disease Is Type 3 Diabetes–Evidence Reviewed. *J. Diabetes Sci. Technol. Online* **2**, 1101–1113 (2008).
3. Duffy, C. M., Hofmeister, J. J., Nixon, J. P. & Butterick, T. A. High fat diet increases cognitive decline and neuroinflammation in a model of orexin loss. *Neurobiol. Learn. Mem.* **157**, 41–47 (2019).
4. Janakiraman, M. & Krishnamoorthy, G. Emerging Role of Diet and Microbiota Interactions in Neuroinflammation. *Front. Immunol.* **9**, (2018).
5. Tengeler, A. C., Kozicz, T. & Kiliaan, A. J. Relationship between diet, the gut microbiota, and brain function. *Nutr. Rev.* **76**, 603–617 (2018).
6. McGeer, E. G. & McGeer, P. L. Neuroinflammation in Alzheimer's disease and mild cognitive impairment: a field in its infancy. *J. Alzheimers Dis. JAD* **19**, 355–361 (2010).
7. McGeer, E. G., Klegeris, A. & McGeer, P. L. Inflammation, the complement system and the diseases of aging. *Neurobiol. Aging* **26 Suppl 1**, 94–97 (2005).
8. Holmes, C. *et al.* Systemic inflammation and disease progression in Alzheimer disease. *Neurology* **73**, 768–774 (2009).
9. Holmes, C., Cunningham, C., Zotova, E., Culliford, D. & Perry, V. H. Proinflammatory cytokines, sickness behavior, and Alzheimer disease. *Neurology* **77**, 212–218 (2011).
10. Perry, V. H. Contribution of systemic inflammation to chronic neurodegeneration. *Acta Neuropathol. (Berl.)* **120**, 277–286 (2010).
11. Semmler, A., Okulla, T., Sastre, M., Dumitrescu-Ozimek, L. & Heneka, M. T. Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *J. Chem. Neuroanat.* **30**, 144–157 (2005).
12. Qin, L. *et al.* Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* **55**, 453–462 (2007).
13. Kumar, A. & Tsao, J. W. Alzheimer Disease. in *StatPearls* (StatPearls Publishing, 2019).
14. Liljegren, M., Landqvist Waldö, M., Rydbeck, R. & Englund, E. Police Interactions Among Neuropathologically Confirmed Dementia Patients: Prevalence and Cause. *Alzheimer Dis. Assoc. Disord.* **32**, 346–350 (2018).
15. Petersen, R. C. How early can we diagnose Alzheimer disease (and is it sufficient)? The 2017 Wartenberg lecture. *Neurology* **91**, 395–402 (2018).
16. Schachter, A. S. & Davis, K. L. Alzheimer's disease. *Dialogues Clin. Neurosci.* **2**, 91–100 (2000).
17. Zhang, Y., Thompson, R., Zhang, H. & Xu, H. APP processing in Alzheimer's disease. *Mol. Brain* **4**, 3 (2011).
18. Murphy, M. P. & LeVine, H. Alzheimer's Disease and the β-Amyloid Peptide. *J. Alzheimers Dis. JAD* **19**, 311 (2010).
19. Selkoe, D. J. Alzheimer's disease: genes, proteins, and therapy. *Physiol. Rev.* **81**, 741–766 (2001).
20. Lambert, M. P. *et al.* Diffusible, nonfibrillar ligands derived from Aβ1–42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci.* **95**, 6448–6453 (1998).
21. Dahlgren, K. N. *et al.* Oligomeric and Fibrillar Species of Amyloid-β Peptides Differentially Affect Neuronal Viability. *J. Biol. Chem.* **277**, 32046–32053 (2002).
22. Lesné, S. *et al.* A specific amyloid-β protein assembly in the brain impairs memory. *Nature* **440**, 352 (2006).
23. Hu, X. *et al.* Tau pathogenesis is promoted by Aβ1-42 but not Aβ1-40. *Mol. Neurodegener.* **9**, 52 (2014).

24. He, Z. *et al.* Amyloid-β plaques enhance Alzheimer's brain tau-seeded pathologies by facilitating neuritic plaque tau aggregation. *Nat. Med.* **24**, 29–38 (2018).
25. Zheng, W.-H., Bastianetto, S., Mennicken, F., Ma, W. & Kar, S. Amyloid beta peptide induces tau phosphorylation and loss of cholinergic neurons in rat primary septal cultures. *Neuroscience* **115**, 201–211 (2002).
26. Ryan, S. D. *et al.* Amyloid-β42 signals tau hyperphosphorylation and compromises neuronal viability by disrupting alkylacylglycerophosphocholine metabolism. *Proc. Natl. Acad. Sci.* **106**, 20936–20941 (2009).
27. Alonso, A., Zaidi, T., Novak, M., Grundke-Iqbal, I. & Iqbal, K. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6923–6928 (2001).
28. Šimić, G. *et al.* Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. *Biomolecules* **6**, (2016).
29. Bierer, L. M. *et al.* Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. *Arch. Neurol.* **52**, 81–88 (1995).
30. Iqbal, K., Liu, F., Gong, C.-X. & Grundke-Iqbal, I. Tau in Alzheimer Disease and Related Tauopathies. *Curr. Alzheimer Res.* **7**, 656–664 (2010).
31. Rosario, E. R., Chang, L., Head, E. H., Stanczyk, F. Z. & Pike, C. J. Brain levels of sex steroid hormones in men and women during normal aging and in Alzheimer's disease. *Neurobiol. Aging* **32**, 604–613 (2011).
32. Asih, P. R. *et al.* Multiple Mechanisms Linking Type 2 Diabetes and Alzheimer's Disease: Testosterone as a Modifier. *J. Alzheimers Dis. JAD* **59**, 445–466 (2017).
33. Pike, C. J., Carroll, J. C., Rosario, E. R. & Barron, A. M. Protective actions of sex steroid hormones in Alzheimer's disease. *Front. Neuroendocrinol.* **30**, 239–258 (2009).
34. Levin-Allerhand, J. A., Lominska, C. E., Wang, J. & Smith, J. D. 17Alpha-estradiol and 17beta-estradiol treatments are effective in lowering cerebral amyloid-beta levels in AbetaPPSWE transgenic mice. *J. Alzheimers Dis. JAD* **4**, 449–457 (2002).
35. Carroll, J. C. & Rosario, E. R. The potential use of hormone-based therapeutics for the treatment of Alzheimer's disease. *Curr. Alzheimer Res.* **9**, 18–34 (2012).
36. Yao, M., Nguyen, T.-V. V., Rosario, E. R., Ramsden, M. & Pike, C. J. Androgens regulate neprilysin expression: role in reducing beta-amyloid levels. *J. Neurochem.* **105**, 2477–2488 (2008).
37. Park, S.-Y. & Ferreira, A. The generation of a 17 kDa neurotoxic fragment: an alternative mechanism by which tau mediates beta-amyloid-induced neurodegeneration. *J. Neurosci. Off. J. Soc. Neurosci.* **25**, 5365–5375 (2005).
38. Everhardt Queen, A., Moerdyk-Schauwecker, M., McKee, L. M., Leamy, L. J. & Huet, Y. M. Differential Expression of Inflammatory Cytokines and Stress Genes in Male and Female Mice in Response to a Lipopolysaccharide Challenge. *PloS One* **11**, e0152289 (2016).
39. Gould, S. J. The Structure Of Evolutionary Theory. 12
40. Cordain, L. *et al.* Origins and evolution of the Western diet: health implications for the 21st century. *Am. J. Clin. Nutr.* **81**, 341–354 (2005).
