THE BEHAVIOURAL EFFECTS OF STRESS AND ALUMINUM TOXICITY ON A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS PARKINSONISM-DEMENTIA COMPLEX

by

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Abstract

Background: The etiology of idiopathic neurodegenerative diseases such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) remain unclear. However, it is likely that some combination of genetic susceptibility, environmental toxins and lifestyle factors precipitate onset in an aging-dependent manner. In order to better understand these complex relationships, this thesis sought to investigate two multi-hit hypotheses. These multi-hit hypotheses involved combining a toxin induced neurodegenerative murine model with an environmental factor, aluminum, and separately with a lifestyle factor, stress.

The model of progressive age-related neurodegenerative disease that was used to assess these multi-hit hypotheses was the stigmasterol beta-D-glucoside (SG) dietary toxin mouse model of amyotrophic lateral sclerosis – parkinsonism dementia complex (ALS-PDC). The SG dietary toxin is found in the cycad tree, a type of which is native to Guam, and whose ingestion is believed responsible for the unusually high levels of neurodegenerative disease which were found there in the mid-twentieth century.

The inclusion of water-borne aluminum as part of a multi-hit model was based on previous research epidemiologically linking high levels of aluminum in water and soil in Guam to the ALS-PDC phenotype. The lifestyle factor which was applied to the ALS-PDC model was exogenously administered chronic stress, which in humans can result in neurodegeneration and impaired neurogenesis. The

prediction was that these two hits, one environmental and one lifestyle related, would exacerbate the behavioural ALS-PDC symptoms.

Results: The combination of water-borne aluminum with the ALS-PDC murine model did not lead to behavioural motor impairments beyond what was seen with aluminum alone. However, anxiogenic phenotypes were observed for both aluminum and the combination of aluminum and the SG toxin relative to controls. The application of chronic restraint stress to the ALS-PDC murine model revealed a significant reduction in gait disturbances relative to what was seen with restraint stress alone.

Conclusion: The results suggest that both restraint stress and aluminum can mediate behavioural outcomes in the SG induced murine model of ALS-PDC. However, the high degree of variability within the results suggests that, as is observed with clinical cases of age-related neurodegenerative disease, individual susceptibility is a core determinant of disease progression.

Preface

No part of the work presented herein has yet been published. Data collection for the light-box test for both the stress and aluminum paradigms was conducted in part by the laboratory research assistant, Dominika Kwok. I was responsible for designing the specifications of the light-dark box apparatus, the setup, some data collection and the eventual data analysis.

All animal work and procedures outlined in this thesis were reviewed and approved by the University of British Columbia Animal Care Committee (ACC). The ACC animal protocol was ACC# A09-863. In accordance with university regulations on animal ethics, I completed the requisite theoretical and practical training. My online theoretical training completion number was GUEL101-08. My practical training certificate numbers were: biology and husbandry RBH-E003-08 & RBH-199-12; anesthesia RA-E001-08 & RA-82-12; and surgery RSHX-E001-08 & RSHX-85-12.

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List of Abbreviations

5-HT	Serotonin
6-OHDA	6-hydroxydopamine
ACC	Animal care committee
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ALS-PDC	Amyotrophic lateral sclerosis-parkinsonism dementia complex
ANOVA	Analysis of variance
APP	Amyloid β-protein precursor-derived amyloid-β
ATP	Adenosine triphosphate
avBNST	Anteroventral bed nucleus of the stria terminalis
AVP	Arginine vasopressin
BBB	Blood brain barrier
BG	Basal ganglia
BMAA	β-N-methylamino-L-alanine
BOAA	β-N-oxalylamino-L-alanine
BSSG	β-sitosterol-D-3-glucoside
CA	Cornu ammonis
CCAC	Canadian council on animal care
CCD	Charge-coupled device
CG	Campesterol glucoside
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropin-releasing hormone
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DA	Dopamine
DAT	Dopamine transporter
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
E	Epinephrine
EPM	Elevated plus maze
fALS	Familial amyotrophic lateral sclerosis
FST	Forced swim test
GABA	Gamma-aminobutyric acid
gALS	Guamanian amyotrophic lateral sclerosis
GC	Glucocorticoid
GFAP	Glial fibrillary acidic protein
GLU	Glutamate
GPA	Gait pattern analysis
GR	Glucocorticoid receptor
HD	Huntington's Disease
HPA	Hypothalamic-pituitary-adrenal
HSD	Honestly significant difference

IGS	International genetic standardization
IL	Infralimbic cortex
i.p.	Intra-peritoneally
L-DOPA	L-3,4-dihydroxyphenylalanine
LB	Lewy body
LDT	Light-dark box test
LMN	Lower motor neuron
LN	Lewy neurite
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MND	Motor neuron disease
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionpiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR	Mineralocorticoid receptor
MWM	Morris water maze
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NFTs	Neurofibrillary tangles
NIH	National Institute of Health
NMDA	N-methyl-D-aspartate
NST	Nucleus of the solitary tract
OFT	Open field test
OXT	Oxytocin
pBNST	Posterior bed nucleus of the stria terminalis
PD	Parkinson's disease
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy
PVN	Hypothalamic paraventricular nucleus
RAM	Radial arm maze
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sALS	Sporadic amyotrophic lateral sclerosis
s.c.	Subcutaneous
SCARL	Statistical Consulting and Research Laboratory
SG	Stigmasterol β-D-glucoside
SN	Substantia nigra
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SNc	Substantia nigra pars compacta
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
TDP-43	Transactivation response DNA-binding protein 43
TH	Tyrosine hydroxylase
UMN	Upper motor neuron
VPS54	Vesicular/vacuolar protein sorting 54
WHT	Wire hang test

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The Statistical Consulting and Research Laboratory (SCARL), operated by the Department of Statistics at the University of British Columbia, provided statistical insight and feedback.

Dr. Doris Doudet and Dr. Chris Shaw reviewed this manuscript and generally facilitated my thesis work over my many years at the University of British Columbia.

A large number of faculty members have been on one or another of my committees (supervisory committee, comprehensive exam committee, thesis committee) over the years. They have provided both insightful feedback and incisive criticism, which has fostered my academic growth.

Dominika Kwok assisted with the collection of data on the light dark test and provided general technical assistance.

Chapter 1 Introduction

1.1 Age-Related Neurodegenerative Disease

The biological process of aging results in molecular and cellular changes that contribute to the onset and progression of most neurodegenerative diseases and represents the main risk factor for neurodegeneration (Niccoli & Partridge 2012). In order to distinguish neurodegenerative diseases for which aging is a risk factor, they are usually termed 'age-related'. There are a large number of age-related neurodegenerative diseases, but three will be discussed in this thesis: ALS, PD, and ALS-PDC.

Aging can be defined as, "a process whereby cells and tissues of somatic lineage deteriorate with time, becoming progressively more vulnerable to environmental insults, eventually resulting in disease and death" (Wills & Brown Jr. 2006). There are over 300 theories of aging, many of which are based on the accumulation of cellular changes over time, though none are without detractors (Ashok & Ali 1999). The free radical theory of aging (later called the oxidative damage theory) first postulated by Harman in the 1950s, is arguably the most popular. The theory proposes that mitochondrial reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, damage macromolecules such as lipids, proteins and mitochondrial deoxyribonucleic acid (DNA) (Harman 1981). The impact of such DNA and protein damage for neurodegenerative diseases is that it could potentially lead to protein aggregates such as amyloid (in Alzheimer's disease, AD), synuclein (in PD) and ubiquitin (in ALS). Another common theory is the somatic mutation theory of aging. This theory points to the capacity for DNA repairs as an important determinant of the rate of

aging at the cellular and molecular levels (Kirkwood 2005). A third popularized theory is the telomere loss theory. This theory argues that the general decline in cellular division seen with age is linked to the telomeres that cap the ends of chromosomes. As one ages, the increasing number of completed replicative cycles leads to a progressive shortening of the ends of the chromosomes until they ultimately prevent further division and repair (Campisi et al. 2001). Interestingly, the rate of telomere loss and shortening is vulnerable to increased oxidative stress which can accelerate telomere shortening (von Zglinicki 2002), suggesting perhaps that a more comprehensive theory of aging may be found by assembling components of existing theories.

There are both genetic and environmental factors which influence age-related neurodegenerative disease. The molecular intersection of signalling pathways for aging and neurodegeneration implies that genetic and environmental factors can act on cellular pathways for one or the other or both to have an effect. This in turn can make assessing what impact a given factor has, if any, on age-related neurodegenerative disease, quite difficult. Thus, while many factors are suspected to impact age-related neurodegenerative disease few have been conclusively confirmed as such.

1.2 Stress and its Impact on Neurodegenerative Disease

One of the lifestyle factors believed to influence age-related disease is stress. However, as with other lifestyle factors, the exact mechanism of how stress acts and reacts with age and neurodegenerative disease in a multi-hit context is not fully understood. As

such, in order to behaviourally explore this relationship, this multi-hit combination was examined in one of the paradigms in this thesis.

1.2.1 The Stress Response

The stress response involves activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Its most basic function is to enable the organism to deal with the threat (perceived or real) through the mobilization of energy reserves while inhibiting nonessential bodily functions. Activation of the SNS results in the rapid stress response and initiates the release of catecholamines including epinephrine (E) and norepinephrine (NE). The HPA axis has a slower response and involves the release of glucocorticoids (GCs) within minutes of stressor onset (Miller & O'Callaghan 2002). In humans the principal GC is cortisol while in rodents it is corticosterone (CORT). A key aspect of the stress response is that it is self-terminating once the stressor has ended. A stress response that fails to terminate can pose a serious threat to health. For instance, while elevated levels of SNS-mediated E lead to prolonged maximized blood flow, which is beneficial for the acute threat, it will increase the risk of heart failure and arteriosclerosis if unchecked. Further, while increased GCs promote energy resource redistribution, they can cause immune dysfunction, disease and inadequate energy supply to some organs if allowed to continue long-term (Conrad 2008).

GCs are steroids produced by the adrenals and receptors for them are found throughout the brain. They can act as transcription factors and regulate gene expression, thus

potentially having long-term effects (Lupien et al. 2009). GCs are also critical in normal brain development and maturation, myelin lipid biosynthesis and cell survival, particularly in the cerebrum (Meyer 1983). There are two types of GC receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). MRs respond to GCs, mineralocorticoids such as aldosterone and deoxycorticosterone, and also progestins. GRs are activated by GCs which, unlike MRs which are predominantly localized in the limbic brain (hippocampus), are present in almost every cell (De Kloet et al. 1998). Importantly, GR and MR expression can be influenced by chronic stress with some studies reporting decreased GR but not MR messenger ribonucleic acid (RNA) levels across the hippocampus (Wright et al. 2006).

The HPA axis is a key element in the body's coordinated physiological response to a stressor (Figure 1-1). Physiological stressors result in activation of the direct pathways whereas psychogenic stressors result in activation of the indirect pathways. All pathways lead to the stimulation of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus, the starting point of the HPA. Concurrent with CRH production there is also arginine vasopressin (AVP) release from the PVN and supraoptic nucleus and oxytocin (OXT) release from the PVN into the bloodstream via neurohypophysis (Bao et al. 2008). Once CRH has entered the portal vasculature it mediates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary which in turn acts on the adrenal cortex resulting in secretion of GCs (Lupien et al. 2009). ACTH simultaneously triggers the release of E and NE from the adrenal medulla. GCs act on numerous internal organs to promote survival including

initiating hepatic glycogenolysis to increase plasma glucose levels, maintaining vascular tone, and suppressing inflammatory processes. The termination of the stressor leads to the activation of feedback loops mediated by GCs that turn off the brain regions driving the HPA and return the axis to its homeostatic start state (Herman 2011).

The direct method of activating the HPA involves stimulation of the CRH neurons in the PVN by ascending NE and E neurons in the nucleus of the solitary tract (NST). The responsiveness of the SNS and the HPA are closely intertwined and catecholamine release following SNS activation results in modulation of HPA function (Plotsky et al. 1989). This catecholamine release follows from physiological stressors such as pain and hypoglycemia (Pacák et al. 1995). There are also a number of direct homeostatic inputs from within the hypothalamus as well those from the circumventricular organs. These include the mediobasal hypothalamus which senses changes in energy, the subfornical organ which excites the HPA via angiotensin II projections, and inflammatory signals which act on the NST (Bell et al. 2000; Saavedra et al. 2000; Rivest 2001).

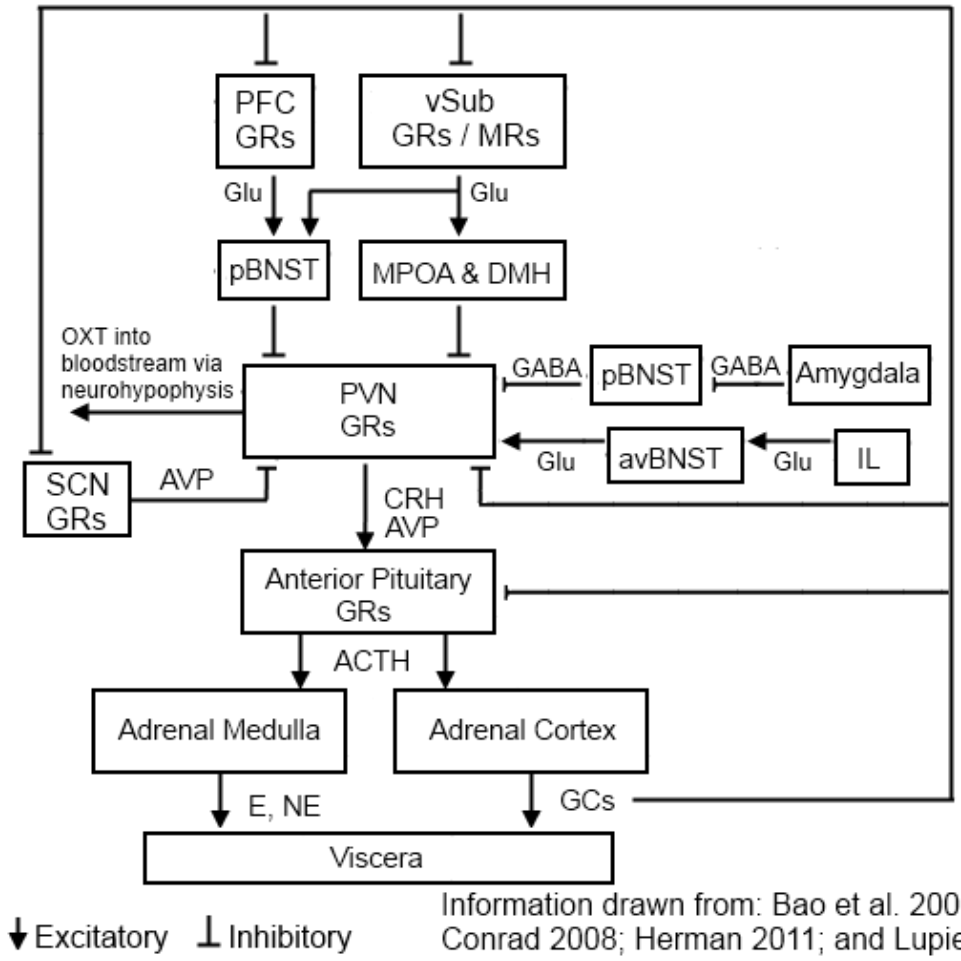
The indirect HPA activation pathway is a mechanism for preparing for anticipated challenges as it responds to psychological stress. Thus its modulating influence on the HPA derives from a combination of memories, threat vigilance, and innate preferences rather than immediate physical risk. This integration requires limbic and associational systems working in tandem to indirectly signal the PVN of the HPA (Jankord & Herman 2008). There are several multisynaptic brain circuits which originate in limbic brain

structures and act on the PVN. The two principle limbic structures are the amygdala and the infralimbic cortex (IL). The amygdala is connected via inhibitory gamma-aminobutyric acid (GABA) projections to the posterior bed nucleus of the stria terminalis (pBNST). The PVN then receives GABA projections from the pBNST. These sequential GABAergic synapses allow for disynaptic dysinhibition of the HPA. The anteroventral bed nucleus of the stria terminalis (avBNST) sends excitatory inputs to the PVN. These inputs, which increase PVN CRH secretion, are suspected extrahypothalamic CRH peptides as suggested by CRH mapping (Drolet & Rivest 2001). The avBNST in turn receives excitatory glutaminergic innervation from the IL thus activating the HPA through sequential excitatory inputs (Herman 2011).

In order to shut down the HPA axis GCs exert negative feedback at all levels. The inhibitory actions of the GCs are predominantly on GRs and not MRs. This is due to MRs having a 10-fold higher affinity for GCs than GRs which results in MRs being largely saturated at basal concentrations of GCs (De Kloet et al. 1998). Thus it is GR activation that suppresses excitability raised by excitatory stimuli and controls feedback loops. MRs do however play a role in maintaining HPA axis homeostasis as MR activation increases cellular responsiveness to excitatory stimuli, controls axis sensitivity and influences behavioural strategies (De Kloet et al. 1991).

Figure 1-1: HPA Axis Neurocircuit

The HPA axis is activated in response to both physiological and psychological stress. In response to a stressor, neurons in the PVN of the hypothalamus release CRH and AVP. These in turn act on the anterior pituitary leading to the secretion of ACTH. ACTH stimulates production of GCs from the adrenal cortex and E and NE from the adrenal medulla. Once the stressor has ceased, GC inhibitory feedback loops return the axis to a homeostatic set point via direct inputs to the PVN and anterior pituitary and indirectly via the frontal cortex and the hippocampus. Psychogenic activation of the HPA can occur through disynaptic pathways from the amygdala and the IL.



Information drawn from: Bao et al. 2008; Bremner 1999; Conrad 2008; Herman 2011; and Lupien et al. 2009.

1.2.2 Chronic Stress and Hippocampal Damage

Chronic stress is widely acknowledged to be detrimental to human health. The hippocampus in particular is vulnerable to chronic stress induced elevations in GCs which leads to neurodegeneration and suppressed neurogenesis (Conrad 2008; Fuchs et al. 1995; Gould et al. 1997). Several hypotheses have been put forward that attempt to explain the relationship between chronic stress and the aging hippocampus. The glucocorticoid cascade hypothesis, described in 1986, endeavoured to explain what was known about hippocampal damage and glucocorticoids at the time (Sapolsky et al. 1986). The hypothesis states that the hippocampus, in response to periods of stress-mediated elevated GCs, becomes desensitized to further GC exposure by down-regulation of GRs. This desensitization is reversible once GCs return to basal levels. However, on occasion the down-regulation of GRs results in hypersecretion of GCs and subsequent hippocampal cell damage. This hippocampal damage is cumulative over time and irreversible. The hippocampus would then become more desensitized with time, resulting in hypercortisolemia and further cellular damage as a person ages (Uno et al. 1994).

Inconsistencies with the glucocorticoid cascade hypothesis led to the formulation of the glucocorticoid vulnerability hypothesis to better explain hippocampal and HPA axis changes in response to aging. The vulnerability hypothesis proposes that a history of chronic stress leaves an imprint on the hippocampus which results in it being susceptible to neurotoxic or metabolic insults such as ibotenic acid, hypoxia, hypoglycemia or ischemia for a period of time following cessation of the chronic stress

(Conrad 2011). Importantly, although the vulnerability hypothesis is specific to the hippocampus, glucocorticoids and their receptors are found throughout the brain (Lupien et al. 2009), which suggests that at least some other brain regions may be similarly predisposed to a chronic stress imprint.

The vulnerability hypothesis argues that as a person ages the risk of them having experienced hippocampal damage increases by virtue of the sheer number of chronic stress events they've potentially experienced. Indeed, the aged hippocampus displays elevated synaptic loss (Nichols et al. 2001). Other age-related factors such as an attenuated GC negative feedback loop could exacerbate hippocampal vulnerability by promoting GC secretion (Mizoguchi et al. 2009). A distinctive difference between the glucocorticoid cascade hypothesis and the vulnerability hypothesis is that the latter does not require GCs to be elevated at the time of the neurotoxic or metabolic challenge for hippocampal damage to occur. This window of vulnerability following elevated GCs has been shown to correspond to the period of cornu ammonis-3 (CA3) dendritic retraction which is observed after chronic stress (Conrad et al. 2007). The dendritic retraction may act as a protective response as it reduces the potential for glutamate (GLU) neurotoxicity (Conrad 2011) which is a foreseeable risk as stress increases hippocampal GLU concentrations (Abrahám et al. 1998). Dendritic atrophy in the CA3 region is also reversible and correlates with behavioural recovery once the chronic stress has ceased (Sousa et al. 2000). Thus by itself changes in dendritic arbors pose no risk, however they create an increased vulnerable state which results in permanent hippocampal neuronal damage following a subsequent insult.

1.2.3 Chronic Restraint Stress and Neurodegenerative Disease

The glucocorticoid vulnerability hypothesis suggests a mechanism through which disease and chronic stress interact: namely through disease mediated alterations in metabolic homeostasis or disease neurotoxins acting on regions made vulnerable through GC exposure. However, the most prominent behavioural symptoms of murine ALS-PDC, the disease model which was combined with restraint stress in this thesis, are motor in nature, and the impact of GCs on the motor cortex is less well studied. Despite this, there is reason to expect that stress will affect motor symptom outcomes as there are substantial densities of GRs in motor regions including the basal ganglia and motor cortex (Ahima et al. 1991). Further, neurons of the substantia nigra (SN), which feature prominently in motor disorders such as PD, are postulated to be vulnerable to sensitization by GCs (Kibel & Drenjančević-Perić 2008). Indeed, Kibel & Drenjančević-Perić's (2008) hypothesis is supported by work by Smith et al. (2008) who, after administering 20mins restraint stress or 5mg CORT daily for 2 weeks, showed that both restraint stress and CORT treatment enhanced motor impairments and exaggerated nigral neuronal loss in 6-hydroxydopamine (6-OHDA)-treated female rats. In contrast, Mo et al. (2014) applied restraint stress for 1 hour daily for 8 weeks to R6/1 transgenic Huntington's disease (HD) mice and while they found enhanced motor activity in male but not female mice, chronic stress did not appear to facilitate a worsening of motor symptomatology. The differing outcomes following restraint stress seen in HD transgenic mice and parkinsonian rats may be a function of the animal models under study or the aspect of the stress response being behaviourally examined.

Indeed, in Smith et al. (2008) behavioural testing occurred 60mins following restraint stress (during the intermediate stress response which lasts from 30mins to several hours), suggesting that the two studies may not have been assessing the same portion of the stress response curve.

The clinical motor phenotypes seen in both PD and HD relate principally to neurodegeneration in the basal ganglia (BG). This means that for chronic restraint stress to affect disease progression in these disorders, its application needs to lead to neurochemical changes, such as GC mediated vulnerabilities, in the BG. In order to ascertain which endogenous molecules contribute to BG vulnerability, Kim et al. (2005) examined the neurochemical impact of chronic immobilization stress on the nigrostriatal system. They found that immobilization stress caused degeneration of the nigrostriatal system and the accumulation of neuromelanin. Nigrostriatal damage was also observed to result from tetrahydrobiopterin induced oxidative damage to dopaminergic neurons (Kim et al. 2005). Smith et al. (2008), in the study described above, found similar motor deficits for chronic immobilization stress and CORT and Metz et al. (2005) using adult female rats reported that chronic immobilization stress (14 days) and oral CORT treatment led to comparable reductions in skilled movement accuracy in reaching and walking and increased performance speed. Together these studies indicate a role for chronic stress in the facilitation of BG-related neurodegenerative disease.

1.3 Aluminum and its Impact on Neurodegenerative Disease

Aluminum is an environmental toxin which is strongly suspected to mediate the course of age-related neurodegenerative disease. Aluminum is ubiquitous on Earth and as such finds its way into nearly every aspect of human life. Despite this, the impact of aluminum's toxic properties on age-related neurodegenerative disease remains poorly understood. This led to its inclusion in this thesis in order to provide insight into the multi-hit combination of aluminum, age and neurodegenerative disease.

1.3.1 Sources of Aluminum Exposure

Aluminum is the third most common element after oxygen and silicon and the most abundant metal in Earth's crust. Aluminum has been identified as having potentially neurotoxic properties since at least the nineteenth century through the parenteral administration of aluminum salts by Orfila in 1814 and animal studies by Siem in 1887, both of whom are referred to by Döllken in a paper in 1897. The same paper demonstrated that aluminum could be used to produce degeneration in rabbit brains (Döllken 1897). Human exposure to aluminum arises from food, water, airborne dust, antiperspirants, immunizations, allergy injections and antacids (Saiyed & Yokel 2005) though food intake is the largest source of aluminum for humans (Yokel & McNamara 2001).

Aluminum can readily enter the human body via soluble aluminum salts which can be absorbed through the stomach with daily human exposure from food products in the range of 3 to 10mg (Yokel & Florence 2008). Aluminum can also be found in drinking

water though its oral bioavailability in water, at around 0.3%, is low (Yokel & McNamara 2001). However, once aluminum from tap water enters the body it has been shown to enter the brain as evidenced in rat brains following gavage of trace amounts (Walton et al. 1995). This latter point is of concern as aluminum is known to build up in the human brain with age (Roider & Drasch 1999). Also of concern is the presence of fluoride in drinking water as it promotes intestinal absorption of aluminum (Varner et al. 1998) and can form aluminofluoride complexes which are extremely toxic to humans as they can act as structural phosphate analogues (Strunecká et al. 2002).

Aluminum that does enter the body's circulatory system via the stomach, transdermal absorption, injection or other means can become deposited as intraneuronal perikaryal aggregates of phosphorylated neurofilament in the neuronal grey matter (Boegman & Bates 1984; Erasmus et al. 1993). This is possible due to carrier-mediated uptake across the blood brain barrier (BBB), which appears to differ for aluminum transferrin and aluminum citrate (Yokel 2002). In the case of aluminum transferrin the aluminum ion may follow the iron acquisition pathway which involves the aluminum transferrin complex interacting with transferrin receptor 1 followed by aluminum release in the endosome and transport to the cytosol (El Hage Chahine et al. 2012). Several candidate BBB transporters for aluminum citrate uptake have been proposed. Among them are an uncharacterized monocarboxylate transporter isoform and an organic anion-transporting protein (Yokel et al. 2002).

The majority of aluminum that enters the brain extracellular fluid is rapidly effluxed, though some aluminum can be retained for extended periods of time (Yokel 2002). Aluminum that is present in the brain extracellular fluid is predicted to be about 90% aluminum citrate and 4% aluminum transferrin (Nurchi et al. 2012). The aluminum in the extracellular fluid is prone towards being deposited in one of the 5 main storage compartments within the human brain for aluminum as identified by Exley & House (2012). These compartments include the BBB, the brain interstitial fluid, neurons, glia, and pathological presentations such as Lewy bodies (LBs), neurofibrillary tangles and senile plaques. Indeed, evidence supports aluminum accumulation in neurons, the BBB, and glial cells (Aremu & Meshitsuka 2005; Lévesque et al. 2000; Reusche et al. 1996).

Aluminum is excreted from the body via urine as has been shown by Day et al. (1991) using a ^{26}Al tracer. Further, glomerular filtration of aluminum from the blood does not appear sufficient to prevent aluminum build up in the body, particularly since chelation therapy using desferrioxamine can elevate aluminum urinary excretion rates even in patients not suffering aluminum overload (Ackrill & Day 1985). Though urine is the predominant mechanism of aluminum excretion, smaller amounts are cleared through feces, skin, hair, nails, sweat, tears and semen (Exley 2008).

1.3.2 Chronic Aluminum Toxicity

Animal studies examining aluminum exposure consistently report toxic outcomes on behaviour and pathology. For instance, aluminum fluoride (AlF_3) given chronically via drinking water to rats resulted in cellular abnormalities in the form of chromatin

clumping, enhanced protein staining, pyknosis, and vacuolation as well as a general reduction of neuronal density in the neocortex relative to controls (Varner et al. 1998). However, despite possible olfactory impairments, chronic aluminum fluoride did not appear to lead to motor deficits in rats (Varner et al. 1994). While these results contribute to the general knowledge of aluminum's toxic profile, it is important to recall that aluminofluoride complexes are extremely toxic by themselves thus making it difficult to firmly attribute the toxic outcomes to aluminum.

Unlike aluminum fluoride, animal studies using aluminum chloride consistently report motor impairments. Chronic aluminum chloride given orally during the perinatal period to mouse dams led to dose-dependent deficits in locomotion at postnatal day 22, learning capability at postnatal day 25 and cognitive behaviour during postnatal days 30 to 36. Concomitant with these behavioural findings were dose-dependent disturbances of dopamine (DA) and serotonin (5-HT) in the forebrain (Abu-Taweel et al. 2012). Erazi et al. (2010) found that chronic aluminum chloride given to rats during adulthood or from intra-uterine age led to significantly reduced locomotor activity. The same rats displayed intense glial fibrillary acidic protein (GFAP) immunoreactivity concurrent with a high density of astrocytes in the cerebral cortex (Erazi et al. 2010). Similarly in mice, aluminum chloride was shown to produce, in a duration-dependent manner, motor coordination impairments (Sahin et al. 1995).

The behavioural results observed with the aluminum chloride and aluminum fluoride salts appear to be distinct not only from each other but also from what is seen with other

aluminum salts. For instance, Connor et al. (1989) reported that chronic water-borne aluminum sulfate led to no locomotion deficits in male rats though an increase was observed in the rate of extinction on a passive avoidance task. A study using aluminum nitrate, which was chronically administered to female rats in their drinking water, revealed no significant motor impairments, however there were deficits in object recognition memory (Azzaoui et al. 2008). Together the evidence appears to suggest that the compound or ion to which aluminum is attached, thereby forming the salt, has a substantial mediating effect on the toxic effect of aluminum. However, it does seem clear that aluminum chloride has a debilitating effect on motor ability in rodents, which is in part why it was chosen for this thesis.

The cognitive impairments associated with aluminum toxicity appear to be, as was seen with motor deficits, linked to the ion or compound to which aluminum is attached. Bilkei-Gorzó (1993) chronically treated rats with aluminum chloride, aluminum hydroxide, aluminum hydroxide + citric acid or sodium chloride + citric acid. The results showed learning impairments on a labyrinth test for the aluminum chloride and aluminum hydroxide + citric acid with the highest elevations of brain aluminum observed for the aluminum chloride group. Of note, the aluminum hydroxide group did not display cognitive deficits (Bilkei-Gorzó 1993). Similar to Bilkei-Gorzó (1993), Walton (2009b) found that chronic administration of aluminum chloride led to functional memory impairments. These deficits, which extended to attention and perseveration, were shown with male rats on a T-maze task. Walton (2009b) also compared rat performance

over the lifespan and showed that rats in the highest aluminum regimen performed the worst once reaching old age.

1.3.3 Aluminum and Neurodegenerative Disease

Chronic aluminum toxicity has long been suspected to facilitate neurodegenerative disease; this is particularly true in the case of AD. The neuropathological hallmarks of AD, senile plaques formed of depositions of amyloid β-protein precursor-derived amyloid-β (APP), and neurofibrillary tangles (NFTs) consisting of aggregates of hyperphosphorylated microtubule-associated protein tau, have been linked to aluminum exposure (Tomljenovic 2010).

The chronic oral administration of aluminum chloride in aged rats has been shown to, in hippocampal and cortical tissue, elevate APP gene expression, generate dense APP deposits in cytoplasm of neural cells and produce APP-immunoreactive neurites (Walton & Wang 2009). Generalized hippocampal damage, indirectly assessed through the area occupied by mossy fibre in the CA3 field, was also found by Fattoretti et al. (2003) following chronic aluminum chloride administration to aged male rats. A follow up histological examination of the rat brains used by Walton & Wang (2009) showed hippocampal lesions consisting of aluminum-rich microtubule-depleted pyramidal cells and a loss of synaptic density (Walton 2009a). Thus, similar to what is seen in AD and what has been reported in animals by others (Kowall et al. 1989; Landsberg et al. 1993; Walton 2006), aluminum toxicity can, in a cell and brain region specific manner, induce neurodegenerative pathology. The fact that specific brain regions are selectively

targeted by aluminum toxicity is likely related to the high densities of transferrin receptors in regions such as the cortex and hippocampus, which allows for limited aluminum transferrin uptake (Edwardson et al. 1992).

An important feature of aluminum induced neurodegeneration is that it appears to be mediated by individual susceptibility. This is pointed to by Walton (2009a), where rats chronically exposed to oral aluminum chloride displayed aluminum accumulation in specific populations of pyramidal neurons in the entorhinal cortex, hippocampal formation and adjoining cortical regions – but only in susceptible aged rats. This genetic susceptibility, whether inherited or triggered by one or more environmental agents and/or lifestyle factors, appears to induce vulnerability to aluminum toxicity in a dose-dependent manner. Indeed, low dose aluminum lactate given as 1mg/g of diet for 120 days, when combined with the Tg 2576 transgenic AD mouse model, did not lead to impaired memory and learning beyond what was seen in either Tg 2576 or aluminum lactate alone (Ribes et al. 2008). By contrast, the same group of researchers conducted a very similar experiment giving aluminum lactate as 11mg/g (an increase of 11x over the previous experiment) of diet for 6 months to Tg 2576 AD mice and found impaired spatial learning, though no effects on neurogenesis were observed (Ribes et al. 2010).

The linkage between aluminum and AD is certainly the most prominent suspected or confirmed instance of aluminum-induced neurodegeneration in an age-related disorder; however it is far from the only one. For instance in Guam and the Kii Peninsula of Japan, the combination of low calcium and magnesium and high aluminum and

manganese in the drinking water and soil has been epidemiologically linked to the ALS-PDC phenotype (Yase et al. 2001; see Section 1.6), which has led to the suggestion of a multi-hit explanation for the diseases etiology.

The relationship between motor symptoms and aluminum has been investigated in rabbits where intracisternal inoculation of aluminum chloride induced motor neuron degeneration marked by intraneuronal neurofilamentous aggregates similar to what is seen in ALS (He & Strong 2000). Selective degeneration of motor neurons was also shown in male mice following 2 subcutaneous (s.c.) doses of aluminum hydroxide; additional motor and spatial memory impairments presented when 6 doses were given (Shaw & Petrik 2009). These findings suggest that ALS-like motor neuron degeneration can be precipitated by aluminum administration.

Aluminum's role in age-related degeneration also appears to encompass PD. Sánchez-Iglesias et al. (2009) found that aluminum chloride given i.p. exacerbated oxidative stress in the dopaminergic system in the 6-OHDA rat model of PD. Indeed, aluminum is known to be involved in the production of ROS, and may through ROS impair mitochondrial function and lead to oxidative stress (Kumar & Gill 2014). Mitochondrial dysfunction has been identified in PD, ALS and other age-related neurodegenerative disorders (Federico et al. 2012), though at present it remains unknown whether oxidative stress is a cause or consequence of these diseases. What is clear however is that aluminum through its adverse actions on mitochondrial energy production could

facilitate disease pathogenesis for age-related disorders, including ALS, PD and ALS-PDC.

1.4 ALS and the Spectrum of Motor Neuron Disease

1.4.1 Clinical Features

The pathology of ALS as a distinct disease was first described by Charcot and his colleague Joffroy in 1869 (Charcot & Joffroy 1869) though it was not until 1874 that it was termed ALS (Charcot 1874). At present ALS is the most common motor neuron disease in adults (Przedborski et al. 2003) with worldwide incidence of 1.8 to 2.1 per 100000 and a lifetime risk of development ALS of 1 in 2000 (Bruijn et al. 2004). Peak incidence for ALS is for individuals from 55 to 65 years with males being more likely to develop the disease (1.6:1). The male:female ratio of ALS incidence varies sharply with age as in the 35 to 44 age group it is as high as 2.5:1 and drops to 1.2:1 in the 65-74 age group; biological changes such as menopause may explain the dramatic change (Manjaly et al. 2010). Nearly a third of new ALS patients are below the age of 45 at symptom onset (Strong 2004). There is evidence to suggest that the ALS disease process has a long preclinical period during which the disease becomes disseminated in the motor system though muscle weakness and atrophy remain hidden due to collateral reinnervation (Swash & Ingram 1988). There has been a steady increase in ALS incidence from the 1970s and 80s which is suspected to be attributable to longer life expectancy, though environmental factors cannot be ruled out (Worms 2001).

ALS is one of a spectrum of motor neuron diseases, each of which possesses some overlapping and distinct qualities. ALS, also called motor neuron disease (MND) in Europe and Lou Gehrig's in the US, is largely limited to the upper and lower motor systems. The most common form of ALS is the variant described by Charcot and this presentation of the disease is termed classical ALS. Other diseases in the spectrum of neurodegenerative syndromes characterised by progressive degeneration of the motor neurons and which have significant clinical overlap with ALS include primary lateral sclerosis (PLS) which afflicts upper motor neurons (UMN) and progressive muscular atrophy (PMA) which targets lower motor neurons (LMN). There are also a number of disorders that present with clinical ALS symptoms but also encompass non-motor symptoms such as frontotemporal dementias, parkinsonism, spinocerebellar ataxias and supranuclear gaze palsy. Lastly there are UMN and LMN disorders that are pathologically distinct from ALS such as the UMN diseases lathyrism and hereditary spastic parapareses and the LMN diseases poliomyelitis and spinal muscular atrophy (Murray & Mitsumoto 2006). It should be noted that while MND is used to describe ALS in Europe, it has also been put forward to describe the spectrum of progressive motor neuron disease (Brain & Walton 1969).