41. Murray, M. J., Murray, A. B. & Murray, N. J. The ecological interdependence of diet and disease in tribal societies. *Yale J. Biol. Med.* **53**, 295–306 (1980).
42. Zampieri, F. *Origins and History of Darwinian Medicine*. **9**, (2009).
43. Kant, A. K. Consumption of energy-dense, nutrient-poor foods by adult Americans: nutritional and health implications. The third National Health and Nutrition Examination Survey, 1988-1994. *Am. J. Clin. Nutr.* **72**, 929–936 (2000).

44. Wang, Y. C., Bleich, S. N. & Gortmaker, S. L. Increasing caloric contribution from sugar-sweetened beverages and 100% fruit juices among US children and adolescents, 1988-2004. *Pediatrics* **121**, e1604-1614 (2008).
45. Slining, M. M. & Popkin, B. M. Trends in intakes and sources of solid fats and added sugars among U.S. children and adolescents: 1994-2010. *Pediatr. Obes.* **8**, 307–324 (2013).
46. Pontzer, H., Wood, B. M. & Raichlen, D. A. Hunter-gatherers as models in public health. *Obes. Rev. Off. J. Int. Assoc. Study Obes.* **19 Suppl 1**, 24–35 (2018).
47. Allison, D. B., Fontaine, K. R., Manson, J. E., Stevens, J. & VanItallie, T. B. Annual deaths attributable to obesity in the United States. *JAMA* **282**, 1530–1538 (1999).
48. Hruby, A. & Hu, F. B. The Epidemiology of Obesity: A Big Picture. *PharmacoEconomics* **33**, 673–689 (2015).
49. Hadrévi, J., Søgaard, K. & Christensen, J. R. Dietary Fiber Intake among Normal-Weight and Overweight Female Health Care Workers: An Exploratory Nested Case-Control Study within FINALE-Health. *J. Nutr. Metab.* **2017**, (2017).
50. Bozzetto, L. *et al.* Dietary Fibre as a Unifying Remedy for the Whole Spectrum of Obesity-Associated Cardiovascular Risk. *Nutrients* **10**, (2018).
51. Kaul, L. & Nidiry, J. High-fiber diet in the treatment of obesity and hypercholesterolemia. *J. Natl. Med. Assoc.* **85**, 231–232 (1993).
52. MU, M., XU, L.-F., HU, D., WU, J. & BAI, M.-J. Dietary Patterns and Overweight/Obesity: A Review Article. *Iran. J. Public Health* **46**, 869–876 (2017).
53. Rush, E. C. & Yan, M. R. Evolution not Revolution: Nutrition and Obesity. *Nutrients* **9**, (2017).
54. Xu, W. L. *et al.* Midlife overweight and obesity increase late-life dementia risk: a population-based twin study. *Neurology* **76**, 1568–1574 (2011).
55. O'Brien, P. D., Hinder, L. M., Callaghan, B. C. & Feldman, E. L. Neurological consequences of obesity. *Lancet Neurol.* **16**, 465–477 (2017).
56. Hildreth, K. L., Van Pelt, R. E. & Schwartz, R. S. Obesity, Insulin Resistance, and Alzheimer's Disease. *Obes. Silver Spring Md* **20**, 1549–1557 (2012).
57. Cersosimo, E. & DeFronzo, R. A. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. *Diabetes Metab. Res. Rev.* **22**, 423–436 (2006).
58. Young, W. S. Periventricular hypothalamic cells in the rat brain contain insulin mRNA. *Neuropeptides* **8**, 93–97 (1986).
59. Devaskar, S. U., Singh, B. S., Carnaghi, L. R., Rajakumar, P. A. & Giddings, S. J. Insulin II gene expression in rat central nervous system. *Regul. Pept.* **48**, 55–63 (1993).
60. Devaskar, S. U. *et al.* Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J. Biol. Chem.* **269**, 8445–8454 (1994).
61. Blázquez, E., Velázquez, E., Hurtado-Carneiro, V. & Ruiz-Albusac, J. M. Insulin in the Brain: Its Pathophysiological Implications for States Related with Central Insulin Resistance, Type 2 Diabetes and Alzheimer's Disease. *Front. Endocrinol.* **5**, (2014).
62. Baura, G. D. *et al.* Insulin Transport From Plasma Into the Central Nervous System Is Inhibited by Dexamethasone in Dogs. *Diabetes* **45**, 86–90 (1996).
63. Baura, G. D. *et al.* Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J. Clin. Invest.* **92**, 1824–1830 (1993).
64. Stafeev, I. S., Vorotnikov, A. V., Ratner, E. I., Menshikov, M. Y. & Parfyonova, Ye. V. Latent Inflammation and Insulin Resistance in Adipose Tissue. *Int. J. Endocrinol.* **2017**, (2017).
65. Wilcox, G. Insulin and Insulin Resistance. *Clin. Biochem. Rev.* **26**, 19–39 (2005).
66. Copps, K. D. & White, M. F. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia* **55**, 2565–2582 (2012).

67. Talbot, K. *et al.* Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J. Clin. Invest.* **122**, 1316–1338 (2012).
68. Willette, A. A. *et al.* Insulin resistance predicts brain amyloid deposition in late middle-aged adults. *Alzheimers Dement. J. Alzheimers Assoc.* **11**, 504-510.e1 (2015).
69. Boucher, J., Kleinridders, A. & Kahn, C. R. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb. Perspect. Biol.* **6**, a009191
70. Kirouac, L., Rajic, A. J., Cribbs, D. H. & Padmanabhan, J. Activation of Ras-ERK Signaling and GSK-3 by Amyloid Precursor Protein and Amyloid Beta Facilitates Neurodegeneration in Alzheimer's Disease. *eNeuro* **4**, ENEURO.0149-16.2017 (2017).
71. Profenno, L. A., Porsteinsson, A. P. & Faraone, S. V. Meta-analysis of Alzheimer's disease risk with obesity, diabetes, and related disorders. *Biol. Psychiatry* **67**, 505–512 (2010).
72. Vanhanen, M. *et al.* Cognitive function in an elderly population with persistent impaired glucose tolerance. *Diabetes Care* **21**, 398–402 (1998).
73. Yaffe, K. *et al.* Diabetes, impaired fasting glucose, and development of cognitive impairment in older women. *Neurology* **63**, 658–663 (2004).
74. de la Monte, S. M. Metabolic derangements mediate cognitive impairment and Alzheimer's disease: role of peripheral insulin resistance diseases. *Panminerva Med.* **54**, 171–178 (2012).
75. Carvalho, C. *et al.* Type 2 diabetic and Alzheimer's disease mice present similar behavioral, cognitive, and vascular anomalies. *J. Alzheimers Dis. JAD* **35**, 623–635 (2013).
76. Thambisetty, M. *et al.* Glucose Intolerance, Insulin Resistance and Alzheimer's Disease Pathology in the Baltimore Longitudinal Study of Aging. *JAMA Neurol.* **70**, 1167–1172 (2013).
77. Choudhary, P., Pacholko, A. G., Palaschuk, J. & Bekar, L. K. The locus coeruleus neurotoxin, DSP4, and/or a high sugar diet induce behavioral and biochemical alterations in wild-type mice consistent with Alzheimers related pathology. *Metab. Brain Dis.* **33**, 1563–1571 (2018).
78. Cao, D., Lu, H., Lewis, T. L. & Li, L. Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *J. Biol. Chem.* **282**, 36275–36282 (2007).
79. Smith, S. M. & Vale, W. W. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin. Neurosci.* **8**, 383–395 (2006).
80. Vicennati, V., Ceroni, L., Gagliardi, L., Gambineri, A. & Pasquali, R. Response of the Hypothalamic-Pituitary-Adrenocortical Axis to High-Protein/Fat and High-Carbohydrate Meals in Women with Different Obesity Phenotypes. *J. Clin. Endocrinol. Metab.* **87**, 3984–3988 (2002).
81. Peng, K. *et al.* 11β-Hydroxysteroid Dehydrogenase Type 1(11β-HSD1) mediates insulin resistance through JNK activation in adipocytes. *Sci. Rep.* **6**, 37160 (2016).
82. Morton, N. M. & Seckl, J. R. 11beta-hydroxysteroid dehydrogenase type 1 and obesity. *Front. Horm. Res.* **36**, 146–164 (2008).
83. Oakley, R. H. & Cidlowski, J. A. Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. *J. Biol. Chem.* **286**, 3177–3184 (2011).
84. Sterner, E. Y. & Kalynchuk, L. E. Behavioral and neurobiological consequences of prolonged glucocorticoid exposure in rats: relevance to depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **34**, 777–790 (2010).
85. Stranahan, A. M. *et al.* Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. *Nat. Neurosci.* **11**, 309–317 (2008).
86. McIntosh, L. J., Hong, K. E. & Sapolsky, R. M. Glucocorticoids may alter antioxidant enzyme capacity in the brain: baseline studies. *Brain Res.* **791**, 209–214 (1998).

87. Frank, M. G., Miguel, Z. D., Watkins, L. R. & Maier, S. F. Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to E. coli lipopolysaccharide. *Brain. Behav. Immun.* **24**, 19–30 (2010).
88. Osmanovic, J. *et al.* Chronic exogenous corticosterone administration generates an insulin-resistant brain state in rats. *Stress Amst. Neth.* **13**, 123–131 (2010).
89. Magariños, A. M. & McEwen, B. S. Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 11056–11061 (2000).
90. Giorgino, F., Almahfouz, A., Goodyear, L. J. & Smith, R. J. Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *J. Clin. Invest.* **91**, 2020–2030 (1993).
91. Landfield, P. W., Blalock, E. M., Chen, K.-C. & Porter, N. M. A New Glucocorticoid Hypothesis of Brain Aging: Implications for Alzheimer's Disease. (2007). doi:info:doi/10.2174/156720507780362083
92. Monte, D. L. & M, S. Triangulated Mal-Signaling in Alzheimer's Disease: Roles of Neurotoxic Ceramides, ER Stress, and Insulin Resistance Reviewed. *J. Alzheimers Dis.* **30**, S231–S249 (2012).
93. Summers, S. A. Ceramides in insulin resistance and lipotoxicity. *Prog. Lipid Res.* **45**, 42–72 (2006).
94. Turpin, S. M. *et al.* Obesity-Induced CerS6-Dependent C16:0 Ceramide Production Promotes Weight Gain and Glucose Intolerance. *Cell Metab.* **20**, 678–686 (2014).
95. Gill, J. M. R. & Sattar, N. Ceramides: A new player in the inflammation–insulin resistance paradigm? *Diabetologia* **52**, 2475–2477 (2009).
96. Kitatani, K., Idkowiak-Baldys, J. & Hannun, Y. A. The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell. Signal.* **20**, 1010–1018 (2008).
97. Bourbon, N. A., Sandirasegarane, L. & Kester, M. Ceramide-induced inhibition of Akt is mediated through protein kinase Czeta: implications for growth arrest. *J. Biol. Chem.* **277**, 3286–3292 (2002).
98. Chalfant, C. E. *et al.* Long chain ceramides activate protein phosphatase-1 and protein phosphatase-2A. Activation is stereospecific and regulated by phosphatidic acid. *J. Biol. Chem.* **274**, 20313–20317 (1999).
99. Woodcock, J. Sphingosine and ceramide signalling in apoptosis. *IUBMB Life* **58**, 462–466 (2006).
100. Cuvillier, O. *et al.* [Sphingolipid-mediated apoptotic signaling pathways]. *J. Soc. Biol.* **197**, 217–221 (2003).
101. Chitturi, S. *et al.* NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatol. Baltim. Md* **35**, 373–379 (2002).
102. Straczkowski, M. *et al.* Increased skeletal muscle ceramide level in men at risk of developing type 2 diabetes. *Diabetologia* **50**, 2366–2373 (2007).
103. Kasumov, T. *et al.* Ceramide as a Mediator of Non-Alcoholic Fatty Liver Disease and Associated Atherosclerosis. *PLOS ONE* **10**, e0126910 (2015).
104. Langeveld, M. & Aerts, J. M. F. G. Glycosphingolipids and insulin resistance. *Prog. Lipid Res.* **48**, 196–205 (2009).
105. Holland, W. L. & Summers, S. A. Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. *Endocr. Rev.* **29**, 381–402 (2008).
106. Arboleda, G. *et al.* Insulin-like growth factor-1-dependent maintenance of neuronal metabolism through the phosphatidylinositol 3-kinase-Akt pathway is inhibited by C2-ceramide in CAD cells. *Eur. J. Neurosci.* **25**, 3030–3038 (2007).
107. de la Monte, S. M. Brain Insulin Resistance and Deficiency as Therapeutic Targets in Alzheimer's Disease. *Curr. Alzheimer Res.* **9**, 35–66 (2012).
108. Maciejczyk, M., Żebrowska, E. & Chabowski, A. Insulin Resistance and Oxidative Stress in the Brain: What's New? *Int. J. Mol. Sci.* **20**, (2019).

109. Katsel, P., Li, C. & Haroutunian, V. Gene expression alterations in the sphingolipid metabolism pathways during progression of dementia and Alzheimer's disease: a shift toward ceramide accumulation at the earliest recognizable stages of Alzheimer's disease? *Neurochem. Res.* **32**, 845–856 (2007).
110. Han, X. Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochim. Biophys. Acta* **1801**, 774–783 (2010).
111. Haughey, N. J., Bandaru, V. V. R., Bae, M. & Mattson, M. P. Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis. *Biochim. Biophys. Acta* **1801**, 878–886 (2010).
112. Mielke, M. M. & Lyketsos, C. G. Alterations of the sphingolipid pathway in Alzheimer's disease: new biomarkers and treatment targets? *Neuromolecular Med.* **12**, 331–340 (2010).
113. de la Monte, S. M., Re, E., Longato, L. & Tong, M. Dysfunctional pro-ceramide, ER stress, and insulin/IGF signaling networks with progression of Alzheimer's disease. *J. Alzheimers Dis. JAD* **30 Suppl 2**, S217-229 (2012).
114. Steen, E. *et al.* Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *J. Alzheimers Dis. JAD* **7**, 63–80 (2005).
115. Lester-Coll, N. *et al.* Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. *J. Alzheimers Dis. JAD* **9**, 13–33 (2006).
116. Tilg, H. & Moschen, A. R. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. *Trends Endocrinol. Metab. TEM* **19**, 371–379 (2008).
117. Stöhr, O. *et al.* Insulin receptor signaling mediates APP processing and β-amyloid accumulation without altering survival in a transgenic mouse model of Alzheimer's disease. *Age Dordr. Neth.* **35**, 83–101 (2013).
118. Kaytor, M. D. & Orr, H. T. The GSK3 beta signaling cascade and neurodegenerative disease. *Curr. Opin. Neurobiol.* **12**, 275–278 (2002).