ALS presents as progressive muscle atrophy and weakness resulting in death within 5 years of symptom onset in most cases (Strong 2004). This progressive muscular paralysis is the result of degeneration of motor neurons in the primary motor cortex, brainstem and spinal cord (Wijesekera & Leigh 2009). LMN specific symptoms include hyporeflexia-areflexia, hypotonicity or flaccidity and muscle cramps while UMN features

include spasticity, hyperreflexia, pathologic reflexes such as Babinski's and pseudobalbar palsy. LMN and UMN symptoms typically occur concurrently though they may not both be clinically evident depending on disease state (Murray & Mitsumoto 2006). The term amyotrophy refers to the pathological wasting of muscles which occurs as their corresponding anterior horn cells degenerate, leading to weakness and fasciculations. Lateral sclerosis refers to the hardening of the anterior and lateral corticospinal tracts with degeneration of motor neurons in these areas and subsequent replacement with gliosis (Rowland & Shneider 2001).

Symptom onset in ALS normally appears as weakness in one extremity followed by gradual spreading to other limbs. This monomelic insidious onset has a predictable spread with the disease progressing first to contralateral limb then into other spinal segments ultimately afflicting all extremities (Murray & Mitsumoto 2006). Coexistence of UMN and LMN signs is typical in late state classical ALS with the bulbar, cervical, thoracic and lumbar regions all affected within the central nervous system (CNS). Death is normally from respiratory failure or other pulmonary complications though survival can often be prolonged through respirator involvement. Interestingly, oculomotor movement can remain unimpaired (Sasaki et al. 1992).

Limb onset as described above is the most common variant of classical ALS, however approximately 25% of ALS cases present with bulbar-onset form. Symptoms include dysarthria of speech which is often apparent after small amounts of alcohol as well as dysphagia and sialorrhoea. Sialorrhoea is due to difficulty swallowing saliva and mild

UMN facial weakness in the lower part of the face (Wijesekera & Leigh 2009). Rarer cases (about 5%) include respiratory failure as a presenting symptom with variable limb or bulbar symptoms. Afflicted patients develop hypercapnic respiratory failure requiring ventilation with diaphragmatic weakness, dyspnoea often with associated orthopnoea, anorexia, disturbed sleep and mood changes (Chen et al. 1996; de Carvalho et al. 1996).

1.4.2 Treatment of ALS

The treatment of clinical ALS is multidisciplinary and aims to control physical disease symptoms such as progressive weakness, respiratory insufficiency and dysphagia in the patient as well as providing care to the family (Ferguson & Elman 2007). However, despite ALS' incurable and progressive physical symptoms, studies show that it is its psychological aspects which most affect quality of life and thus psychological, social and spiritual support is crucial (Neudert et al 2004). As respiratory failure is the leading cause of mortality in ALS patients, respiratory function is closely monitored following diagnosis. The respiratory state of the patient is assessed using measurements of the forced vital capacity which in turn determine when ventilator support is required. Once the forced vital capacity declines to 50% of the predicted value non-invasive ventilation is recommended, though a higher threshold value is often appropriate dependent upon respiratory symptoms (Gruis et al. 2005). Dysphagia in ALS patients poses risks of malnutrition and breathing. Thus treatment includes a specific dietary and nutritional regime, particularly in light of the hyper metabolic state of ALS patients which requires an increased caloric intake (Muscaritoli et al. 2012).

There have been over 20 clinical trials in patients with ALS since the late 1990s, which while adding to the general body of knowledge on ALS have been largely unsuccessful in finding pharmacological treatments for the disease (Lau et al. 2006). To date only the drug riluzole has been shown to extend ALS patient life with an increased lifespan of around 2 to 3 months (Miller et al. 2012). Although it remains the subject of investigation it is suggested that riluzole has four primary effects at clinically relevant doses: inhibition of repetitive firing, inhibition of persistent Na^+ current, potentiation of calcium-dependent K^+ current, and inhibition of neurotransmitter release (Bellingham 2011). These effects are believed to result in neuronal consequences including interference with N-methyl-D-aspartate (NMDA) receptor mediated responses and inhibition of GLU release from pre-synaptic terminals (Distad et al. 2008).

1.4.3 Pathogenesis of ALS

The onset of clinical ALS symptoms is accompanied by and is suspected to be preceded by histopathological changes. Perhaps the most preeminent among these changes is the presence of protein aggregates which are immunoreactive to antibodies against ubiquitin. These ubiquitin positive skein and/or spherical shaped aggregates are found in virtually all ALS cases (Piao et al. 2003), though as with protein aggregates in other neurodegenerative diseases, it remains unknown whether they are a primary cause of the disease, a harmless product or possibly a result of a cellular defense mechanism (Caughey & Lansbury 2003).

A further cellular characteristic of ALS is alterations in mitochondrial respiratory complex activity. Mitochondria are an early target in the pathogenesis of ALS and present with morphological and functional defects both in human patients and mice overexpressing mutant copper-zinc superoxide dismutase 1 (SOD1) (Shi et al. 2010). Indeed mitochondria, which are responsible for the generation of adenosine triphosphate (ATP), appear to confer susceptibility to motor neurons to energy deficits, calcium mishandling and oxidative stress following dysfunction (Cozzolino & Carri 2012). Of note, these dysfunction driven changes appear to be only part of the mitochondrial problem as axonal transport of mitochondria along microtubules is also disrupted in SOD1 mutants (Williamson & Cleveland 1999), which may help explain the 'dying back' progression of ALS in motor neurons. This 'dying back' pathophysiology in ALS, along with changes at the neuromuscular junction such as mitochondrial dysfunction, has given rise to the proposition that ALS is a distal axonopathy. This hypothesis states that axonal defects occur prior to cell death and the loss of axonal function correlates with functional motor neuron decline (Moloney et al. 2014).

The histopathological changes present in ALS include as noted above changes and disruptions in intracellular transport. Axonal calibre and the framework for axonal transport are heavily dependent on neurofilaments, which are around 10nm in size and composed of light, medium and heavy subunits (Barber & Shaw 2007). These neurofilaments are transported along microtubules by the motor proteins kinesin and the dynein/dynactin-1 complex. The accumulation of neurofilaments in axons in somata,

which is characteristic of motor neuron diseases, results from damage to the motor proteins (Ikenaka et al. 2012).

Histochemically, the pathogenesis of ALS is defined by oxidative stress. Oxidative stress is the process by which the normal reducing conditions within the cell are disrupted by ROS such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, which are generated as the by-products of aerobic metabolism (Barber & Shaw 2007). In human ALS spinal tissue, elevated levels of free radicals as well as oxidative damage to proteins, lipids and DNA have been reported (Shibata et al. 2001) which, as noted above, may be a consequence of early mitochondrial dysfunction in the disease (Federico et al. 2012). In early ALS, the increasing levels of ROS form a positive feedback loop which induces more ROS release, contributes to upregulated intraterminal cacium ions and diminishes the neurotransmitter pool via the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) protein, Snap25 (Pollari et al. 2014). In late ALS, the impact of ROS is exacerbated by several factors including extracellular ATP activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the terminal Schwann cells and elevated inflammation due to loss of neuroprotective trophic factors (Pollari et al. 2014). Interestingly, physiological concentrations of nitric oxide, which modulate neurological activity through cyclic guanosine monophosphate synthesis and promote motor neuron survival, may initiate and amplify oxidative damage in ALS by diffusion limited reactions with superoxide to produce peroxynitrite (Drechsel et al. 2012).

1.4.4 Animal Models

The first animal model of ALS was the result of a spontaneous mutation transmitted by a single recessive gene (wr). Termed the Wobbler mouse, it arose from a C57BL/Fa strain and was first described by Falconer (1956). Mice with the wobbler gene display progressive muscular weakness, wasting and degeneration of motor nerve cells in both brain stem and spinal cord (Duchen & Strich 1968). The Wobbler phenotype is linked to a point mutation in the vesicular/vacuolar protein sorting 54 (VPS54) gene and has a neurological phenotype which in some ways resembles ALS (Schmitt-John et al. 2005). The model is argued to be a useful contribution to understanding the complex interactions that take place in ALS because of similarities in molecular pathology (Moser et al. 2013). However, clinical examination suggests that VPS54 is not a candidate disease gene in ALS patients (Meisler et al. 2008) which may limit the usefulness of the Wobbler model for understanding ALS in humans.

In order to study ALS a number of transgenic murine models based on SOD1 mutations have also been developed. These models enable the study of the cellular and molecular basis of the disease and have resulted from the identification of mutations in the SOD1 gene associated with familial ALS (fALS) (Rockenstein et al. 2007). The first model developed for ALS was the transgenic mouse overexpressing mutant SOD1, the G93A. Gurney et al. (1994) developed this mouse model by a substitution of glycine to alanine at position 93 (hence G93A) which resulted in motor neuron disease through gain-of-function mutations in SOD1. Following the development of the G93A a number of other transgenic mouse lines that overexpressed human SOD1 were produced

including the G37R, the G85R, the G86R and the D90A which all showed a similar phenotype to the G93A (Van Den Bosch 2011). To date there are at least 15 transgenic mutant SOD1 mouse models (Turner & Talbot 2008).

The G93A transgenic murine model is the most commonly studied ALS animal model. Mice in this model develop hind limb tremor and weakness leading to eventual paralysis in one or more limbs and premature death by 5 or 6 months (Gurney et al. 1994). Pathological examination showed that at 47 days, 40% of motor end-plates were denervated while at 80 days 60% of ventral root axons were lost. Further, distal axon degeneration preceded microglial and astrocytic activation around motor neurons which is suggestive that this mouse model of ALS may be a distal axonopathy, at least for lumbar motor neurons (Fischer et al. 2004). G93A spinal motor neurons present a number of distinct disease related processes including the aggregation of insoluble protein complexes several months before motor neuron pathology is detectable (Johnston et al. 2000), mitochondrial vacuolation through intermembrane space expansion (Higgins et al. 2003), and impairment of axonal transport in the axon hillock and initial segment (Sasaki et al. 2005).

SOD1 mouse models have been extensively criticized for their failure to lead to successful translational research and pharmacological interventions for human ALS (Benatar 2007). For instance, minocycline, a second-generation tetracycline which inhibits microglial activation, was found to slow disease progression in SOD1 G37R mice (Kritz et al. 2002). Following similar findings in SOD1 mouse models by other

independent research groups, a clinical study was conducted which found that minocycline was an ineffective ALS treatment and further had a harmful effect on patients (Gordon et al. 2007). This and similar clinical failures based on the transgenic ALS models led to criticisms such as most animal studies were underpowered and largely focused on presymptomatic onset of treatment (Benatar 2007). Benatar (2007) also points out that SOD1 mice may only represent an animal model of familial ALS, or perhaps only familial ALS brought on by SOD1 mutations. Indeed, SOD1 mouse models differ from what is seen in humans as there is SOD1 overexpression leading to artificial mitochondrial loading (Bergemalm et al. 2006). There are also many general considerations which are frequently disregarded including pharmacokinetics (higher metabolism in mice), altered corticospinal neuroanatomy between humans and mice, physiology (shortened lifespan in mice), examination of a dose-response relationship and the ability to generalise from a fALS gene to sporadic amyotrophic lateral sclerosis (sALS) (Rothstein 2003; Turner & Talbot 2008; Van Den Bosch & Robberecht 2006).

1.5 Parkinson's Disease

1.5.1 Clinical Features

Parkinson's Disease (PD) was first clinically identified by James Parkinson in 1817 through an essay on shaking palsy (Parkinson 1817; reviewed in Kempster et al. 2007). The next step in understanding PD came in 1919 with the recognition that PD patients have neuron loss in the SN. This was followed much later (1960) with the finding that DA is depleted in the BG of individuals with PD. This in turn led to the commencement of successful treatment of PD with L-3,4-dihydroxyphenylalanine, also called levodopa

(L-DOPA, the immediate precursor to DA), in order to replace the lost DA (Yahr et al. 1969).

There are four cardinal features of PD, these being tremor at rest, rigidity, akinesia (or bradykinesia), and postural instability (Jankovic 2007). The most common among these features is the rest tremor which initially occurs unilaterally, is easily identifiable, and is most prominent in the distal part of an extremity. The rest tremor will normally disappear with action or sleep. Bradykinesia is a slowness of movement and presents as difficulty with preparing and executing commands to move, inability to perform simultaneous tasks, and abnormalities in sensorimotor integration (Berardelli et al. 2001). Bradykinesia has been closely linked to BG dysfunction (Kühn et al. 2009) and as such is often considered a hallmark of such disorders (Jankovic 2007). Rigidity in PD is described as increased passive stiffness in muscles which leads to rigidity in joints. This is commonly quantified by evaluation of stiffness in the wrist and/or elbow (Sepehri et al. 2007). Postural instability normally occurs late in the disease course and most commonly presents as an impaired postural reflex. This abnormal reflex is tested by pulling or pushing a patient by the shoulders and assessing their ability to stop their direction of motion. Postural instability, along with freezing, is the primary cause of falls in PD patients and is identified as the most disabling feature of the disease (Bloem 1992). These four features of PD form the traditional motor symptomatology of the disease; however there are also a number of non-motor characteristics that although often overlooked, are quite common.

The non-motor symptoms of PD can broadly be divided into four groupings: sleep disorders, autonomic dysfunction, cognitive disturbances and sensory abnormalities (Zesiewicz et al. 2006). Sleep disorders in PD patients are immensely detrimental to quality of life. These sleep disturbances take many forms with insomnia, daytime sleepiness with sleep attacks, restless-legs syndrome and REM sleep behaviour disorders being the most common (Schrempf et al. 2014). A second category of non-motor symptoms in PD relates to autonomic dysfunction. These most commonly include orthostatic hypotension, sialorrhea, sexual dysfunction, urinary complications and constipation (Perez-Lloret et al. 2013). Importantly, clinical presentation of symptoms arising from peripheral autonomic neuron degeneration could act as early premotor biomarkers, given their early onset (Palma & Kaufmann 2014), but as yet none have emerged for the PD phenotype.

The non-dementia cognitive disturbances in PD have substantial individual variability, and the severity of these deficits is correlated with education level (Pfeiffer et al. 2014). The most common cognitive impairments from a cohort of early-stage PD patients were deficits in episodic memory, executive function, language, visuospatial processing and attention/working memory (Pfeiffer et al. 2014). As PD progresses into later stages, the cognitive disturbances become more severe potentially leading to dementia, psychosis, depression and apathy (Coelho & Ferreira 2012).

Sensory symptoms such as hyposmia, paresthesia, akathisia and pain are frequently reported by PD patients, and tend to occur early in the disease process (Defazio et al.

2013; Yoshii 2012). The pain associated with PD is thought to follow a bimodal distribution with the first peak before or at the onset of clinical PD and the second peak occurring in conjunction with the development of motor fluctuations or dyskinesia (Yoshii 2012). As with autonomic system dysfunction, the onset of olfactory bulb degeneration antedates motor symptoms and along with changes to other sensory processes is considered a possible early biomarker (Xiao et al. 2014).

1.5.2 Treatment of PD

Despite advances in the understanding of PD, there remains no therapy which targets the underlying neurodegenerative processes (Sprenger & Poewe 2013). The principle pharmacological treatment for PD motor symptoms in the vast majority of clinical cases is, as it has been for nearly 50 years, L-DOPA. There have however been some advances in treatment. For instance, the side effects which arise with L-DOPA induced dyskinesias are increasingly managed through modifications in the doping regime and/or by supplementation with other medications such as monoamine oxidase type B inhibitors, catechol-O-methyltransferase inhibitors or DA agonists (Connolly & Lang 2014).

Non-motor symptoms are prominent in the advanced stages of PD, shorten life span and generally impact quality of life. There are some non-motor symptoms which can be treated with varying degrees of success with existing drugs and these include depression, constipation, pain, genitourinary problems and sleep disorders (Chaudhuri

et al. 2006). However, most non-motor symptoms are refractory in nature and will require the development novel pharmacotherapeutics for treatment.

1.5.3 Pathogenesis of PD

The neuropathological diagnosis of PD, whether in the subclinical or clinical phases, requires the identification of specific inclusions in nerve cells. These filamentous inclusions, while are largely comprised of aggregated α-synuclein protein, develop as spindle- or thread-like Lewy neurites (LNs) in cellular processes or as globular LBs in neuronal perikarya (Spillantini & Goedert 2000). The relationship between Lewy pathology and clinical symptoms is well established, however it remains unknown how these proteinaceous inclusions are initiated in vulnerable cell populations (Luk & Lee 2014). However, strong evidence indicates that once Lewy pathology has become ingrained in a cell, it can propagate to neighbouring cells through a prion-like intercellular transfer of misfolded α-synuclein (Chauhan & Jeans 2015), which suggests a mechanism for PD progression.

The brain pathology of PD, specifically in relation to LB and LN formation, is perhaps best explained by Braak's staging, as described in a seminal paper in 2003 (Braak et al. 2003). In brief, there are 6 stages described for PD pathology. Stages 1 and 2 illustrate a brain pathology which commences in the dorsal IX/X motor nucleus and/or adjoining intermediate reticular zone and follows an ascending course into additional brain stem nuclei eventually reaching the cerebral cortex. Frequently, concurrent with brain stem pathology, lesions also appear in the anterior olfactory nucleus. In stages 3 and 4,

midbrain and prosencephalic lesions emerge with the substantia nigra pars compacta (SNc) being particularly affected. Stages 5 and 6 are predominated by a worsening of damage in subcortical and mesocortical structures previously affected. Further, the number of LBs and LNs gradually decreases in the SN while the number of extraneuronal neuromelanin aggregations increases. By stage 6 there is pathological involvement in nearly the entire neocortex with the final cortical pathology extending into the first order sensory association areas, premotor fields and occasionally even the primary sensory and motor fields.

1.5.4 Animal Models

There are several neurotoxic models of Parkinson's which are used extensively to study the pathogenesis of the disease. The most well known of these are the 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models (for review: Bové & Perier 2012). Ongoing genetic advances have also led to a number of genetic models including Parkin (PARK2), DJ-1 (PARK7), and PINK1 (PARK6); however none of these models demonstrate the typical degeneration of dopaminergic neurons seen in PD (Goldberg et al. 2003; Goldberg et al. 2005; Kitada et al. 2007). This limitation of genetic models has meant that neurotoxic *in vivo* paradigms remain the most common mechanism for studying PD processes (Bové et al. 2005). In contrast to genetic models, toxin induced models replicate most of the pathological and phenotypic features of PD which enables the testing of pharmacologic agents or therapeutic strategies aimed at ameliorating deficits resulting from a nigrostriatal lesion (Blandini & Armentero 2012).

The MPTP neurotoxin model of PD was brought to the fore in the early 1980s by street preparation and injection of 1-methyl-4-phenyl-4-propionpiperidine (MPPP) by drug users in California. The drug users presented with an acute state of akinesia and showed neurological signs very similar to those in PD. The scientific community took a particular interest in determining what compound had led to the effects seen in the drug users, given the PD-like symptomatology, and analysis eventually revealed that MPTP had been accidentally synthesized during the preparation of MPPP (Bové et al. 2005).

MPTP toxicity reproduces in humans and non-human primates the tetrad of Parkinson's features: tremor at rest, bradykinesia, postural instability, and rigidity. These behavioural symptoms are accompanied by cognitive impairments including verbal fluency and executive function deficits in humans and object retrieval detour task errors in monkeys (Stern & Langston 1985; Vezoli et al. 2011). The severe and irreversible symptomatology of MPTP has been shown to be indistinguishable from idiopathic Parkinson's. However, while pathological analysis has identified nigrostriatal degeneration in the MPTP model, there is no evidence of LB inclusions that are typical of sporadic Parkinson's (Forno et al. 1993).

Toxicity to MPTP varies widely among vertebrates with rat dopaminergic neurons being far more resistant than those of primates. Mouse sensitivity is strain dependent with C57BL/6 being identified as highly sensitive (Kitamura et al. 2000). The difference in MPTP vulnerability between rats and mice necessitates MPTP administration by systemic injection in mice and intracerebral injection in rats (Dauer & Przedborski 2003).

After systemic injection MPTP crosses the BBB and is converted in astrocytes to the toxic 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (MAO-B). MPP+ is then released into the extracellular plasma by plasma membrane transporter Oct3 and enters into dopaminergic neurons via the dopamine transporter (DAT) (Kopin 1987). Once in DA neurons MPP+ inhibits complex I of the mitochondria which in turn impairs oxidative phosphorylation and generates ROS (Baunez & Gubellini 2010). The production of ROS leads to apoptosis and results in dopaminergic neuron loss (Gubellini et al. 2010).

MPP+, unlike MPTP, cannot cross the BBB (Kitamura et al. 2000), meaning that once MPTP is converted to MPP+ in the brain, the clearance rate for this toxic compound is low. Interestingly, its formation can be prevented with MAO-B inhibitors which have been shown to prevent the toxic effects of MPTP (Kopin 1987). However, once an acute MPTP insult has occurred, a self-sustained cascade of cellular and molecular events takes place which results in long term consequences (Bové et al. 2005). One hypothesis for this self-perpetuation, as seen in humans, is that it results from peroxidases originating in the insoluble aggregates (LBs). LBs contain a significant amount of bound cytochrome c, and the hypothesis is that it remains functioning as an active peroxidase exerting oxidative damage and preventing repair (Everse & Coates 2009).

Like the MPTP model, 6-OHDA acts on mitochondrial complex I and additionally acts on complex IV, resulting in ROS and dopaminergic cell death, although differences in

bioenergetic dysfunction between the two toxins suggest that cell death mechanisms are at least partially distinct (Giordano et al. 2012). Also like the MPTP model, a major flaw in the 6-OHDA model is the lack of LB formation. Despite this, the 6-OHDA neurotoxin model remains the most extensively used since its initial development over 30 years ago by Mendez & Finn (1975).

While the 6-OHDA toxin can induce permeability changes in the BBB, it poorly crosses the BBB itself (Carvey et al. 2005). Thus, if the desired intent is degeneration of the nigrostriatal DA system, 6-OHDA is administered directly into the brain, often through stereotaxic intraventricular, intracisternal or intracerebral lesions. This administration is typically unilateral as bilateral lesions are frequently terminal with death resulting from a combination of aphagia, adipsia, akinesia and seizures (Dunnett et al. 1981). A striking behavioural result of the unilateral lesion is an asymmetric circling whose magnitude directly correlates with the severity of the nigrostriatal lesion (Von Voigtlander & Moore 1973). The strength and direction of this circling behaviour can be altered by drugs that stimulate dopaminergic receptors (Hefti et al. 1980), and this property of the 6-OHDA model has been used extensively to test new drugs for PD.

1.6 ALS-PDC

1.6.1 Overview

The Western Pacific including Guam and other islands in the Mariana Group has been shown to have an extremely high incidence of neurodegenerative disease (Galasko et al. 2002). The neurological symptoms can resemble classical ALS, parkinsonism

(atypical PD), dementia (atypical AD), or a combination (Hirano et al. 1961). When the symptoms present as a combination the disorder is termed amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC). The term ALS-PDC can also be used to refer to the spectrum of disease which includes disorders, like Kii ALS-PDC, which have similar though not identical pathogenesis and symptomatology. On Guam, ALS-PDC has predominantly affected the indigenous Chamorro population with ALS known locally as lytico and PDC as bodig (Trojanowski et al. 2002). The disorders were most prevalent in the 1940s and 1950s with Guamanian ALS (gALS) incidence around 50-100 times higher than the rest of the world (Plato et al. 2002). From the 1960s onwards there was a rapid decline in incidence of gALS while PDC levels also decreased albeit to a far lesser extent (Waring et al. 2004). During the same period both disorders saw increases in the age of onset by approximately a decade (Wiederholt 1999). This rapid decline in incidence over such a relatively short period of time, along with the increased age of onset, was suggestive of a changing environmental factor to which the native Chamorros were exposed.

While the possibility of a genetic factor linked to the Chamorro genotype cannot be entirely excluded, Yanagihara et al. (1983) showed through epidemiological surveillance that both the islands Guam and Rota had comparable age-adjusted mortality rates of ALS and PD while Saipan had substantially lower mortality rates. All three islands are part of the Mariana Islands and the Chamorros who inhabit them were, at least 30 years ago, genetically indistinguishable, suggesting that an environmental exposure was a determining factor in the high incidence of disease (Yanagihara et al. 1983).

1.6.2 Sterol Glucosides

The environmental factor in question appears to be the ingestion of seeds from the cycad tree (*Cycas micronesica*) which contain a number of neurotoxins (Khabazian et al. 2002) and whose consumption is linked through epidemiological evidence to ALS-PDC (Borenstein et al. 2007; Whiting 1963). The initial research focus was on toxic amino acids such as cycasin, methyl-azoxymethanol β-D-glucoside, β-N-oxalylamino-L-alanine (BOAA), and β-N-methylamino-L-alanine (BMAA) (Ross & Spencer 1987; reviewed in Ly et al. 2007). However, all of these toxins are water-soluble and thus would be removed through the normal cycad washing process as performed by the Chamorro people, and further none reproduce the behavioural and pathological deficits seen in ALS-PDC (Shaw & Wilson 2003). Given the strong evidence pointing to toxins in the cycad tree, researchers then examined washed cycad flour for insoluble neurotoxins which led to three sterol β-D-glucosides being identified: campesterol glucoside (CG), SG, and β-sitosterol-D-3-glucoside (BSSG). Chemically, these neurotoxins belong to the steryl glucoside molecular family which is characterized by a carbohydrate moiety attached to tetracyclic carbon backbone (Ly et al. 2007).

Of the three neurotoxins which were isolated from cycad flour, the most common of the three, CG, was found to be the least toxic while the least common, SG, was the most (Khabazian et al. 2002). Subsequent *in vivo* work showed that mice fed BSSG developed significant motor neuron loss, gliosis, decreased tyrosine hydroxylase (TH) labelling in the striatum and SN and behavioural deficits after 15 weeks (Tabata et al.

2008). Similarly, when BSSG was fed to rats they displayed progressive neurological and behavioural deficits including striatal DA loss, loss of dopaminergic neurons in the SNc and the development of LB-like α-synuclein aggregates (Van Kampen et al. 2014). Deficits were also shown in mice fed SG with marked loss of motor neurons, astrocyte and microglia proliferation, and reduced TH labelling in the striatum and SN (Tabata 2008). These effects were further shown to be sex-dependent for both BSSG and SG exposure with decreased TH labelling in the nigro-striatal pathway, increased apoptosis and gliosis, and lipid accumulation all found in male but not female mice (Banjo 2009).

BSSG and SG can both induce aspects of ALS-PDC but the latter more accurately reflects the correct pathology in mice. Mice fed SG for 15 weeks displayed reduced overall movements and impaired reflexes comparable to BSSG fed mice; however SG fed animals were weaker and less coordinated (Tabata 2008). SG fed animals also exhibited a broader spectrum of motor neuron degeneration, whereas in BSSG neurodegeneration was principally limited to large motor neurons. Importantly, the SG mice showed aggregation of either tau or transactivation response DNA-binding protein with molecular weight 43 kD (TDP-43) (Tabata 2008). ALS-PDC is characterized neuropathologically by intracytoplasmic filamentous tau inclusions (Hirano & Llena 1986), and thus SG's ability to reproduce this feature along with more representative behavioural and motor neuron deficits led to its use over BSSG in the experiments outlined herein.

Interestingly, the oral panax ginseng extract, G115, has been shown to prevent the development of BSSG induced locomotor deficits and reduce dopaminergic cell loss, microgliosis and the accumulation of α-synuclein aggregates (Van Kampen et al. 2014). Further, intracerebroventricular ginseng injections in mice have been shown to inhibit stress induced plasma CORT levels (Kim et al. 1998). Given that panax ginseng has known anti-inflammatory sterol glucoside constituents (Kim et al. 2013), it may be that it acts to interfere with other sterol glucoside pathways, such as the synthesis of CORT from cholesterol (Davies & MacKenzie 2003). These findings suggest that the neuroprotective role of gisenosides in neurodegenerative disease may be a valuable line of future research.

1.6.3 Clinical and Neuropathological Features

Lytico or gALS was in the mid-twentieth century responsible for 1 in 10 Chamorro deaths over the age of 25 while PDC was responsible for another 1 in 10 (Brody 1971). This incidence rate for gALS was more than 50 times the comparable rate in the US and led to substantial academic interest in the disorder.

The clinical features of gALS share similarities with fALS and sALS. All three exhibit the primary symptoms of ALS, namely steadily progressive muscular weakness with atrophy and fasciculations. Spasticity is slightly more frequent in gALS with around 15% cases having a clinical presentation, and the mean age of onset, at 46, is comparable to fALS (at 47) and lower than sALS (ranges from 52-60) (Gibbs Jr. & Gajdusek 1972). Distinguishing neuropathological features of gALS are the frequent appearance of

neurofibrillary changes before the age of 60 and the occasional presentation of granulovacuolar bodies, both of which are rare in sALS and fALS (Gibbs Jr. & Gajdusek 1972).

PDC shares many clinical features with PD and AD. Indeed, PDC patients present with symptoms including tremors, akinesia, rigidity and a shuffling gait (Kurland et al. 1968), which are clinically comparable to those seen in PD. Further, the disease is accompanied by progressive dementia (Hirano et al. 1961) and olfactory dysfunction which is clinically indistinguishable from olfactory deficits seen in PD and AD (Doty et al. 1991). Neuropathologically, PDC presents with alpha-synuclein pathology in 37% of patients, which is often co-localized within neurons harbouring NFTs (Forman et al. 2002). Indeed, both gALS and PDC are characterized by widespread NFTs containing insoluble aggregates of tau which are morphologically and biochemically identical to those found in AD (Trojanowski et al. 2002). A comparative immunohistochemical analysis of the basal ganglia in PDC, PD and AD patients revealed that while the striatal output systems are preserved in all three disorders, there is a severe reduction (greater than 90%) in the number of DA neurons in the lateral and medial portions of the SN in PDC (Goto et al. 1990).

1.6.4 Caveats

An interesting caveat to the cycad-ALS-PDC linkage is that despite widespread cycad seed ingestion by the Guamanian population, many exposed individuals never displayed symptomatology. One explanation may lie in the variability in the toxic

elements found within the cycad trees on different parts of the island (Marler et al. 2005) which could explain some geographic differences in susceptibility. Another possibility is that a combination of genetic predisposition along with additional environmental or lifestyle factors determined individual vulnerability. There is no clear picture of what any additional factors might be; however they may be common to the Pacific region as suggested by other foci of similar disease.

For instance, on Guadeloupe, both PDC (clinically comparable to PDC on Guam) and a progressive supranuclear palsy-like disorder have been correlated with the mitochondrial complex I inhibitor annonacin, which is contained in the fruit and leaves of the *Annona muricata* plant (Lannuzel et al. 2007). This contrasts with the Kii peninsula of Japan where no genetic or environmental cause has been identified for the high incidence of PDC there (Kuzuhara 2011). Interestingly, and unlike other regions where PDC is present, the PDC incidence in Kii rose dramatically in the late twentieth century and largely replaced the formerly high incidence of ALS (Muro disease). Along with this change came a much later disease onset and longer survival (Kuzuhara 2007). In Papua, Indonesia the high prevalence of ALS, which frequently overlaps with parkinsonism and less frequently with dementia-like impairments, has dramatically declined since the 1960s and 70s, though it remains higher than the global average (Okumiya et al. 2014). Similar to the Kii peninsula, while an environmental factor seems likely to mediate the high ALS incidence in Papua, none has been identified. These different foci of disease seem to strengthen the argument for investigations of multi-hit

combinations of environmental and lifestyle factors to better understand neurodegenerative disease risk factors.

1.7 The Multi-Hit Hypothesis

1.7.1 The Multi-Hit Hypothesis

There is wide consensus among PD researchers, that aside from a small proportion of rare genetic mutations, interplay between environmental and genetic factors likely influences the risk of developing PD (Bronstein et al. 2009). This is reinforced by the recognition that there are many determinants within each individual which lead to PD risk (Bronstein et al. 2009) and that a multifactorial model best encompasses all the factors involved in the disease etiology (Taylor et al. 1999). This idea that neurodegenerative disease, whether it be PD, ALS or another disorder, is caused by a complex interaction between genetic predisposition, aging and environmental toxins forms the basis of the dual or multi-hit hypothesis (Boger et al. 2010).

Genetic predisposition as defined in the multi-hit context can take the form of early life events which alter gene regulation or activate epigenetic mechanisms, such as infections, stress and pesticides (Logroscino 2005). These initial hits, in a multi-hit model, are triggers which through altered gene regulation or epigenetic mechanisms lead to secondary effects such as inflammation, mitochondrial malfunction or other processes which promote vulnerability to later life events (Diederich & Parent 2012). In the case of environmental toxins, it is theorized that the early sites for pathogenic invasion, at least in the case of PD, are the olfactory bulb and the enteric plexus of the

stomach – given that these are the earliest sites of LB pathology (Hawkes et al. 2007). Given the marked olfactory deficits present in ALS-PDC (Ahlskog et al. 1998), and the presumed gene-environment interplay of the underlying causes (Lynch et al. 2008), it may be that the olfactory route also forms the basis for an initial hit in its etiology.

Researchers have long argued that in order for animal models of PD and other neurodegenerative disorders to fully recapitulate the pathology seen in humans, more than one risk factor would likely need to be applied (Carvey et al. 2006). There has been a gradual increase in recognition of this need with moves towards developing animal models which combine genetic vulnerabilities with environmental toxins, such as administering MPTP to DJ-1 gene deficient mice (Manning-Boğ et al. 2007). Indeed, there is already evidence which points towards the summative and synergistic effects that multiple hits can have. Ling et al. (2004) showed that in the case of PD rats, prenatal exposure to lipopolysaccharide (LPS) results in increased sensitivity to low dose rotenone infusion. This suggests that a pre-existing inflammatory state can act to facilitate the effects of an environmental neurotoxin. In fact, this use of a prenatal bacterial insult such as LPS has in rodents led to what is now called the endotoxin-induced neuroinflammation model of PD (Tufekci et al. 2011), and offers one line of future multi-hit exploration. However, the domain of possible multi-hit animal models remains largely understudied, despite the wide recognition that it is the way forward for animal models of neurodegenerative disease. This paucity of data, along with lines of evidence suggesting a multifactorial role for restraint stress and aluminum in age-related neurodegenerative disorders, as well as research which has shown that

aluminum and restraint stress can produce epigenetic effects (Alexandrov et al. 2013; Babenko et al. 2012), led to the two multi-hit experiments which were behaviourally examined in this thesis.

1.7.2 SG + Restraint Stress Paradigm

The first multi-hit experiment involved the application of chronic restraint stress to mice first exposed to a progressive neurodegenerative disease-inducing toxin. The toxin to which mice were exposed, SG, is (as described in Section 1.6) known to induce an ALS-PDC phenotype which recapitulates the main features of the human condition seen in Guam. Similar to what was shown by Smith et al. (2008), it was anticipated that the combination of chronic restraint stress with the disease-inducing toxin would lead to a promotion of both the severity and the rate of progression of motor, anxiogenic and cognitive behavioural deficits relative to mice treated with either SG or restraint stress alone.

Thus, mouse behaviour was assessed not only for its motor component but also for anxiety, firstly to confirm that restraint stress mice were indeed displaying elevated levels of stress and secondly to test the hypothesis that mice experiencing chronic restraint stress combined with the SG toxin would display quantifiably higher levels of behavioural stress than mice receiving restraint stress or SG alone. This hypothesis is premised on two points: 1) previous anecdotal observations of motor behaviour in mice following SG toxin administration suggest an increased anxiogenic phenotype (Tabata 2008) and 2) that SG-induced motor deficits would be inherently stressful, and that this

disability stress would be at least partially cumulative when combined with the stress arising from the restraint procedure.

In order to further understand cognition in the toxin induced SG model, the ability of SG to induce cognitive deficits was behaviourally examined. At present, the potential for SG to impair cognition is unknown. However, ALS-PDC possesses a dementia syndrome component, similar to what is seen in AD (Lee 2011). Given this, spatial working memory and spatial reference memory were tested for all treatment groups with the expectation that SG mice would exhibit deficits relative to controls. As chronic stress has been reported to facilitate memory degradation in AD (Carroll et al. 2011), it was anticipated that chronic restraint stress alone would exacerbate memory deficits. As the behavioural test used in this thesis to assess cognitive performance was the appetitively motivated RAM, which assess spatial reference and spatial working memory, it was hypothesized that mice administered chronic restraint stress would specifically display spatial reference but not spatial working memory deficits. In addition, it was predicted that mice receiving both the SG toxin and chronic restraint stress together would show an increase in severity in spatial reference memory deficits beyond what was seen with either SG or restraint stress alone.