119. Phukan, S., Babu, V., Kannoji, A., Hariharan, R. & Balaji, V. GSK3β: role in therapeutic landscape and development of modulators. *Br. J. Pharmacol.* **160**, 1–19 (2010).
120. Hernandez, F., Lucas, J. J. & Avila, J. GSK3 and tau: two convergence points in Alzheimer's disease. *J. Alzheimers Dis. JAD* **33 Suppl 1**, S141-144 (2013).
121. Parr, C., Mirzaei, N., Christian, M. & Sastre, M. Activation of the Wnt/β-catenin pathway represses the transcription of the β-amyloid precursor protein cleaving enzyme (BACE1) via binding of T-cell factor-4 to BACE1 promoter. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **29**, 623–635 (2015).
122. Hooper, C., Killick, R. & Lovestone, S. The GSK3 hypothesis of Alzheimer's disease. *J. Neurochem.* **104**, 1433–1439 (2008).
123. Beurel, E., Grieco, S. F. & Jope, R. S. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol. Ther.* **148**, 114–131 (2015).
124. Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nat. Med.* **19**, 983–997 (2013).
125. Nixon, R. A. Autophagy in neurodegenerative disease: friend, foe or turncoat? *Trends Neurosci.* **29**, 528–535 (2006).
126. Nixon, R. A. Endosome function and dysfunction in Alzheimer's disease and other neurodegenerative diseases. *Neurobiol. Aging* **26**, 373–382 (2005).
127. Avrahami, L. & Eldar-Finkelman, H. GSK-3 and lysosomes meet in Alzheimer's disease. *Commun. Integr. Biol.* **6**, e25179 (2013).
128. Avrahami, L. *et al.* Inhibition of glycogen synthase kinase-3 ameliorates β-amyloid pathology and restores lysosomal acidification and mammalian target of rapamycin activity in the Alzheimer disease mouse model: in vivo and in vitro studies. *J. Biol. Chem.* **288**, 1295–1306 (2013).

129. Kraft, A. D. & Harry, G. J. Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. *Int. J. Environ. Res. Public. Health* **8**, 2980–3018 (2011).
130. Catorce, M. N. & Gevorkian, G. LPS-induced Murine Neuroinflammation Model: Main Features and Suitability for Pre-clinical Assessment of Nutraceuticals. *Curr. Neuropharmacol.* **14**, 155–164 (2016).
131. Cherry, J. D., Olschowka, J. A. & O'Banion, M. K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J. Neuroinflammation* **11**, 98 (2014).
132. Landreth, G. E. & Reed-Geaghan, E. G. Toll-like receptors in Alzheimer's disease. *Curr. Top. Microbiol. Immunol.* **336**, 137–153 (2009).
133. Colton, C. A. & Gilbert, D. L. Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.* **223**, 284–288 (1987).
134. Liu, B. *et al.* Role of nitric oxide in inflammation-mediated neurodegeneration. *Ann. N. Y. Acad. Sci.* **962**, 318–331 (2002).
135. Zick, Y. Role of Ser/Thr kinases in the uncoupling of insulin signaling. *Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes.* **27 Suppl 3**, S56-60 (2003).
136. Medzhitov, R. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1**, 135–145 (2001).
137. Aguirre, V., Uchida, T., Yenush, L., Davis, R. & White, M. F. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J. Biol. Chem.* **275**, 9047–9054 (2000).
138. Maes, M., Leonard, B. E., Myint, A. M., Kubera, M. & Verkerk, R. The new '5-HT' hypothesis of depression: cell-mediated immune activation induces indoleamine 2,3-dioxygenase, which leads to lower plasma tryptophan and an increased synthesis of detrimental tryptophan catabolites (TRYCATs), both of which contribute to the onset of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35**, 702–721 (2011).
139. Dantzer, R., O'Connor, J. C., Lawson, M. A. & Kelley, K. W. Inflammation-associated depression: from serotonin to kynurenine. *Psychoneuroendocrinology* **36**, 426–436 (2011).
140. Leonard, B. & Maes, M. Mechanistic explanations how cell-mediated immune activation, inflammation and oxidative and nitrosative stress pathways and their sequels and concomitants play a role in the pathophysiology of unipolar depression. *Neurosci. Biobehav. Rev.* **36**, 764–785 (2012).
141. Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. & Kelley, K. W. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* **9**, 46–56 (2008).
142. Barnes, P. J. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. Lond. Engl. 1979* **94**, 557–572 (1998).
143. Vinson, G. P. The adrenal cortex and life. *Mol. Cell. Endocrinol.* **300**, 2–6 (2009).
144. Wang, Z.-J. *et al.* Glucocorticoid receptors in the locus coeruleus mediate sleep disorders caused by repeated corticosterone treatment. *Sci. Rep.* **5**, (2015).
145. Ishii, Y. *et al.* Anti-inflammatory effects of noradrenaline on LPS-treated microglial cells: Suppression of NFκB nuclear translocation and subsequent STAT1 phosphorylation. *Neurochem. Int.* **90**, 56–66 (2015).
146. O'Neill, E. & Harkin, A. Targeting the noradrenergic system for anti-inflammatory and neuroprotective effects: implications for Parkinson's disease. *Neural Regen. Res.* **13**, 1332–1337 (2018).
147. Zarow, C., Lyness, S. A., Mortimer, J. A. & Chui, H. C. Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch. Neurol.* **60**, 337–341 (2003).
148. Marien, M. R., Colpaert, F. C. & Rosenquist, A. C. Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res. Brain Res. Rev.* **45**, 38–78 (2004).

149. Harik, S. I., LaManna, J. C., Light, A. I. & Rosenthal, M. Cerebral norepinephrine: influence on cortical oxidative metabolism in situ. *Science* **206**, 69–71 (1979).
150. Hargrave, S. L., Davidson, T. L., Zheng, W. & Kinzig, K. P. Western diets induce blood-brain barrier leakage and alter spatial strategies in rats. *Behav. Neurosci.* **130**, 123–135 (2016).
151. Basharat, S. *et al.* Leptin fails to blunt the lipopolysaccharide-induced activation of the hypothalamic-pituitary-adrenal axis in rats. *J. Endocrinol.* **221**, 229–234 (2014).
152. DiCarlo, G., Wilcock, D., Henderson, D., Gordon, M. & Morgan, D. Intrahippocampal LPS injections reduce Aβ load in APP+PS1 transgenic mice. *Neurobiol. Aging* **22**, 1007–1012 (2001).
153. Yirmiya, R. & Goshen, I. Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain. Behav. Immun.* **25**, 181–213 (2011).
154. Magaki, S., Mueller, C., Dickson, C. & Kirsch, W. Increased production of inflammatory cytokines in mild cognitive impairment. *Exp. Gerontol.* **42**, 233–240 (2007).
155. Galimberti, D. *et al.* Serum MCP-1 levels are increased in mild cognitive impairment and mild Alzheimer's disease. *Neurobiol. Aging* **27**, 1763–1768 (2006).
156. Tarkowski, E., Andreasen, N., Tarkowski, A. & Blennow, K. Intrathecal inflammation precedes development of Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry* **74**, 1200–1205 (2003).
157. Kwak, Y.-D. *et al.* Differential regulation of BACE1 expression by oxidative and nitrosative signals. *Mol. Neurodegener.* **6**, 17 (2011).
158. Guix, F. X. *et al.* Modification of γ-secretase by nitrosative stress links neuronal ageing to sporadic Alzheimer's disease. *EMBO Mol. Med.* **4**, 660–673 (2012).