1.7.3 SG + Aluminum Paradigm

The second multi-hit experiment involved the application of oral aluminum to mice first given SG. In line with previous research in our lab, SG was expected to produce motor deficits. Chronic oral aluminum chloride exposure has been shown to impair locomotor

performance (Erazi et al. 2011; also see Section 1.3.2), and thus motor deficits were also anticipated for the aluminum treatment group. Further, chronic aluminum chloride, given i.p. in rats, has been shown to enhance the ability of 6-OHDA to cause oxidative stress and neurodegeneration in the DA system (Sánchez-Iglesias et al. 2009). As SG administration, like 6-OHDA, affects the dopaminergic pathways into the striatum, as evidence by decreased TH labeling and microglia proliferation (Tabata 2008), it was hypothesized that the combination of chronic oral aluminum and the SG toxin would lead to an incremental facilitation of both severity and progression of motor deficits.

Anxiety was also behaviourally measured in this experiment. Chronic aluminum administration can lead to both anxiolytic and anxiogenic outcomes (Erazi et al. 2010; Sharma et al. 2013), which appear dependent on rodent age and method of aluminum administration. Here we hypothesize that chronic oral aluminum, when combined with the SG toxin, will not lead to anxiogenic or anxiolytic behaviours beyond what is seen with SG or aluminum alone. Further, it is hypothesized that mice receiving only SG and mice receiving only aluminum (but not the combination) will display an increase in anxiogenic behaviours relative to control mice.

Furthermore, based on aluminum and SG's established effects on memory (Bhalla et al. 2010; Rebai & Djebli 2008; see Sections 1.3.2 and 1.7.2), all treatment groups were behaviourally assessed for memory deficits. Given the predicted deficits for both SG and aluminum treatment groups, it was hypothesized that the combination of aluminum

and the SG toxin would lead to measurable spatial working memory deficits beyond what is seen with aluminum or SG alone.

Chapter 2 Methods

2.1 Animals

All procedures were approved by the University of British Columbia Animal Care Council (Protocol: A09-0863) and conducted in accordance with the regulations of the Canadian Council on Animal Care (CCAC). Humane endpoints were enumerated and mouse phenotypes were recorded such that mice were euthanized if they showed signs of suffering or impaired function to the extent that they could no longer perform in the behavioural tests. Mice were also euthanized if they lost significant body weight (>20%) or if they experienced any illness which resulted in a loss of welfare, such as a loss of appetite, and could not be treated effectively. Motor deficits which caused difficulty in eating or drinking but did not warrant euthanizing led to food and/or transgel (Charles River Laboratories International Inc., Wilmington, MA) being placed on the cage floor as needed. Following commencement of invasive procedures or toxin administration, animals were monitored for the next 48 hours by trained members of our lab. At any point in the experiment when an animal was observed to be suffering or experiencing difficulties which did not meet humane endpoints, regular monitoring and recording (minimum 3 times daily) of the animal's phenotype was performed using a mouse monitoring record as shown in Table 1. A score summarizing the animal's present observable state was achieved following each completion of the mouse monitoring record. If at any point a score of 5 or higher was recorded, the mouse was euthanized immediately.

Table 2-1: Mouse Monitoring Record

Mouse Monitoring Record: Mouse Strain CD-1					
Mouse ID:		**DOB:**		**Sex:**	**M / F**
			Date:		
			Experimenter Initials:		
Score			9:00 AM	1:00 PM	6:00 PM
0	***Nothing Abnormal***				
Body Weight					
1	5-10% loss				
2	10-15% loss				
4	15-20% loss				
5	>20 % loss				
Behaviour/Activity					
2	slow moving, decreased alertness				
2	decreased grooming (greasy/oily coat)				
4	abnormal gait, isolated, hyperactive				
4	decreased activity, fearful				
4	Rash >1cm patch on skin, ruffled, hunched				
5	severe self-mutilation/trauma				
5	immobile, constantly shaking, vocalizations				
5	no response when stimulated				
Clinical Signs					
1	increased respiratory effort, circling				
5	irregular/gasping respirations, labored				
5	paresis/paralysis of hind or all limbs				

All mice used were experimentally naive CD-1 IGS (International Genetic Standardization), 7-7.5 month old retired male breeders from Jackson Laboratory (Bar Harbor, ME). CD-1 mice were selected since previous research in our lab has examined both cycad toxicity and the ALS-PDC mouse model in this strain (Tabata et al. 2008; Wilson et al. 2002). Male mice were chosen to reduce estrogen related complications that arise with behavioural testing in female mice, particularly in the CD-1 strain (LaBuda et al. 2002). Estrogen also alters dendritic morphology in the medial prefrontal cortex in stress paradigms as shown with chronic restraint stress in rats (Garrett & Wellman 2009) suggesting that results from one sex would not be comparable to the other. The use of middle age adult mice (~7.5 months) was intended to better reflect actual ALS-PDC onset in Guam as well as onset times of other neurological diseases, which typically occur in mid-life but can arise much earlier (Plato et al. 2003). Further, specific to the restraint stress experiment, the CD-1 mouse has been used to demonstrate the potential for restraint stress to enhance the effects of ingested toxins (Colomina et al. 1999). This latter point suggests that the CD-1 mouse is a viable vehicle for examining stress effects on a dietary toxin induced disease phenotype.
A total of 61 mice were used which were randomly divided amongst 6 groups: 11 control, 10 SG, 10 restraint stress, 10 SG + restraint stress, 10 aluminum and 10 SG + aluminum. The control and SG groups were common to both the restraint stress and aluminum paradigms for analysis purposes; however they did not undergo any additional procedures beyond what they would have as part of one experimental protocol alone. Mice were given a one-week rest period to acclimatise to the new environment upon arrival. Mouse body weights at first weighing ranged from

approximately 35 to 45g. Mice were individually housed at the Jack Bell Research Centre (Vancouver, BC) with a 12 hour light:dark cycle with lights on at 0600hrs. The ambient colony room temperature was 22 +/- 1°C, which while substantially cooler than recommended for individually housed adult CD-1 mice (Gordon et al. 1998), was maintained constant throughout. The mouse home cages contained a small plastic object under which they could hide/nest, material from which to build a nest and corn cob bedding with a depth of approximately 2cm. Food (Purina Rodent Chow, Purina Mills LLC, Gray Summit, MO) and water were available *ad libitum* except prior to the radial arm maze (RAM) test as described below. Most behavioural testing was conducted in the evening during the colony room dark phase, which since mice are nocturnal, is their active phase. Some tests such as the light-dark box test (LDT) were however conducted during colony room light phase due to resource constraints. This is not expected to significantly impact results since recent research in mice suggests that light phase testing can yield results as robust as those obtained in the dark phase (Yang et al. 2008). Also, the behaviour room in which all testing occurred was continuously lit with white light during all experiments for proper camera operation.

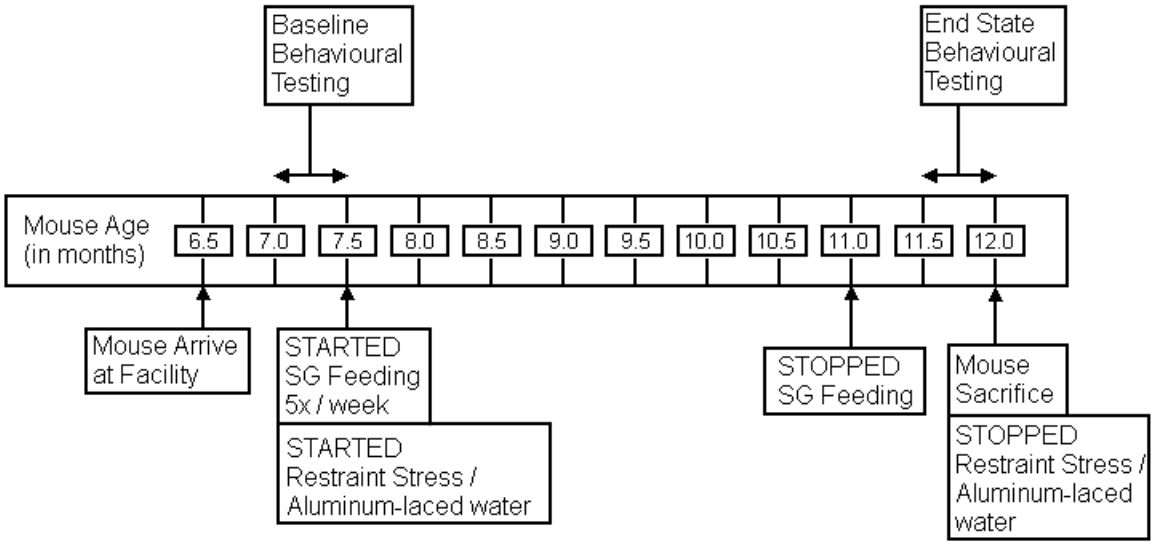
All mice were weighed every second week in a clean, plastic container placed on top of a scale. Weighing was performed following behavioural testing to minimize the influence on scored behaviours of experimenter induced handling stress.

2.2 SG Feeding

SG was procured from Neurodyn Inc. (Charlottetown, PE) and mixed with ground Purina rodent chow to create mouse pellets weighing 1g and containing 3mg of SG. In order to form the mouse pellets double distilled water (ddH_2O) was added to lightly dampen the mixture. The amount of SG was chosen based on previous research in our lab which has utilized 42mg of steryl glucoside per kilogram of body weight per day over a 52 week period. The timeframe for the *in vivo* portion of this study was intended to be much shorter with only 14 weeks of feeding, thus a higher quantity of SG was used. For a 40g mouse, 3mg of SG equates to 75mg of steryl glucoside per kilogram body weight. The lethality of this higher SG quantity given as part of daily food intake was not a concern as higher amounts have being used in previous unpublished lab work.

SG feeding began approximately 5 weeks after mouse arrival allowing time for mouse acclimatisation and baseline behavioural measurements to be conducted. Once SG feeding commenced, it occurred 5 times per week for 14 weeks with the SG, SG + aluminum and SG + restraint stress groups receiving pellets. There was an additional 5 weeks post-feeding to conduct final observations and behavioural testing prior to sacrifice. Visual verification was used to confirm that all SG pellets were consumed each day. Figure 2-1 depicts the experimental timeline for the restraint stress and aluminum paradigms.

Figure 2-1: Experimental Timeline for both Restraint Stress and Aluminum Paradigms



2.3 Restraint Stress Procedure

The chronic restraint stress paradigm and variants thereof have been used extensively to reveal the effects of chronic stress on the murine brain (McLaughlin et al. 2007). This experiment sought to use the restraint stress procedure in conjunction with a neurodegenerative disease inducing environmental toxin to examine the resulting effects on behavioural disease outcomes.

Mice in stress groups (restraint stress, SG + restraint stress) were placed in 50mL conical tubes on a flat surface in an illuminated room with their tail extending out the back of the tube through a hole cut in the tube cap. The tip of the conical tube was cut off to allow for easier breathing. The restraint duration was kept constant at 1 hour; however the time of day and the interval between restraint sessions was not in order to avoid habituation, a concern with repeated restraint (Grissom et al. 2008). The mice were placed in restraint stress approximately twice a week for 18 weeks starting in the same week as SG feeding and lasting for 4 weeks after it had ceased. The conical tubes were washed with Alconox powdered detergent (Structure Probe Inc., West Chester, PA) after each use. The tubes were also marked with numbers such that a given mouse was always restrained in the same tube.

Struggling during restraint has been found to vary according to prior stress experience with repeated restraint leading to habituation and prior repeated swim facilitating struggling behaviour (Grissom et al. 2008). This suggests that a number of factors mediate struggling response, including not only experience but also health, social

contact, and environment. The possibility that deteriorating health in toxin fed mice might alter struggling behaviour relative to controls led to the restraint stress procedure being recorded for mobility scoring. However, due to mouse weight gains in the latter half of the experiment, 50mL conical tubes were replaced for a number of mice with 75mL tubes. This resulted in substantial changes in the average mobility scores across testing sessions making comparisons using this metric across testing sessions and between groups difficult, and for this reason they are not reported.

2.4 Aluminum-laced Water

Auminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON), was dissolved in ddH_2O and placed in the mouse cage bottles of aluminum and SG + aluminum groups at 40mg/L. The mouse cage water was available *ad libitum* and changed weekly as with non-aluminum groups. Aluminum-laced water began approximately 5 weeks after mouse arrival (starting the same day as SG feeding). Aluminum-laced water was given until sacrifice which resulted in chronic exposure over approximately 4.5 months. Visual verification was used to confirm that mice drank the aluminum-laced water. Previous research has shown that rats will drink aluminum chloride laced water at normal consumption levels under chronic exposure conditions (Walton 2007) and thus it was expected that mouse water intake would be unaffected by the metal additive.

The choice of 40mg/L $AlCl_3 \cdot 6H_2O$ concentration was based on comparable concentrations used by Walton (2007) and resulted in an estimated average aluminum

intake per mouse of 1.03mg aluminum/kg bodyweight/day. Further, this amount is comparable to aluminum intake levels used by Walton (2009b) at which behavioural deficits were observed.

2.5 Behavioural Tests and Data Collection

2.5.1 Wire Hang Test

The wire hang test (WHT) is used to assess mouse grip strength and endurance, often in neurological disease models that involve motor irregularities (McDonald et al. 2001; Petrik et al. 2007). The procedure used was performed as described previously (Karl et al. 2003; Lee et al. 2009), with mice being placed right side up on a wire mesh which was then inverted over a pail at a height of approximately 60cm. The WHT apparatus was a circular wire mesh platform which could readily be inverted by means of rods attached at the polar ends. The latency for the mice to fall was measured up to a maximum time of 60 seconds and timing began once the mouse was fully inverted. The mouse was given a second attempt if it fell or jumped off during the first wire hang with the better of the two times recorded. Although the test was conducted every second week, approximately 10% of the mice appeared to habituate to the test by jumping off the wire mesh within seconds of the test starting. The data from these mice was still included as there was no discernible way of ascertaining whether the jumping behaviour was related to neuromuscular strength deficits since the jumping mice were spread across experimental groups. The wire mesh was cleaned with 70% ethanol (Vancouver General Hospital, Vancouver, BC) after each mouse.

2.5.2 Open Field Test

The open field test (OFT) is used to evaluate both spontaneous locomotor activity (van Gaalen & Steckler 2000) and stress-related responses (Choleris et al. 2001). Stress related responses are assessed by measuring time spent in the perimeter of the field (thigmotaxis or wall-following behaviour) versus the centre. This measure of the stress response has been shown to be independent of any effect on locomotion (Choleris et al. 2001).

The OFT apparatus consisted of a flat, circular arena with a diameter of approximately 1m. The arena walls were approximately 50cm in height which prevented both escape and the influence of room spatial cues on mouse behaviour. The floor and walls of the arena were blackened to provide better contrast with the albino CD-1 mice and thereby enhance camera motion detection. The testing session duration was 5 minutes which was considered sufficient to permit an assessment of motor function and anxiety-like behaviours (Prut & Belzung 2003). The test was started by placing mice into the centre of the arena and starting the camera. Room lighting was adjusted to minimize glare and to ensure an even distribution of the light intensity throughout the test area. After each testing session the arena was cleaned with 70% ethanol.

2.5.3 Radial Arm Maze

The RAM is a test for spatial reference and working memory that has been validated in mice (Hiraga & Iwasaki 1983; Pick & Yanai 1983) and is suitable for repeated measures (Hodges 1996). The test involves a mouse being placed on a platform at the centre of 8

arms radiating outward each 45° offset from the two arms closest to it. 4 of the arms are baited and prior to testing the mouse is habituated both to the maze and the location of the food pellets. The test is stopped when all of the baits have been 'found' or when 10 minutes have passed.

The maze was constructed of plexiglass and entirely enclosed with removable lids. The arm dimensions were approximately 40 x 5.5 x 7cm (length x width x height) and the diameter of the central platform was approximately 20cm. The entire maze was elevated off the ground by an attached plastic pillar roughly 50cm in height in order to decrease the distance between the ceiling mounted charge-coupled device (CCD) camera and the maze. At the end of each maze arm there was a slight cavity in the plexiglass floor with a depth of 1cm for placement of the 20mg dustless sugar reward pellet (Bio-Serv, Frenchtown, NJ). Placing the pellet in the cavity alleviated the problem of the mouse visually identifying the pellet and thus ignoring spatial cues.

The test was started by placing the mouse in the centre of the maze, replacing the lid, and commencing recording. The night prior to each test, whether for habituation or for a scored test, mice were given tiny fragments of the sugar reward pellets to ensure that they would still readily eat the pellets and to re-familiarize them with the taste. The pellets fragments were always eaten prior to testing the following day. Further, to ensure mice were motivated to retrieve the food pellets, all food was removed from the mouse cages 12-16 hours prior to testing. The RAM habituation phase consisted of 3 habituation testing sessions of 10 minutes in duration in the partially baited radial maze;

these occurred prior to baseline testing. During the testing phase testing sessions were also 10 minutes in duration but were terminated earlier if the mouse found all four sugar pellets. Note that for the mouse to find the pellets it only had to explore the end portion of the arm where the pellet cavity was located, it did not need to consume the pellet. Colourful posters were deliberate placed on walls near the maze and distinctive room objects proximal to the maze were always returned to the same positions prior to testing as rodents have been shown to use extra-maze rather than intra-maze cues for spatial orientation in the RAM (Olton & Samuelson 1976). The maze was thoroughly cleaned with 70% ethanol after each use.

The types of spatial memory that the RAM design allows to be quantitatively assessed are working memory and reference memory (Wenk 2004). Working memory is measured by counting the total number of repeat entries (entries beyond the first) into all maze arms on during a testing session. A sufficiently motivated mouse seeking to find the food rewards attempts to find the food pellets with the least number of arm entries. Thus repeated arm entries are treated as short-term or working memory errors. Reference memory is measured by counting the total number of entries into non-baited arms. Mice sufficiently habituated to the maze will recall which arms are baited and optimize their arm entry strategy to collect them as rapidly as possible. Thus entries into non-baited arms are treated as longer-term memory recall errors.

2.5.4 Light-Dark Box

The LDT is used to assess rodent anxiety. Mice initially placed in the dark chamber delay entry into the light area (Costall et al. 1989), and this latency is reported as a measure of mouse stress. Conversely, anxiolytic behaviour results in a decrease in this latency to enter the light area as well as an increase in the time spent in the illuminated area (Chaouloff et al. 1997). The inclusion of the LDT allowed for a novel assessment of behavioural anxiety which can act as a proxy measure for underlying disease.