159. Tanzi, R. E. & Bertram, L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. *Cell* **120**, 545–555 (2005).
160. Bordji, K., Becerril-Ortega, J., Nicole, O. & Buisson, A. Activation of extrasynaptic, but not synaptic, NMDA receptors modifies amyloid precursor protein expression pattern and increases amyloid-ß production. *J. Neurosci. Off. J. Soc. Neurosci.* **30**, 15927–15942 (2010).
161. Liu, S. *et al.* TLR2 is a primary receptor for Alzheimer's amyloid β peptide to trigger neuroinflammatory activation. *J. Immunol. Baltim. Md 1950* **188**, 1098–1107 (2012).
162. Wirz, K. T. S. *et al.* Cortical beta amyloid protein triggers an immune response, but no synaptic changes in the APPswe/PS1dE9 Alzheimer's disease mouse model. *Neurobiol. Aging* **34**, 1328–1342 (2013).
163. Stewart, C. R. *et al.* CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat. Immunol.* **11**, 155–161 (2010).
164. Karran, E., Mercken, M. & De Strooper, B. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat. Rev. Drug Discov.* **10**, 698–712 (2011).
165. Macdonald, A. *et al.* A feasibility and tolerability study of lithium in Alzheimer's disease. *Int. J. Geriatr. Psychiatry* **23**, 704–711 (2008).
166. Hampel, H. *et al.* Lithium trial in Alzheimer's disease: a randomized, single-blind, placebo-controlled, multicenter 10-week study. *J. Clin. Psychiatry* **70**, 922–931 (2009).
167. Forlenza, O. V. *et al.* Disease-modifying properties of long-term lithium treatment for amnestic mild cognitive impairment: randomised controlled trial. *Br. J. Psychiatry J. Ment. Sci.* **198**, 351–356 (2011).
168. Nunes, P. V., Forlenza, O. V. & Gattaz, W. F. Lithium and risk for Alzheimer's disease in elderly patients with bipolar disorder. *Br. J. Psychiatry J. Ment. Sci.* **190**, 359–360 (2007).
169. Ryves, W. J. & Harwood, A. J. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. *Biochem. Biophys. Res. Commun.* **280**, 720–725 (2001).
170. Lie, D.-C. *et al.* Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370–1375 (2005).
171. Siopi, E. *et al.* Anxiety- and Depression-Like States Lead to Pronounced Olfactory Deficits and Impaired Adult Neurogenesis in Mice. *J. Neurosci. Off. J. Soc. Neurosci.* **36**, 518–531 (2016).

172. Liu, L. *et al.* Resveratrol counteracts lipopolysaccharide-induced depressive-like behaviors via enhanced hippocampal neurogenesis. *Oncotarget* **7**, 56045–56059 (2016).
173. Tamura, Y. *et al.* Noninvasive Evaluation of Cellular Proliferative Activity in Brain Neurogenic Regions in Rats under Depression and Treatment by Enhanced [18F]FLT-PET Imaging. *J. Neurosci.* **36**, 8123–8131 (2016).
174. Hollands, C., Bartolotti, N. & Lazarov, O. Alzheimer's Disease and Hippocampal Adult Neurogenesis; Exploring Shared Mechanisms. *Front. Neurosci.* **10**, (2016).
175. Ridler, C. Exercise wards off Alzheimer disease by boosting neurogenesis and neuroprotective factors. *Nat. Rev. Neurol.* **14**, 632 (2018).
176. Moon, R. T., Kohn, A. D., De Ferrari, G. V. & Kaykas, A. WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5**, 691–701 (2004).
177. Nelson, W. J. & Nusse, R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303**, 1483–1487 (2004).
178. Gordon, M. D. & Nusse, R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J. Biol. Chem.* **281**, 22429–22433 (2006).
179. Wexler, E. M., Geschwind, D. H. & Palmer, T. D. Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol. Psychiatry* **13**, 285–292 (2008).
180. Gould, T. D. *et al.* Beta-catenin overexpression in the mouse brain phenocopies lithium-sensitive behaviors. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **32**, 2173–2183 (2007).
181. Dell'Osso, L., Del Grande, C., Gesi, C., Carmassi, C. & Musetti, L. A new look at an old drug: neuroprotective effects and therapeutic potentials of lithium salts. *Neuropsychiatr. Dis. Treat.* **12**, 1687–1703 (2016).
182. Hille, B. The Permeability of the Sodium Channel to Metal Cations in Myelinated Nerve. *J. Gen. Physiol.* **59**, 637–658 (1972).
183. Christensen, B. M. *et al.* αENaC-Mediated Lithium Absorption Promotes Nephrogenic Diabetes Insipidus. *J. Am. Soc. Nephrol. JASN* **22**, 253–261 (2011).
184. Thomsen, K. & Leyssac, P. P. Effect of dietary sodium content on renal handling of lithium. *Pflüg. Arch.* **407**, 55–58 (1986).
185. James, W. P., Ralph, A. & Sanchez-Castillo, C. P. The dominance of salt in manufactured food in the sodium intake of affluent societies. *Lancet Lond. Engl.* **1**, 426–429 (1987).
186. Shirley, D. G., Walter, S. J. & Noormohamed, F. H. Natriuretic effect of caffeine: assessment of segmental sodium reabsorption in humans. *Clin. Sci. Lond. Engl. 1979* **103**, 461–466 (2002).
187. Schrauzer, G. N. & Shrestha, K. P. Lithium in drinking water and the incidences of crimes, suicides, and arrests related to drug addictions. *Biol. Trace Elem. Res.* **25**, 105–113 (1990).
188. Ishii, N. *et al.* Low risk of male suicide and lithium in drinking water. *J. Clin. Psychiatry* **76**, 319–326 (2015).
189. Dawson, E. B., Moore, T. D. & McGanity, W. J. The mathematical relationship of drinking water lithium and rainfall to mental hospital admission. *Dis. Nerv. Syst.* **31**, 811–820 (1970).
190. Adams, J. B., Holloway, C. E., George, F. & Quig, D. Analyses of toxic metals and essential minerals in the hair of arizona children with autism and associated conditions, and their mothers. *Biol. Trace Elem. Res.* **110**, 193–209 (2006).
191. Schrauzer, G. N., Shrestha, K. P. & Flores-Arce, M. F. Lithium in scalp hair of adults, students, and violent criminals. Effects of supplementation and evidence for interactions of lithium with vitamin B12 and with other trace elements. *Biol. Trace Elem. Res.* **34**, 161–176 (1992).
192. Kling, M. A., Manowitz, P. & Pollack, I. W. Rat brain and serum lithium concentrations after acute injections of lithium carbonate and orotate. *J. Pharm. Pharmacol.* **30**, 368–370 (1978).

193. Smith, D. F. & Schou, M. Kidney function and lithium concentrations of rats given an injection of lithium orotate or lithium carbonate. *J. Pharm. Pharmacol.* **31**, 161–163 (1979).
194. Nieper, H. A. The clinical applications of lithium orotate. A two years study. *Agressol. Rev. Int. Physio-Biol. Pharmacol. Appl. Aux Eff. Agression* **14**, 407–411 (1973).
195. Nieper, A. Recalcification of bone metastases by calcium diorotate. *Agressol. Rev. Int. Physio-Biol. Pharmacol. Appl. Aux Eff. Agression* **11**, 495–500 (1970).
196. Pauzé, D. K. & Brooks, D. E. Lithium toxicity from an internet dietary supplement. *J. Med. Toxicol.* **3**, 61–62 (2007).
197. Carvalho, C. *et al.* Metabolic alterations induced by sucrose intake and Alzheimer's disease promote similar brain mitochondrial abnormalities. *Diabetes* **61**, 1234–1242 (2012).
198. Jope, R. S. Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends Pharmacol. Sci.* **24**, 441–443 (2003).
199. Freland, L. & Beaulieu, J.-M. Inhibition of GSK3 by lithium, from single molecules to signaling networks. *Front. Mol. Neurosci.* **5**, (2012).
200. Dong, H. *et al.* Lithium ameliorates lipopolysaccharide-induced microglial activation via inhibition of toll-like receptor 4 expression by activating the PI3K/Akt/FoxO1 pathway. *J. Neuroinflammation* **11**, 140 (2014).
201. Sullivan, G. M. & Feinn, R. Using Effect Size—or Why the P Value Is Not Enough. *J. Grad. Med. Educ.* **4**, 279–282 (2012).
202. Nahm, F. S. What the P values really tell us. *Korean J. Pain* **30**, 241–242 (2017).
203. Bartolucci, A. A., Tendera, M. & Howard, G. Meta-analysis of multiple primary prevention trials of cardiovascular events using aspirin. *Am. J. Cardiol.* **107**, 1796–1801 (2011).
204. Manly, J. J. *et al.* Endogenous estrogen levels and Alzheimer's disease among postmenopausal women. *Neurology* **54**, 833–837 (2000).
205. Herrera, A. Y. & Mather, M. Actions and interactions of estradiol and glucocorticoids in cognition and the brain: Implications for aging women. *Neurosci. Biobehav. Rev.* **55**, 36–52 (2015).
206. Capeau, J. Insulin resistance and steatosis in humans. *Diabetes Metab.* **34**, 649–657 (2008).
207. Lyn-Cook, L. E. *et al.* Hepatic Ceramide May Mediate Brain Insulin Resistance and Neurodegeneration in Type 2 Diabetes and Non-alcoholic Steatohepatitis. *J. Alzheimers Dis. JAD* **16**, 715–729 (2009).
208. Lieber, C. S. *et al.* Model of nonalcoholic steatohepatitis. *Am. J. Clin. Nutr.* **79**, 502–509 (2004).
209. Landfield, P. W., Blalock, E. M., Chen, K.-C. & Porter, N. M. A New Glucocorticoid Hypothesis of Brain Aging: Implications for Alzheimer's Disease. *Curr. Alzheimer Res.* **4**, 205–212 (2007).
210. Correia, S. C. *et al.* Insulin-resistant brain state: the culprit in sporadic Alzheimer's disease? *Ageing Res. Rev.* **10**, 264–273 (2011).
211. Beurel, E., Grieco, S. F. & Jope, R. S. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol. Ther.* **148**, 114–131 (2015).
212. Shimobayashi, M. *et al.* Insulin resistance causes inflammation in adipose tissue. *J. Clin. Invest.* **128**, 1538–1550 (2018).
213. Wellen, K. E. & Hotamisligil, G. S. Inflammation, stress, and diabetes. *J. Clin. Invest.* **115**, 1111–1119 (2005).
214. Burchfield, J. G. *et al.* High dietary fat and sucrose result in an extensive and time-dependent deterioration in health of multiple physiological systems in mice. *J. Biol. Chem.* **293**, 5731–5745 (2018).
215. Davidson, T. L. *et al.* The effects of a high-energy diet on hippocampal-dependent discrimination performance and blood-brain barrier integrity differ for diet-induced obese and diet-resistant rats. *Physiol. Behav.* **107**, 26–33 (2012).

216. Kanoski, S. E., Zhang, Y., Zheng, W. & Davidson, T. L. The effects of a high-energy diet on hippocampal function and blood-brain barrier integrity in the rat. *J. Alzheimers Dis. JAD* **21**, 207–219 (2010).
217. Fox, J. *et al.* The Mouse in Biomedical Research, 2nd Edition. *Elsevier* **3**, 637–672 (2007).
218. Dutta, S. & Sengupta, P. Men and mice: Relating their ages. *Life Sci.* **152**, 244–248 (2016).
219. Chen, T.-C. *et al.* The glucocorticoid-Angptl4-ceramide axis induces insulin resistance through PP2A and PKCζ. *Sci Signal* **10**, eaai7905 (2017).
220. Wright, R. L., Lightner, E. N., Harman, J. S., Meijer, O. C. & Conrad, C., D. Attenuating corticosterone levels on the day of memory assessment prevents chronic stress-induced impairments in spatial memory. *Eur. J. Neurosci.* **24**, 595–605 (2006).
221. Yau, J. L., Olsson, T., Morris, R. G., Meaney, M. J. & Seckl, J. R. Glucocorticoids, hippocampal corticosteroid receptor gene expression and antidepressant treatment: relationship with spatial learning in young and aged rats. *Neuroscience* **66**, 571–581 (1995).
222. Prut, L. & Belzung, C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur. J. Pharmacol.* **463**, 3–33 (2003).
223. Christmas, A. J. & Maxwell, D. R. A comparison of the effects of some benzodiazepines and other drugs on aggressive and exploratory behaviour in mice and rats. *Neuropharmacology* **9**, 17–29 (1970).
224. Bailey, K. R. & Crawley, J. N. Anxiety-Related Behaviors in Mice. in *Methods of Behavior Analysis in Neuroscience* (ed. Buccafusco, J. J.) (CRC Press/Taylor & Francis, 2009).
225. Saad, M. J. A., Santos, A. & Prada, P. O. Linking Gut Microbiota and Inflammation to Obesity and Insulin Resistance. *Physiol. Bethesda Md* **31**, 283–293 (2016).
226. Leonard, B. L., Watson, R. N., Loomes, K. M., Phillips, A. R. J. & Cooper, G. J. Insulin resistance in the Zucker diabetic fatty rat: a metabolic characterisation of obese and lean phenotypes. *Acta Diabetol.* **42**, 162–170 (2005).
227. de la Monte, S. M. *et al.* Ceramide-mediated insulin resistance and impairment of cognitive-motor functions. *J. Alzheimers Dis. JAD* **21**, 967–984 (2010).
228. Scheiblich, H. *et al.* Activation of the NLRP3 inflammasome in microglia: the role of ceramide. *J. Neurochem.* **143**, 534–550 (2017).
229. Soscia, S. J. *et al.* The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PloS One* **5**, e9505 (2010).
230. Kumar, D. K. V. *et al.* Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* **8**, 340ra72 (2016).
231. Miklossy, J. *et al.* Beta-amyloid deposition and Alzheimer's type changes induced by Borrelia spirochetes. *Neurobiol. Aging* **27**, 228–236 (2006).
232. Ishida, N. *et al.* Periodontitis induced by bacterial infection exacerbates features of Alzheimer's disease in transgenic mice. *NPJ Aging Mech. Dis.* **3**, 15 (2017).
233. Pardon, M. C. Lipopolysaccharide hyporesponsiveness: protective or damaging response to the brain? *Romanian J. Morphol. Embryol. Rev. Roum. Morphol. Embryol.* **56**, 903–913 (2015).
234. O' Neill, C. PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. *Exp. Gerontol.* **48**, 647–653 (2013).
235. Zähringer, U., Lindner, B. & Rietschel, E. T. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. *Adv. Carbohydr. Chem. Biochem.* **50**, 211–276 (1994).
236. Opal, S. M. Endotoxins and other sepsis triggers. *Contrib. Nephrol.* **167**, 14–24 (2010).
237. de Vos, A. F. *et al.* In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands. *J. Immunol. Baltim. Md 1950* **183**, 533–542 (2009).