The light-dark box was made of transparent plexiglass on the 'light' side and black plexiglass on the 'dark' side. The dimensions of the light side were 30 x 30 x 21cm (length x width x height) and for the dark side were 15 x 30 x 21cm (length x width x height). Thus the light area was twice the size of the dark area which is consistent with the original light-dark behavioural test proposed by Crawley & Goodwin (1980). The opening connecting the light and dark areas was 7 x 7cm. The dark area was enclosed with a lid while the light area was not.

The light area of the apparatus was brightened beyond normal ambient room light by means of an additional ceiling mounted 100W incandescent bulb with light focused directly at the light area. This was necessary to ensure that the light area was sufficiently aversive for the mice and to create a differential between the two chambers as described by Bourin & Hascoët (2003). The test was started with the mouse being placed in the dark area and the CCD camera simultaneously started. The test duration

was 5 minutes after which the apparatus was cleaned with 70% ethanol prior to the next use.

1	

2.5.5 Elevated Plus Maze

The elevated plus maze (EPM) is, like the light-dark box, a task which examines innate anxiety. The tests themselves do not capture the same aspects of anxiety (van Gaalen & Steckler 2000) and therefore incorporating both will enable a better parsing of stressor related effects. The EPM uses a mouse's natural aversion to high, exposed space to test anxiety (Belzung & Griebel 2001). As with the light-dark box, time spent in the illuminated area and entries into the light area are considered key measures of anxiety.

The EPM consisted of 4 arms of dimensions 40 x 5.5 x 7cm (length x width x height) which radiated out along cardinal points from a central platform of diameter 20cm. Two of the arms were made from opaque, black plexiglass and were enclosed except for an opening into the central platform. The other two arms consisted solely of the bottom portion of the arms with no walls or top covering, and were made of a transparent plexiglass. The EPM was illuminated above ambient lighting conditions by means of a ceiling mounted incandescent light. The testing session, which was 5 minutes in duration, was started by placing the test mouse in the centre of the EPM and starting recording. The apparatus was cleaned with 70% ethanol following each use.

2.5.6 Gait Pattern Analysis

Gait anomalies are a recognized behavioural feature of cycad toxicity in both rats and mice (Shen et al. 2010; Wilson et al. 2005). Mouse gait patterns were assessed through use of a transparent, enclosed, automated treadmill which records mouse gait dynamics, kinematics and posture via a camera placed below the illuminated and transparent moving walkway (Mouse Specifics Inc., Boston, MA). The use of an automated treadmill has been shown as a means of capturing early functional impairments in motor neuron disease (Wooley et al. 2005) symptoms and pathology of which can also be seen in ALS-PDC (Kaji et al. 2012). Gait videos were captured at 150 frames/s for 3 to 4s. Treadmill speed was adjustable and was initially set at 20cm/s based on previous laboratory observations in this mouse model. However, as individual mouse performance deteriorated, whether due to disease, weight or other considerations, the speed was lowered to 10 cm/s. Any mice that were unable to perform at 10 cm/s were omitted from the test as it is difficult to obtain consistent gait patterns at slower treadmill speeds (Wooley et al. 2005). As part of the gait analysis and prior to the behaviours being scored it is necessary for the experimenter to visually inspect each video and ensure that the software is correctly identifying each paw print as the correct paw. In the advent of software paw attribution errors the experimenter must attempt to edit which paw the software is recognizing, adding some subjectivity into the data. The gait pattern analysis (GPA) was performed with DigiGait 9.9 software (Mouse Specifics Inc., Boston, MA) which scores a large number of behaviours. Differences in treadmill speed were automatically accounted for when the behaviours

were computed by the DigiGait software. The apparatus was cleaned with 70% ethanol after each use.

2.5.7 Forced Swim Test

The forced swim test (FST) has been shown as a behavioural assessment tool to measure depression in rodents, particularly during the first 5 to 10 minutes of the test (Kitada et al. 1981). Depression is known to be closely linked to stress both in humans and rodents. In order to better understand behavioural changes arising from disease-related stress, the FST was included.

The FST involves a mouse being placed in a container of water for a fixed period of time. The edge of the cylinder must be high enough that the mouse cannot climb out, and the water deep enough that the mouse cannot balance its tail on the bottom (Crawley 2000). Mice will initially actively seek to escape the swim chamber by pawing at the walls and engaging in exploratory swimming. After a period of time most mice will cease swimming and float, conserving energy. This time the mouse spends in immobility, often called learned helplessness, is reduced by anti-depressants and is the key metric being measured (Yan et al. 2010).

The test apparatus consisted of transparent plexiglass cylinders which measured 30cm in height and 20cm in diameter. The cylinders were filled with tap water to a height of 20cm with a temperature of 25 +/- 1°C as measured by thermometer. Mice were then placed in the middle of the water filled cylinder and their behaviours recorded for 10

minutes. Mice were monitored by an experimenter throughout. Cylinders were cleaned with Alconox powdered detergent and the water was changed after each use.

2.6 Behavioural Scoring

Behaviours were recorded using an overhead CCD camera. Camera data was analysed using Ethovision 3.1 software (Noldus Information Technology Inc., Leesburg, VA) through automated assessment of behaviours. Automated scoring is normally reported as being more accurate than observer scoring, such as in the case of mouse mobility measurements in the FST (De Pablo et al. 1989). Automated scoring utilizes algorithms to analyze pixel changes from frame to frame and thus quantify the changes according to the behaviour that most appropriately describes it (Spink et al. 2001). Pre-defined behavioural variables as well as variables defining specific regions within each apparatus were used for the tests described. The behavioural variables were selected *a priori* based on previous observations and findings in this mouse model (Cruz-Aguado et al. 2006; Tabata et al. 2008) and the mouse ethogram (Grant & Mackintosh 1963).

2.7 Behavioural Testing Schedule

The behavioural testing schedule was intense due to the large battery of tests being conducted. In order to minimize habituation, behavioural tests were run at regular intervals of 2 to 3 weeks with the frequencies of each test given in Table 2. Although mice were not repeatedly tested using the same behavioural paradigm back to back, they were tested numerous days each week with different tests, raising the spectre of test fatigue. This risk could not be entirely eliminated, although it was mitigated by

ensuring that mice were always given 2 to 3 days of rest each week. The order of tests was also considered with subsequent behavioural testing not occurring for 24 hours after restraint stress or the FST.

Water immersion, such as the FST, is inherently stressful to rodents (Moore et al. 2012) and could potentially impact mouse behaviour on tests conducted a week later (Andreatini & Bacellar 1999). Further, restraint stress results in acute stress, which necessitated that it be followed by rest block in which no behavioural testing occurred. The choice of a 24 hour rest period was intended as an experimental compromise which ensured that the intermediate stress response, which begins around 30 minutes after a stress event and lasts for hours (Dallman 2000), had terminated, but which fell well short of a 1 week rest period following a FST. While desirable, a 1 week rest period was not experimentally feasible, and thus it was anticipated that there would be a degree of background ‘noise’ due to above average stress levels.

Table 2-2: Frequency of Behavioural Testing and Data Collection

Frequency	Behavioural Test or Data Collected
Twice Weekly	Restraint Stress
Every 2 Weeks	Wire hang test, light-dark box, weighing
Every 3 Weeks	Open field test, radial arm maze, elevated plus maze, gait pattern analysis, forced swim test

2.8 Analysis

All statistical analysis has been performed using SigmaStat v3.5 and all graphs generated with SigmaPlot v10.0 (Systat Software, Inc., San Jose, CA). Data is presented as means +/- standard error of the means. Behavioural data for all tests was analyzed using 2-way repeated measures ANOVA with Testing Session as the repeated factor. This allowed an examination of main effects, their interaction, pairwise comparisons and post hoc analysis as appropriate. Post hoc comparisons consisted of Tukey's Honestly Significant Difference (HSD) test. Additionally, t-tests were conducted both within and between groups to explore significant main effects. For a difference to be accepted as statistically significant and greater than would be expected by chance alone, the level of significance (α) was set at 5%. This level of significance is considered to set an appropriate balance between false positives and false negatives for this type of behavioural data and has been published by researchers from our lab on ALS-PDC data (Lee et al. 2009; Tabata et al. 2008; Wilson et al. 2002). Levels of significance of 1% and 0.1% are also indicated. Non-significant findings, when reported, are expressed as exact values. With respect to rounding, in instances where a level of achieved significance ended in a 5, it was rounded upwards as this was the more conservative approach.

For all tests, homogeneity and equal variance were assessed to determine if the data distribution was parametric. When data failed the requirements for parametric statistics the non-parametric Kruskal-Wallis ANOVA on ranks was used. This was followed by post hoc comparisons using the non-parametric Wilcoxon rank-sum test and where

appropriate paired tests using the Wilcoxon signed-rank test. Over the course of the experiment there was some shrinkage of the experimental groups due to mortality and disease-necessitated euthanasia (most were due to severe dermatitis and/or tumours). None of the animals were euthanized because of experimental endpoints. Tables 3 and 4 reflect the number of mice that were used for behavioural statistics at sacrifice as compared to baseline for both the restraint stress and aluminum experiments. Note that the control and SG groups are common to both experiments. The Statistical Consulting and Research Laboratory (SCARL) at the University of British Columbia provided advice on the statistical analysis.

Table 2-3: Number of Mice per Group in Restraint Stress Experiment

Group	Baseline	Mid-point	Sacrifice
Control	11	11	9
SG	10	10	9
Restraint Stress	10	10	9
SG + Restraint Stress	10	9	8
	41	40	35

Table 2-4: Number of Mice per Group in Aluminum Experiment

Group	Baseline	Mid-point	Sacrifice
Control	11	11	9
SG	10	10	9
Aluminum	10	9	9
SG + Aluminum	10	9	8
	41	39	35

Chapter 3 Results

3.1 Wire Hang Test

3.1.1 Restraint Stress – Motor Findings

The WHT assessed motor differences in neuromuscular strength as measured by the latency for a mouse to fall from the wire mesh. An analysis of the latency to fall data failed to reveal any significant between or within group differences (Figure 3-1).

The main effect of Testing Session was significant for the WHT, ($F(8, 275) = 2.90$, $p < .01$), while both the main effect of Treatment ($F(3,37) = 1.14$, $p = .35$) and the factor interaction ($F(24, 275) = 1.05$, $p = .40$), were not. Pairwise comparisons failed to identify significant findings.

3.1.2 Aluminum – Motor Findings

There were no significant treatment differences in neuromuscular strength as measured by the latency to fall on the WHT (Figure 3-2). There was a significant interaction ($F(24, 274) = 1.75$, $p < .05$) and main effect of Testing Session ($F(8, 274) = 3.41$, $p < .001$) which led to post hoc analyses. These analyses revealed no significant within or between treatment group differences. There was no main effect of treatment ($F(3, 37) = .51$, $p = .68$).

Figure 3-1: Wire Hang Test – Restraint Stress

There were no significant between or within group differences in neuromuscular grip strength as measured by the latency to fall from the wire mesh. This was partly due to high within group variability. [Error bars represent SEM.]

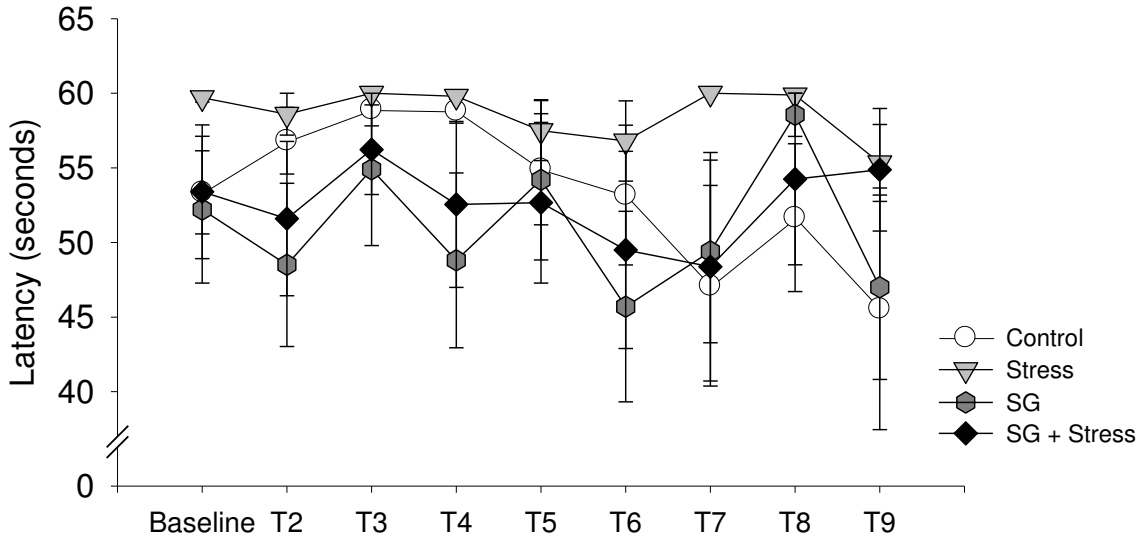
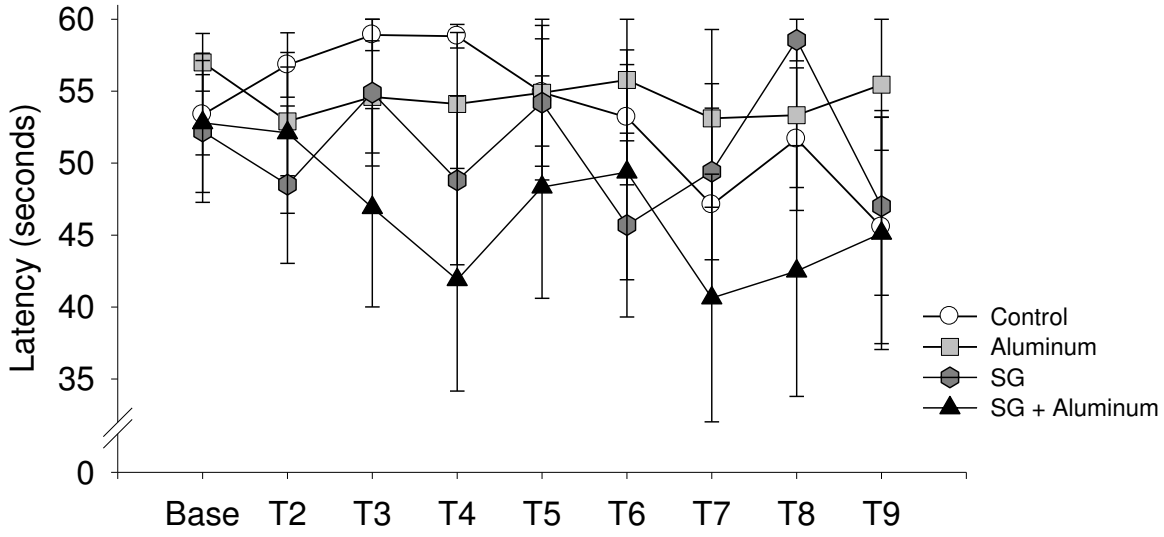


Figure 3-2: Wire Hang Test - Aluminum

There were no significant differences between treatment groups for grip strength as measured by the latency to fall from the wire hang apparatus. [Error bars represent SEM.]



3.2 Open Field Test

3.2.1 Restraint Stress – Motor Findings

The OFT was used to assess mouse locomotor activity by quantifying the amount of time spent in horizontal movement. Analysis of the results revealed a significant reduction in locomotor activity for the SG group relative to its baseline activity (Figure 3-3).

There was a significant main effect of Testing Session for horizontal movement in the OFT ($F(6, 206) = 7.55$, $p < .001$), which pairwise comparisons revealed to be in part explained by a significant reduction in movement by the SG treatment group ($q(7) = 4.45$, $p < .05$) from baseline. This reduced horizontal movement seen with the SG group relative to its baseline activity was not found in the SG + stress group ($q(7) = 2.10$, $p = .76$). There was no interaction effect ($F(18, 206) = 1.23$, $p = .24$) or main effect for Treatment ($F(3, 37) = .91$, $p = .45$) for the horizontal movement measure.

3.2.2 Restraint Stress – Anxiety Findings

The OFT measured anxiogenic behaviour through time spent in thigmotaxis, or by its corollary, time spent in the centre of the field. Analysis of OFT time in centre showed that the stress group had a significant increase across testing sessions in time spent in the centre of the open field. This led to the stress group having a trend higher time in the centre than the control group on the last testing session. Interestingly, the SG + stress group did not exhibit a similar increase in time in the centre of the field,

suggesting that the SG toxin has suppressed a stress mediated increase in anxiolytic behaviour (Figure 3-4).

A statistical examination of the time spent in the centre of the OFT revealed a significant interaction effect ($F(18, 206) = 1.71$, $p < .05$) as well as a significant main effect of Testing Session ($F(6, 206) = 2.54$, $p < .05$). These significant effects were followed with pairwise comparisons which showed that the stress group had a significant increase across testing sessions in time spent in the centre of the open field ($q(7) = 5.25$, $p < .01$). There was no main effect of Treatment ($F(3, 37) = 1.06$, $p = .38$). As an increased time in the centre of the OFT is suggestive of reduced anxiety, and given that only the stress group but not the SG + stress group displayed this increase, it may be that SG suppressed the anxiolytic effect seen with restraint stress alone.

3.2.3 Aluminum – Motor Findings

In rats it has been shown that aluminum, given i.p. as aluminum chloride, results in significant reductions in locomotor and exploratory behaviour (Abdel-Aal et al. 2011b). Dietary intake of aluminum has also been shown to reduce motor activity in mice (Golub et al. 1989). This suggests that aluminum treated mice should display horizontal movement deficits in the OFT, as this was used as a proxy measure for locomotor and exploratory behaviour. Indeed, it was found that the aluminum treatment group, as well as the SG treatment group, displayed significant decreases in horizontal movement (Figure 3-5). While the SG + aluminum treatment group also demonstrated decreased horizontal movement across testing sessions, this decrease was not significant,

suggesting a possible ameliorating effect on locomotion for the multi-hit combination. This latter point, that the SG + aluminum group did not have a comparable decrease in horizontal movement to what was seen with aluminum alone, evinces that gait anomalies such as the increased ataxia described in the GPA results (Section 3.6) are unlikely to wholly explain the reduced horizontal movement for the aluminum group.

All treatment groups showed decreases in horizontal movement from baseline behavioural testing to end state. The decreases largely paralleled one another leading to no Treatment differences ($F(3, 37) = .23$, $p = .88$). However, there was a main effect of Testing Session ($F(6, 205) = 15.22$, $p < .001$) and post hoc analysis revealed that both the aluminum and SG groups had significant decreases in time spent in horizontal movement from baseline to the last testing session (aluminum group: $q(7) = 5.78$, $p < .001$; SG group $q(7) = 4.46$, $p < .05$). There was no significant decrease from baseline to the last testing session for either the control group or the SG + aluminum group. There was no interaction of the factors ($F(18, 205) = 1.41$, $p = .13$).