238. Merkel, S. M., Alexander, S., Zufall, E., Oliver, J. D. & Huet-Hudson, Y. M. Essential role for estrogen in protection against Vibrio vulnificus-induced endotoxic shock. *Infect. Immun.* **69**, 6119–6122 (2001).

239. Saxena, K. *et al.* T102. Plasma Interleukin 1 Beta Positively Correlates With Anxiety Scores in Youths With Bipolar Disorder. *Biol. Psychiatry* **83**, S168 (2018).
240. Rossi, S. *et al.* Interleukin-1β causes anxiety by interacting with the endocannabinoid system. *J. Neurosci. Off. J. Soc. Neurosci.* **32**, 13896–13905 (2012).
241. Vogelzangs, N., de Jonge, P., Smit, J. H., Bahn, S. & Penninx, B. W. Cytokine production capacity in depression and anxiety. *Transl. Psychiatry* **6**, e825 (2016).
242. Vogelzangs, N., Beekman, A. T. F., de Jonge, P. & Penninx, B. W. J. H. Anxiety disorders and inflammation in a large adult cohort. *Transl. Psychiatry* **3**, e249 (2013).
243. Rapaport, M. H. & Stein, M. B. Serum cytokine and soluble interleukin-2 receptors in patients with panic disorder. *Anxiety* **1**, 22–25 (1994).
244. Hoge, E. A. *et al.* Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. *Depress. Anxiety* **26**, 447–455 (2009).
245. Tükel, R. *et al.* Decreased IFN-γ and IL-12 levels in panic disorder. *J. Psychosom. Res.* **73**, 63–67 (2012).
246. Vieira, M. M. M. *et al.* Enhanced Th17 phenotype in individuals with generalized anxiety disorder. *J. Neuroimmunol.* **229**, 212–218 (2010).
247. Barone, E. *et al.* Impairment of biliverdin reductase-A promotes brain insulin resistance in Alzheimer disease: A new paradigm. *Free Radic. Biol. Med.* **91**, 127–142 (2016).
248. Medina, M. & Avila, J. Glycogen synthase kinase-3 (GSK-3) inhibitors for the treatment of Alzheimer's disease. *Curr. Pharm. Des.* **16**, 2790–2798 (2010).
249. Hu, S. *et al.* GSK3 inhibitors show benefits in an Alzheimer's disease (AD) model of neurodegeneration but adverse effects in control animals. *Neurobiol. Dis.* **33**, 193–206 (2009).
250. Nassar, A. & Azab, A. N. Effects of Lithium on Inflammation. *ACS Chem. Neurosci.* **5**, 451–458 (2014).
251. Yuskaitis, C. J. & Jope, R. S. GLYCOGEN SYNTHASE KINASE-3 REGULATES MICROGLIAL MIGRATION, INFLAMMATION, AND INFLAMMATION-INDUCED NEUROTOXICITY. *Cell. Signal.* **21**, 264–273 (2009).
252. Eskalith, Lithobid (lithium) dosing, indications, interactions, adverse effects, and more. Available at: https://reference.medscape.com/drug/eskalith-lithobid-lithium-342934. (Accessed: 13th May 2019)
253. Fryar, C. D., Kruszon-Moran, D., Gu, Q. & Ogden, C. L. Mean Body Weight, Height, Waist Circumference, and Body Mass Index Among Adults: United States, 1999-2000 Through 2015-2016. *Natl. Health Stat. Rep.* 1–16 (2018).
254. Chae, B. S. Pretreatment of Low-Dose and Super-Low-Dose LPS on the Production of In Vitro LPS-Induced Inflammatory Mediators. *Toxicol. Res.* **34**, 65–73 (2018).
255. Chen, Z. *et al.* Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury Is Independent of Hematogenous TLR4. *J. Neurosci. Off. J. Soc. Neurosci.* **32**, 11706–11715 (2012).
256. Duque, E. de A. & Munhoz, C. D. The Pro-inflammatory Effects of Glucocorticoids in the Brain. *Front. Endocrinol.* **7**, (2016).
257. Medzhitov, R. Inflammation 2010: New Adventures of an Old Flame. *Cell* **140**, 771–776 (2010).
258. Vandevyver, S., Dejager, L., Tuckermann, J. & Libert, C. New Insights into the Anti-inflammatory Mechanisms of Glucocorticoids: An Emerging Role for Glucocorticoid-Receptor-Mediated Transactivation. *Endocrinology* **154**, 993–1007 (2013).
259. Newton, R. Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55**, 603–613 (2000).
260. Harvey, B. H., Carstens, M. E. & Taljaard, J. J. Evidence that lithium induces a glutamatergic: nitric oxide-mediated response in rat brain. *Neurochem. Res.* **19**, 469–474 (1994).
261. Ghasemi, M. & Dehpour, A. R. The NMDA receptor/nitric oxide pathway: a target for the therapeutic and toxic effects of lithium. *Trends Pharmacol. Sci.* **32**, 420–434 (2011).

262. Alvarez, G. *et al.* Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. *FEBS Lett.* **453**, 260–264 (1999).
263. Alvarez, G. *et al.* Regulation of tau phosphorylation and protection against beta-amyloid-induced neurodegeneration by lithium. Possible implications for Alzheimer's disease. *Bipolar Disord.* **4**, 153–165 (2002).
264. Chiu, C.-T. *et al.* The Mood Stabilizer Lithium Potentiates the Antidepressant-Like Effects and Ameliorates Oxidative Stress Induced by Acute Ketamine in a Mouse Model of Stress. *Int. J. Neuropsychopharmacol.* **18**, (2015).
265. An, Y. *et al.* Subchronic lithium treatment increases the anxiolytic-like effect of mirtazapine on the expression of contextual conditioned fear. *Eur. J. Pharmacol.* **747**, 13–17 (2015).
266. Rouillon, F. & Gorwood, P. The use of lithium to augment antidepressant medication. *J. Clin. Psychiatry* **59 Suppl 5**, 32–39; discussion 40-41 (1998).
267. Bschor, T. Lithium in the Treatment of Major Depressive Disorder. *Drugs* **74**, 855–862 (2014).
268. Faria, M. S. & Teixeira, N. A. Reversal of learned helplessness by chronic lithium treatment at a prophylactic level. *Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Medicas E Biol.* **26**, 1201–1212 (1993).
269. Teixeira, N. A., Pereira, D. G. & Hermini, A. H. Chronic but not acute Li+ treatment prevents behavioral depression in rats. *Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Medicas E Biol.* **28**, 1003–1007 (1995).
270. Yu, F. *et al.* Lithium Ameliorates Neurodegeneration, Suppresses Neuroinflammation, and Improves Behavioral Performance in a Mouse Model of Traumatic Brain Injury. *J. Neurotrauma* **29**, 362–374 (2012).
271. Martinez, F. O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Front. Biosci. J. Virtual Libr.* **13**, 453–461 (2008).
272. Park, M.-J., Yeo, H.-L., You, M.-J., Kim, B. & Kwon, M.-S. PS206. Persistent glucocorticoid receptor activation reduces M2-like microglia phenotypes without inflammatory signaling. *Int. J. Neuropsychopharmacol.* **19**, 75–76 (2016).
273. Frank, M. G., Hershman, S. A., Weber, M. D., Watkins, L. R. & Maier, S. F. Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. *Psychoneuroendocrinology* **40**, 191–200 (2014).
274. Liu, H. *et al.* Involvement of GSK3β/β-catenin signaling in the impairment effect of ketamine on spatial memory consolidation in rats. *Neurobiol. Learn. Mem.* **111**, 26–34 (2014).
275. Zhang, F., Phiel, C. J., Spece, L., Gurvich, N. & Klein, P. S. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J. Biol. Chem.* **278**, 33067–33077 (2003).
276. Lipina, T. V. *et al.* Inhibition of Glycogen Synthase Kinase 3 Prevents Synaptic Long- Term Depression and Facilitates Cognition in C57bl/6J Mice. *Opera Medica Physiol.* **2**, 87–102 (2016).
277. Hernández, F., Borrell, J., Guaza, C., Avila, J. & Lucas, J. J. Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. *J. Neurochem.* **83**, 1529–1533 (2002).
278. Ge, Y. *et al.* Hippocampal long-term depression is required for the consolidation of spatial memory. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 16697–16702 (2010).
279. Hines, G. Effects of lithium and rubidium on shock-induced changes in open-field activity. *Psychopharmacology (Berl.)* **88**, 209–212 (1986).
280. Hines, G. & Poling, T. H. Lithium effects on active and passive avoidance behavior in the rat. *Psychopharmacology (Berl.)* **82**, 78–82 (1984).
281. O'Donnell, K. C. & Gould, T. D. The Behavioral Actions of Lithium in Rodent Models. *Neurosci. Biobehav. Rev.* **31**, 932–962 (2007).

282. Khan, M. S. *et al.* Lithium ameliorates lipopolysaccharide-induced neurotoxicity in the cortex and hippocampus of the adult rat brain. *Neurochem. Int.* **108**, 343–354 (2017).
283. Guo, W. *et al.* Inhibition of GSK3β improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome. *Hum. Mol. Genet.* **21**, 681–691 (2012).
284. Deters, N., Ittner, L. M. & Götz, J. Substrate-specific reduction of PP2A activity exaggerates tau pathology. *Biochem. Biophys. Res. Commun.* **379**, 400–405 (2009).
285. Oddo, S., Billings, L., Kesslak, J. P., Cribbs, D. H. & LaFerla, F. M. Aβ Immunotherapy Leads to Clearance of Early, but Not Late, Hyperphosphorylated Tau Aggregates via the Proteasome. *Neuron* **43**, 321–332 (2004).
286. Serrano-Pozo, A., Frosch, M. P., Masliah, E. & Hyman, B. T. Neuropathological Alterations in Alzheimer Disease. *Cold Spring Harb. Perspect. Med.* **1**, (2011).
287. Kaushik, D. K. & Basu, A. A friend in need may not be a friend indeed: role of microglia in neurodegenerative diseases. *CNS Neurol. Disord. Drug Targets* **12**, 726–740 (2013).
288. Quartini, A., Iannitelli, A. & Bersani, G. Lithium: from mood stabilizer to putative cognitive enhancer. *Neural Regen. Res.* **11**, 1234–1235 (2016).
289. Post, R. M. The New News about Lithium: An Underutilized Treatment in the United States. *Neuropsychopharmacology* **43**, 1174–1179 (2018).
290. Martin, M., Rehani, K., Jope, R. S. & Michalek, S. M. Toll-like receptor—mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* **6**, 777–784 (2005).
291. Farrer, L. A. *et al.* Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* **278**, 1349–1356 (1997).
292. Liu, C.-C., Kanekiyo, T., Xu, H. & Bu, G. Apolipoprotein E and Alzheimer disease: risk, mechanisms, and therapy. *Nat. Rev. Neurol.* **9**, 106–118 (2013).
293. Colucci-D'Amato, L., Bonavita, V. & di Porzio, U. The end of the central dogma of neurobiology: stem cells and neurogenesis in adult CNS. *Neurol. Sci. Off. J. Ital. Neurol. Soc. Ital. Soc. Clin. Neurophysiol.* **27**, 266–270 (2006).
294. Abrous, D. N., Koehl, M. & Le Moal, M. Adult neurogenesis: from precursors to network and physiology. *Physiol. Rev.* **85**, 523–569 (2005).
295. Anderson, M. L., Sisti, H. M., Curlik, D. M. & Shors, T. J. Associative learning increases adult neurogenesis during a critical period. *Eur. J. Neurosci.* **33**, 175–181 (2011).
296. Gould, E., Beylin, A., Tanapat, P., Reeves, A. & Shors, T. J. Learning enhances adult neurogenesis in the hippocampal formation. *Nat. Neurosci.* **2**, 260–265 (1999).
297. Leuner, B. *et al.* Learning enhances the survival of new neurons beyond the time when the hippocampus is required for memory. *J. Neurosci. Off. J. Soc. Neurosci.* **24**, 7477–7481 (2004).
298. Koehl, M. & Abrous, D. N. A new chapter in the field of memory: adult hippocampal neurogenesis. *Eur. J. Neurosci.* **33**, 1101–1114 (2011).
299. Rodríguez, J. J. & Verkhratsky, A. Neurogenesis in Alzheimer's disease. *J. Anat.* **219**, 78–89 (2011).
300. Donovan, M. H. *et al.* Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. *J. Comp. Neurol.* **495**, 70–83 (2006).
301. Zhang, C., McNeil, E., Dressler, L. & Siman, R. Long-lasting impairment in hippocampal neurogenesis associated with amyloid deposition in a knock-in mouse model of familial Alzheimer's disease. *Exp. Neurol.* **204**, 77–87 (2007).
302. Demars, M., Hu, Y.-S., Gadadhar, A. & Lazarov, O. Impaired neurogenesis is an early event in the etiology of familial Alzheimer's disease in transgenic mice. *J. Neurosci. Res.* **88**, 2103–2117 (2010).
303. Chuang, D.-M. Neuroprotective and neurotrophic actions of the mood stabilizer lithium: can it be used to treat neurodegenerative diseases? *Crit. Rev. Neurobiol.* **16**, 83–90 (2004).

304. Malhi, G. S., Tanious, M., Das, P., Coulston, C. M. & Berk, M. Potential mechanisms of action of lithium in bipolar disorder. Current understanding. *CNS Drugs* **27**, 135–153 (2013).
305. Rowe, M. K. & Chuang, D.-M. Lithium neuroprotection: molecular mechanisms and clinical implications. *Expert Rev. Mol. Med.* **6**, 1–18 (2004).
306. Zhou, K. *et al.* Lithium protects hippocampal progenitors, cognitive performance and hypothalamus–pituitary function after irradiation to the juvenile rat brain. *Oncotarget* **8**, 34111–34127 (2017).
307. Tooyama, I., Kimura, H., Akiyama, H. & McGeer, P. L. Reactive microglia express class I and class II major histocompatibility complex antigens in Alzheimer's disease. *Brain Res.* **523**, 273–280 (1990).
308. Frautschy, S. A. *et al.* Microglial response to amyloid plaques in APPsw transgenic mice. *Am. J. Pathol.* **152**, 307–317 (1998).
309. Martin, E., Boucher, C., Fontaine, B. & Delarasse, C. Distinct inflammatory phenotypes of microglia and monocyte-derived macrophages in Alzheimer's disease models: effects of aging and amyloid pathology. *Aging Cell* **16**, 27–38 (2017).
310. Ahmed, Z. *et al.* Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. *J. Histochem. Cytochem. Off. J. Histochem. Soc.* **55**, 687–700 (2007).
311. Sasaki, Y., Ohsawa, K., Kanazawa, H., Kohsaka, S. & Imai, Y. Iba1 is an actin-cross-linking protein in macrophages/microglia. *Biochem. Biophys. Res. Commun.* **286**, 292–297 (2001).