3.2.4 Aluminum – Anxiety Findings

An examination of the OFT anxiety results revealed a trend decrease in time spent in the centre of the open field for the aluminum group (Figure 3-6).

Analysis of the duration spent in the centre of the open field revealed both a significant interaction ($F(18, 205) = 3.01$, $p < .001$) and a significant main effect of Testing Session ($F(6, 205) = 4.03$, $p < .001$), while the main effect of Treatment was not significant ($F(3,$

37) = .05, p = .98). Pairwise comparisons using Tukey's HSD showed that the aluminum group had a trend towards a decrease from baseline to the last testing session (q(7) = 3.93, p = .08). Given the possibility that this trend towards a decrease from baseline to the last testing session may have been an artefact of a difference between baseline levels of time spent in the centre of the OFT, treatment groups were compared at baseline. The difference between the aluminum group and the control group at baseline was found to be non-significant (t(19) = 1.66, p = .11) and therefore baseline differences do not explain the trend decrease for the aluminum group across testing sessions.

Figure 3-3: Open Field Test Horizontal Movement – Restraint Stress

The SG group displayed a significant decrease in locomotor activity across testing sessions. This decrease was not seen in any of the other treatment groups. [Error bars represent SEM; * = $p < .05$]

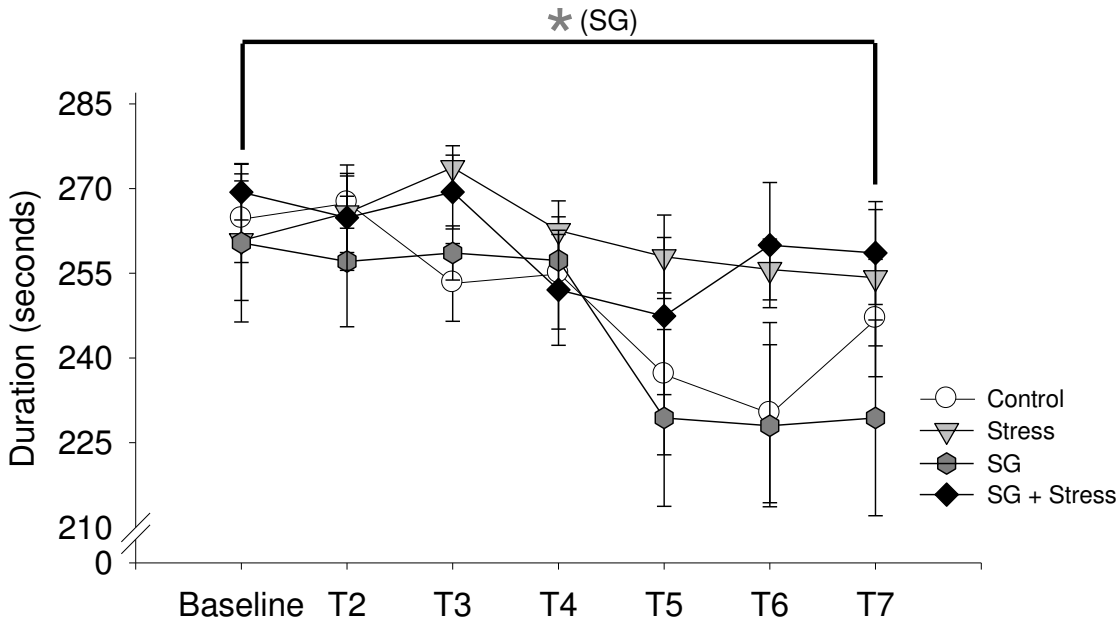


Figure 3-4: Open Field Test Duration in Centre – Restraint Stress

The stress group displayed a significant increase across testing sessions in time spent in the centre of the open field relative to baseline. Of note, the SG + stress group did not display a similar increase in time in the centre of the open field, suggesting that the SG toxin suppressed the increase seen with stress alone. [Error bars represent SEM; ** = $p < .01$]

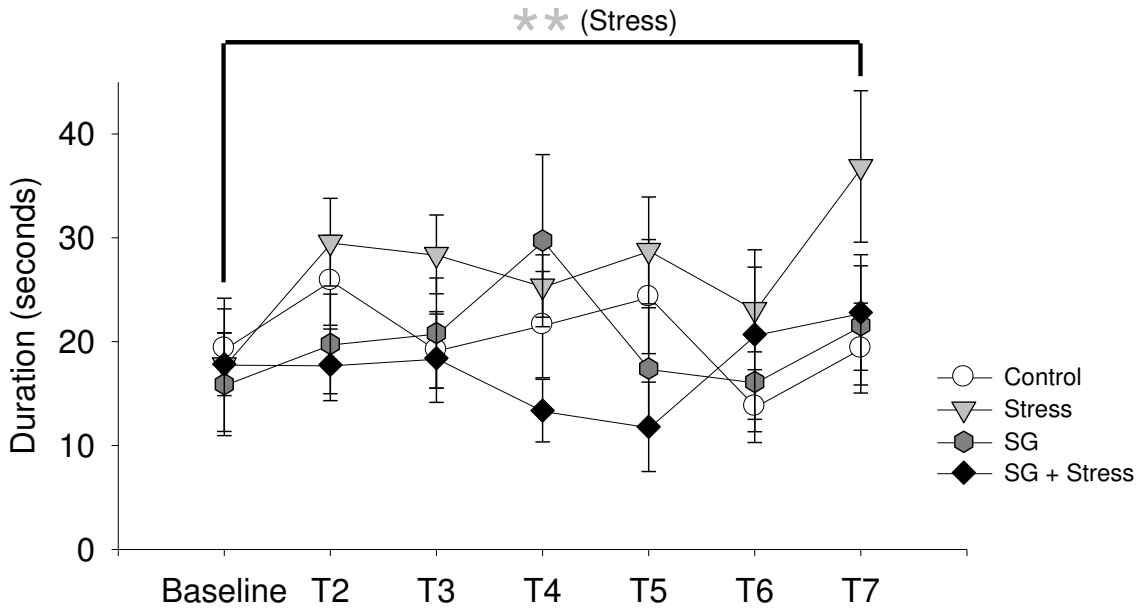


Figure 3-5: Open Field Test Horizontal Movement - Aluminum

All groups showed decreases in horizontal movement from baseline. However, only for the aluminum group and the SG group was this decrease significant to the last testing session. [Error bars represent SEM; * = $p < .05$, *** = $p < .001$]

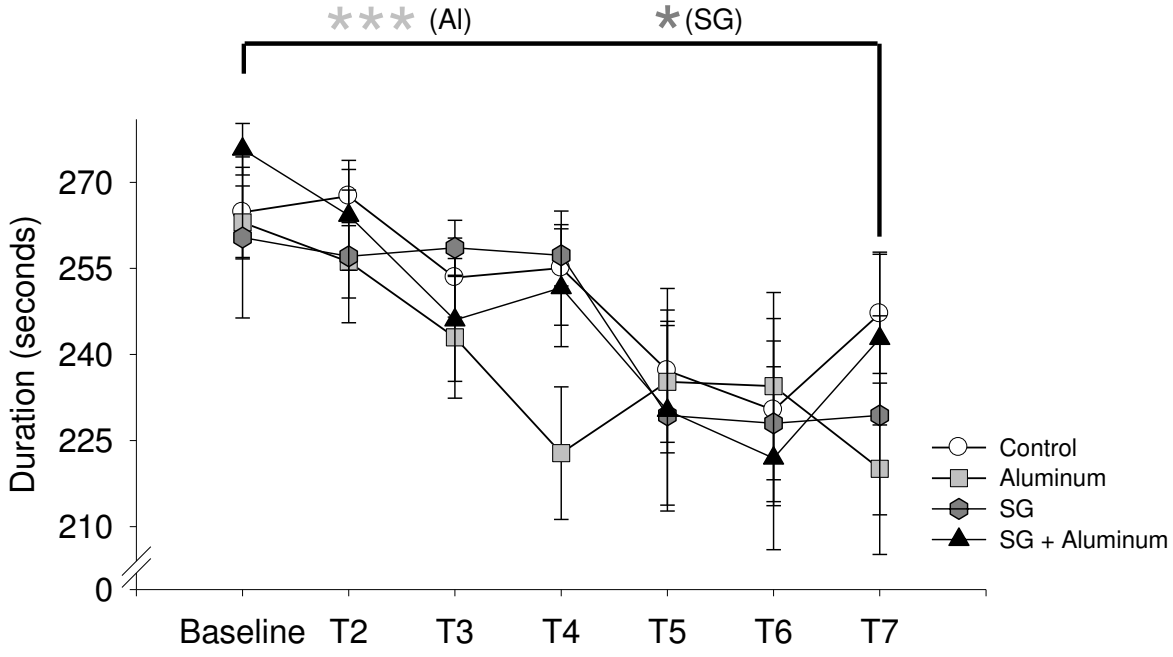
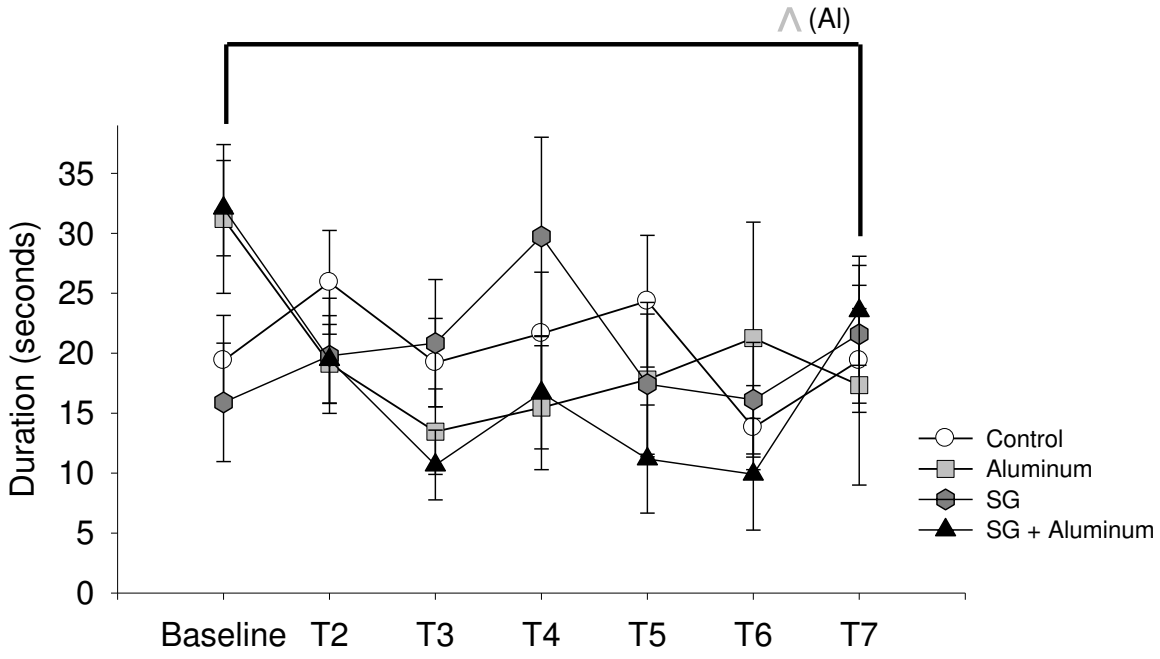


Figure 3-6: Open Field Test Duration in Centre - Aluminum

There was a trend towards a decrease in time spent in the centre of the open field for the aluminum treatment group. [Error bars represent SEM; ^ = .05 ≤ p < .10]



3.3 Radial Arm Maze

3.3.1 Restraint Stress – Memory Findings

An examination of working memory in the RAM uncovered no significant within group differences, however the SG + stress group had significantly more errors than the stress group on the last testing session (Figure 3-7). Similarly for reference memory, there were no significant within group differences, however on the last testing session the SG + stress group had significantly more errors than the stress group (Figure 3-8). This seems to suggest that SG when combined with stress, but not SG alone, promotes memory impairment relative to stress alone. However, given the high variability between testing sessions and the lack of a confirmatory trend in the preceding testing sessions, this finding should be interpreted cautiously.

Statistical analysis of working memory errors in the RAM exposed a significant main effect for Testing Session ($F(6, 201) = 4.06$, $p < .001$). Post hoc pair wise comparisons revealed no significant within group differences. However, *a priori* planned t-tests on the last testing session showed a significant increase in errors for the SG + stress group relative to the stress group ($t(15) = -2.40$, $p < .05$). There was no interaction effect ($F(18, 201) = 1.11$, $p = .35$) nor main effect of Treatment ($F(3, 37) = .34$, $p = .80$).

The analysis of reference memory errors in the RAM showed a significant main effect for Testing Session ($F(6, 201) = 3.25$, $p < .01$). This was followed with post hoc tests which revealed no significant between or within group differences. There was no main

effect for Treatment ($F(3, 37) = .22$, $p = .88$) nor interaction effect ($F(18, 201) = 1.06$, $p = .39$).

3.3.2 Aluminum – Memory Findings

Chronic oral intake of aluminum has been shown to progressively impair cognitive function in rats in an age and dose-dependent manner on a hippocampal-dependent T-maze task (Walton 2009b). This led to the suspicion that other behavioural tests, including the RAM, would identify hippocampal-dependent memory deficits in a paradigm involving chronic intake of aluminum-laced water. However, an analysis of the RAM results indicated a lack of cognitive deficits in aluminum treated groups as measured by working memory and reference memory errors.

There were no interaction or main effects for either working (Figure 3-9) or reference (Figure 3-10) memory errors suggesting that this experimental paradigm, which included CD-1 males in mid-life, aluminum exposure in water (40 mg/L $AlCl_3 \cdot 6H_2O$) over a period of 4.5 months, and RAM testing every 3 weeks amidst a dense battery of behavioural tests was not sufficient to identify cognitive deficits of this nature. For working memory errors the interaction ($F(18, 201) = 1.02$, $p = .44$), main effect of Treatment ($F(3, 37) = .19$, $p = .91$), and main effect of Testing Session ($F(6, 201) = 1.39$, $p = .22$) were all non-significant. Similarly for reference memory errors, the interaction ($F(18, 201) = 1.15$, $p = .31$), main effect of Treatment ($F(3, 37) = .08$, $p = .97$), and main effect of Testing Session ($F(6, 201) = 1.31$, $p = .25$) were non-significant.

Figure 3-7: Radial Arm Maze Working Memory Errors – Restraint Stress

The SG + stress group had a significantly higher number of working memory errors on the last testing session as compared to the stress group. However, given the lack of a confirmatory trend in the preceding testing sessions, this finding must be interpreted cautiously from the perspective that it may be an experimental outlier. [Error bars represent SEM; * = $p < .05$]

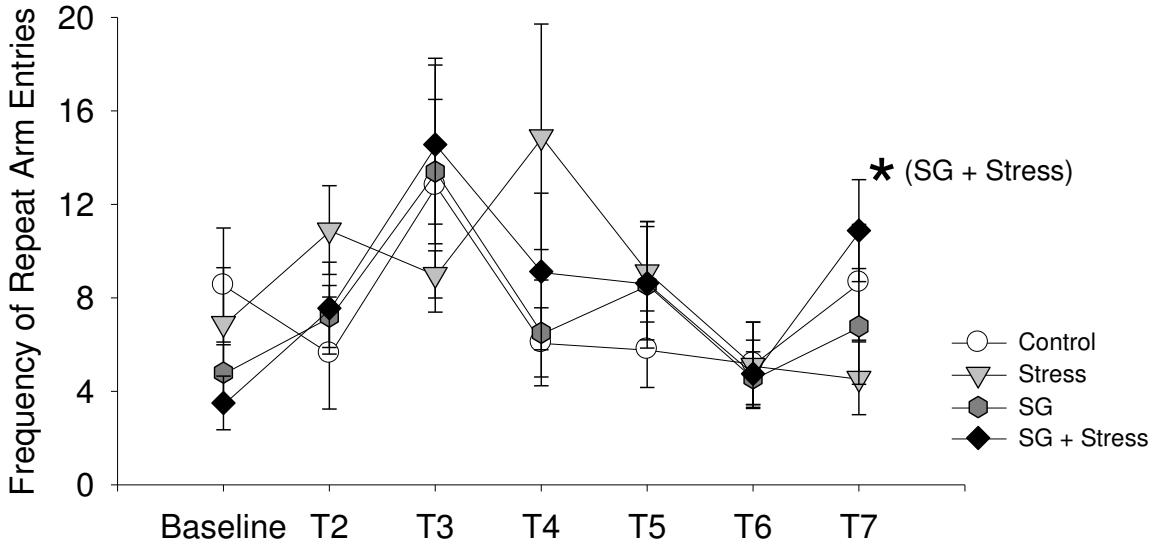


Figure 3-8: Radial Arm Maze Reference Memory Errors – Restraint Stress

There were no significant main effects for spatial reference memory in the RAM. [Error bars represent SEM]

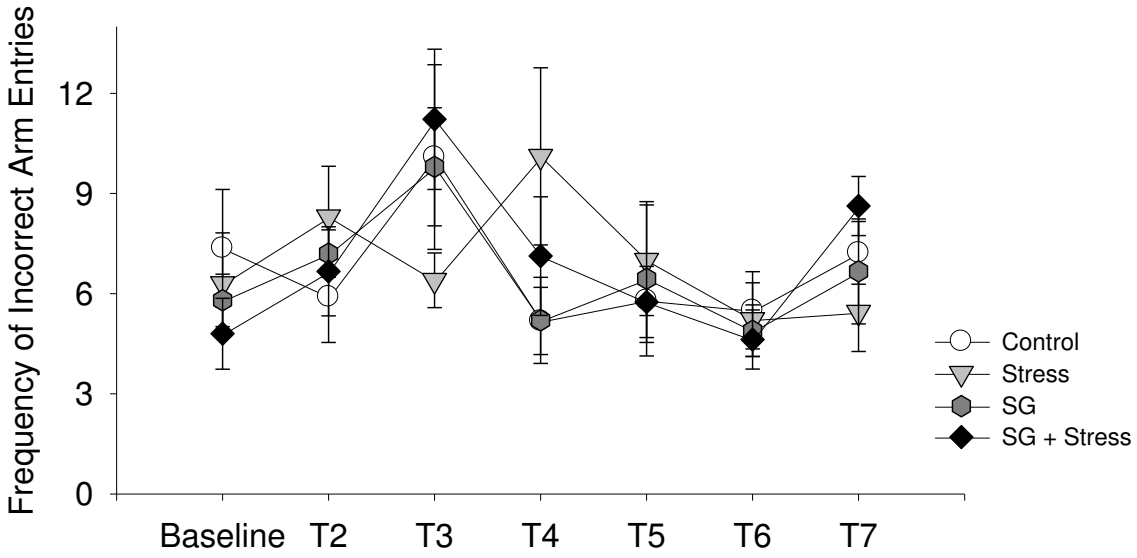


Figure 3-9: Radial Arm Maze Working Memory Errors – Aluminum

There were no significant main effects for spatial working memory in the RAM. [Error bars represent SEM.]

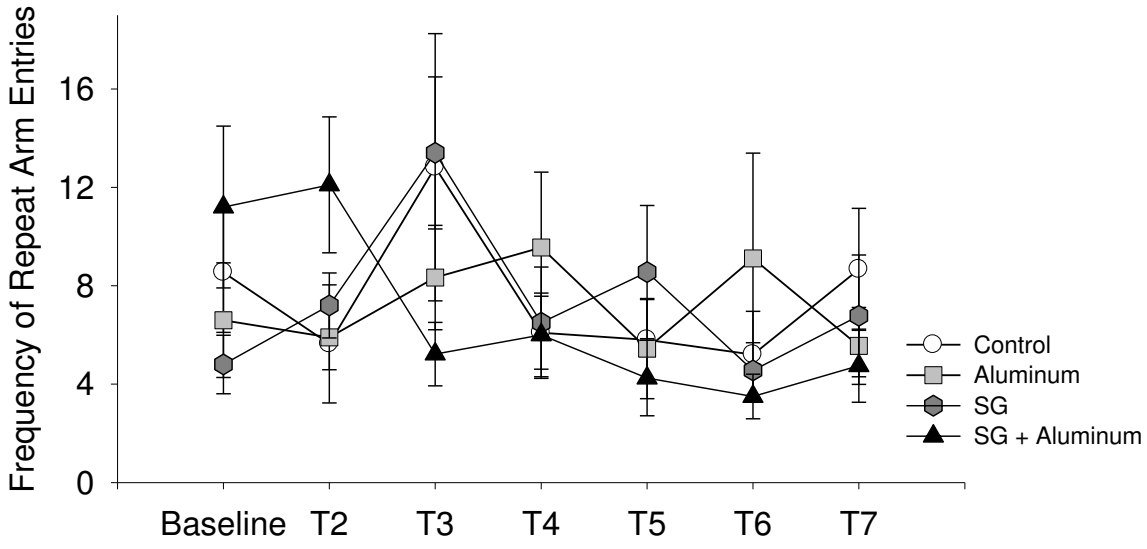
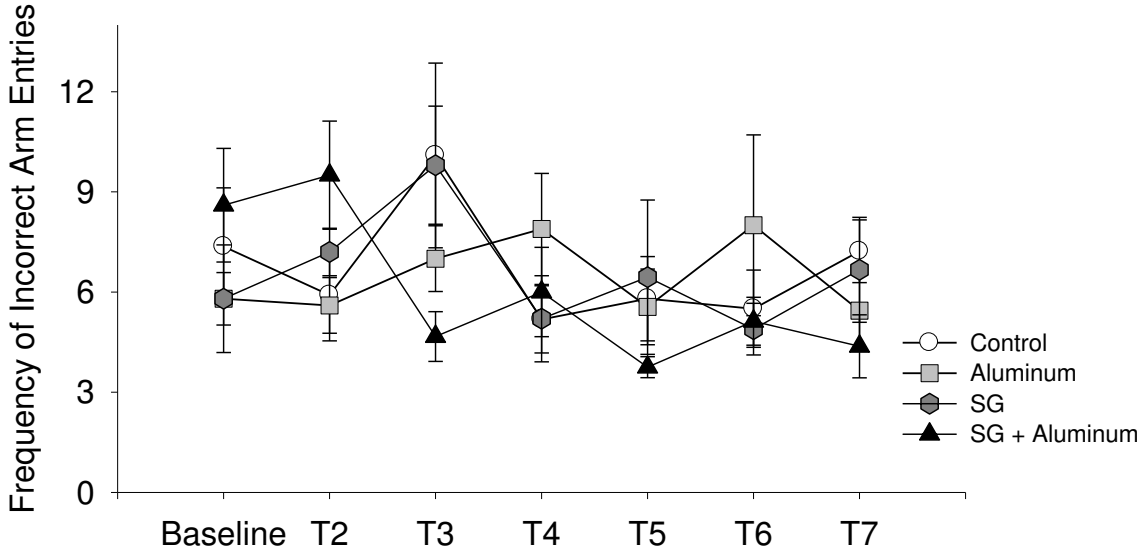


Figure 3-10: Radial Arm Maze Reference Memory Errors – Aluminum

There were no significant between or within Treatment differences for reference memory errors. The lack of a significant increase in errors for either reference or working memory errors (Figure 3-9) for either aluminum treatment group in the RAM suggests that if cognitive deficits were present, the RAM was unable to capture them. [Error bars represent SEM.]



3.4 Light-Dark Box

3.4.1 Restraint Stress – Anxiety Findings

The LDT assessed anxiogenic behaviour through a comparison of a rodent's desire to stay in a dark enclosed space with its desire to explore a novel environment. The two LDT metrics which best captured this comparison were the latency for the test mouse to enter the illuminated light portion of the box and the total duration spent in the illuminated area. Analysis of the LDT results revealed that the SG and the stress groups exhibited increased anxiogenicity as shown by increased latency to enter the light area (Figure 3-11). Further, the SG and control groups displayed a significant decrease in the time spent in the illuminated portion of the LDT (Figure 3-12). Interestingly, these anxiogenic behaviours which were observed with the SG toxin were not exacerbated when combined with chronic restraint stress.

An examination of the latency to enter the light area of the LDT revealed a significant main effect of Testing Session ($F(7, 241) = 5.00$, $p < .001$). Post hoc pairwise comparisons showed that only the SG group had a significant increase in latency to enter the light area by the last testing session relative to its baseline ($q(8) = 4.34$, $p < .05$). This increase in latency by the last testing session for the SG group was a trend higher than the SG + stress group ($T(9)=53.50$, $p = 0.08$) but not the control group ($T(9)=67.00$, $p = 0.11$). Interestingly, though the latency for the stress group did not change significantly across testing sessions relative to its baseline value, its latency was significantly higher than the SG + stress group latency on the last testing session ($T(9) = 49.00$, $p < .05$). As the latencies for both the SG group and the stress group were

higher than the SG + stress group on the last testing session, this is suggestive of a latency suppressing effect resulting from the combination. There was no main effect of Treatment ($F(3, 37) = 1.88$, $p = .15$) or interaction effect ($F(21, 241) = 1.13$, $p = .31$).

Analysis of the time spent in the illuminated portion of the LDT showed a significant main effect of Testing Session ($F(7, 241) = 10.08$, $p < .001$). Post hoc comparisons revealed that both the SG group ($q(8) = 4.44$, $p < .05$) and the control group ($q(8) = 4.14$, $p < .05$) had spent significantly less time in light area of the box across testing sessions. Indeed, all groups showed declines in the time spent in the light area across testing sessions, however as these decreases occurred at different rates, there were significant findings by the last testing session. By the last testing session the SG group had significantly less time in the light area as compared to the control group ($T(9)=63.00$, $p < .05$) and showed a trend compared to the SG + stress group ($T(9)=92.00$, $p = .06$). Further, the stress group also displayed a trend towards less duration in the light area on the last testing session as compared to the SG + stress group ($T(9)=92.00$, $p = .06$). The reduced time spent in the light area for both the SG group and the stress group relative to the SG + stress group on the last testing session is suggestive of a cumulative restorative effect on time in the light area when stress and SG are combined. There was no main effect of Treatment ($F(3, 37) = 1.70$, $p = .18$), nor significant interaction ($F(21, 241) = 1.27$, $p = .20$).

3.4.2 Aluminum – Anxiety Findings

The LDT results showed increased latencies to enter the light area for the aluminum and SG + aluminum groups relative to baseline. All groups were found to have had a decreased preference for the light area across testing sessions.

All groups spent less time in the light area of the light-dark box by end-state behavioural testing relative to baseline (Figure 3-13). This decreased preference for the light area was significant for the control group ($q(8) = 5.20$, $p < .01$) and SG group ($q(8) = 5.23$, $p < .01$) and a trend for the aluminum group ($q(8) = 4.17$, $p = .06$) and the SG + aluminum group ($q(8) = 4.01$, $p = .09$). These post hoc comparisons followed from a significant main effect of Testing Session ($F(7, 240) = 10.78$, $p < .001$). There were no treatment differences and the main effect of Treatment was not significant ($F(3, 37) = 1.34$, $p = .28$). However, the control group did spend on average more time in the light area ($\bar{x}$ = 54s, SEM = 8s), particularly compared to the SG group ($\bar{x}$ = 32s, SEM = 8s) and the aluminum group ($\bar{x}$ = 38s, SEM = 9s), which is suggestive of a reduced level of anxiety in the control group. There was no interaction between the main effects ($F(21, 240) = .60$, $p = .92$). A separate assessment of the horizontal movement time (figure not shown) revealed results which mirrored (both statistically and in absolute terms) those just described for the time spent in the light area. This indicates that mice from all treatment groups, while in the light area, were in near continuous horizontal movement.

Analysis of the latency to enter light area (Figure 3-14) revealed increases in latency up to Testing Session #7 which returned to within baseline levels on Testing Session #8.

This longer latency from baseline to Testing Session #7 was significant for the aluminum group ($q(8) = 5.73$, $p < .001$) and a trend for the SG + aluminum group ($q(8) = 4.18$, $p = .06$). These pairwise comparisons were conducted after the finding of a significant main effect of Testing Session ($F(7, 240) = 7.75$, $p < .001$). There was no effect of Treatment ($F(3, 37) = 1.22$, $p = .32$), however the control group on average had a much shorter latency ($\bar{x}$ = 19s, SEM = 12s) than the other treatment groups: aluminum ($\bar{x}$ = 51s, SEM = 14s), SG ($\bar{x}$ = 41s, SEM = 13s), and SG + aluminum ($\bar{x}$ = 40s, SEM = 14s). There was no factor interaction for the latency to enter the light area ($F(21, 240) = 1.27$, $p = .20$).

Figure 3-11: Light-Dark Box Latency to Enter Light Area – Restraint Stress

The SG group displayed a significantly increased latency to enter the light area of the box by the last testing session relative to its baseline latency. This increase in latency on the last testing session was significantly higher than the SG + stress group and the control group. Further, the stress group had a trend higher latency to enter the light area on the last testing session as compared to the SG + stress, though this latency for the stress group was not significantly different from its baseline value. As both the SG and stress groups had higher latencies to enter the light area than the SG + stress group, this is suggestive of a cumulative effect when SG and stress are combined. [Error bars represent SEM; * = $p < .05$]

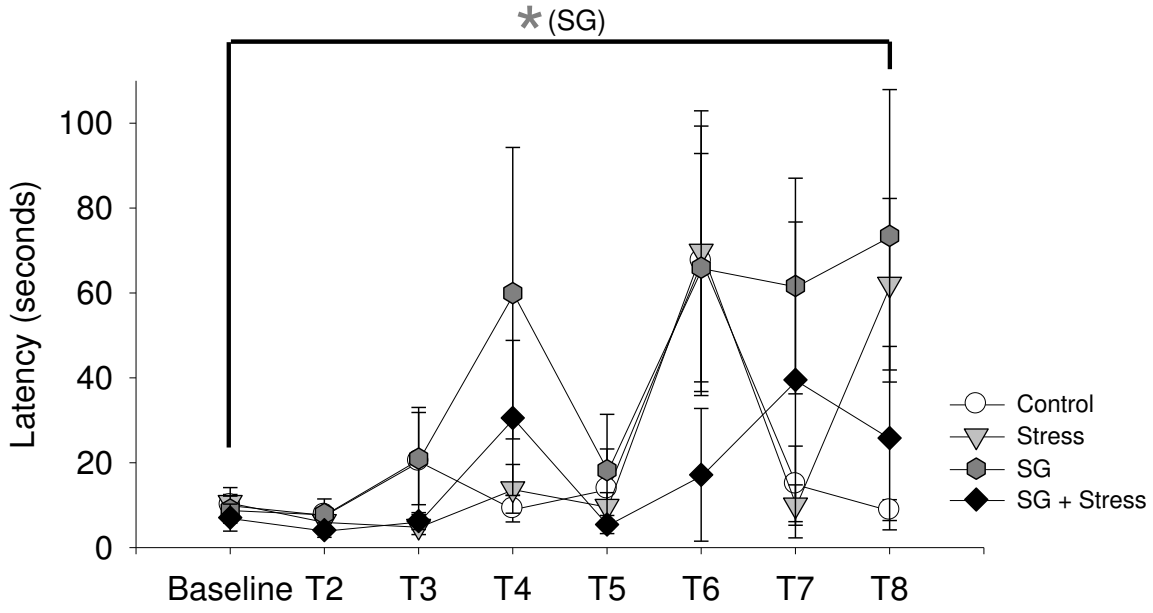


Figure 3-12: Light-Dark Box Duration in Light Area – Restraint Stress

There was a significant decrease across testing sessions in time spent in the light area of the box for both the SG group and the control group. This decrease led to the SG group having a significantly reduced time in the light area on the last testing session compared to the control group and a trend lower than the SG + stress group. Further, the stress group also displayed a trend lower duration in the light area on the last testing session as compared to the SG + stress group. The lower durations on the last testing session for the SG group and the stress group, relative to their combination, are suggestive of an anxiety suppressing effect for the combination. [Error bars represent SEM; * = $p < .05$]

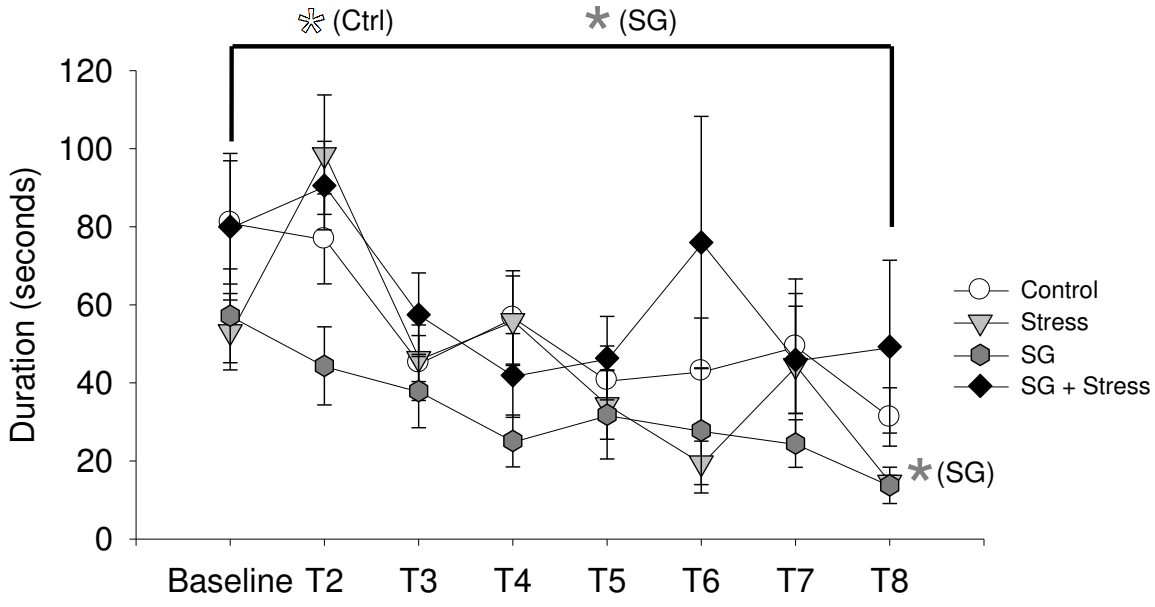


Figure 3-13: Light-Dark Box Duration in Light Area – Aluminum

There were significant decreases in time spent in the light area from baseline for the control and SG groups while there was a trend to decrease for the aluminum and SG + aluminum groups. [Error bars represent SEM; ^ = .05 ≤ p < .10, ** = p < .01]

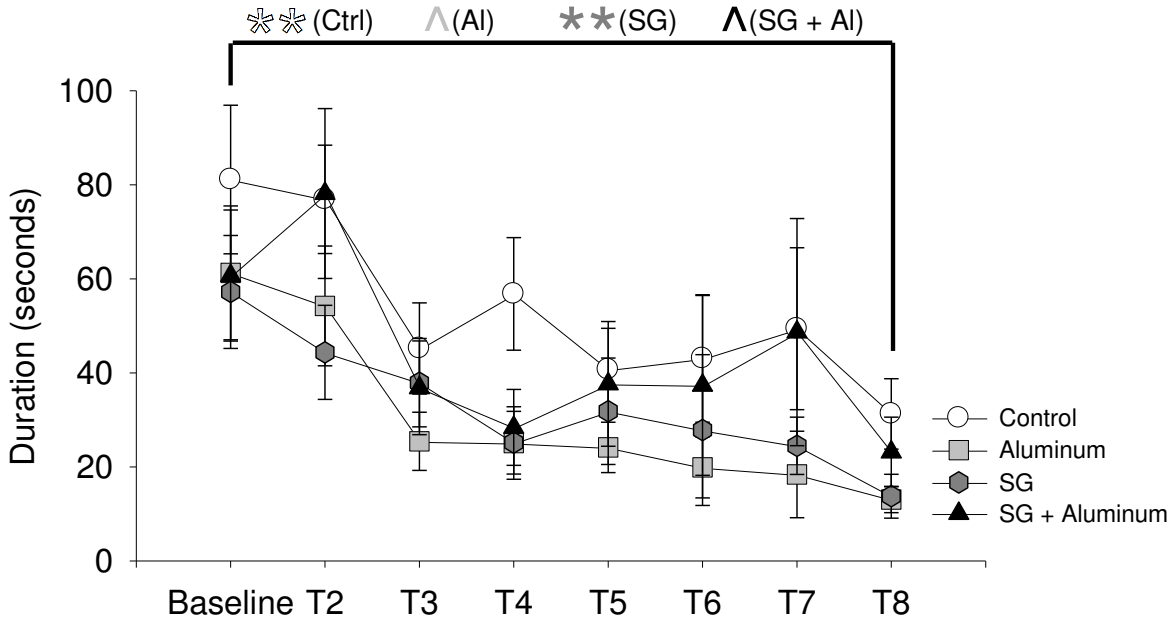
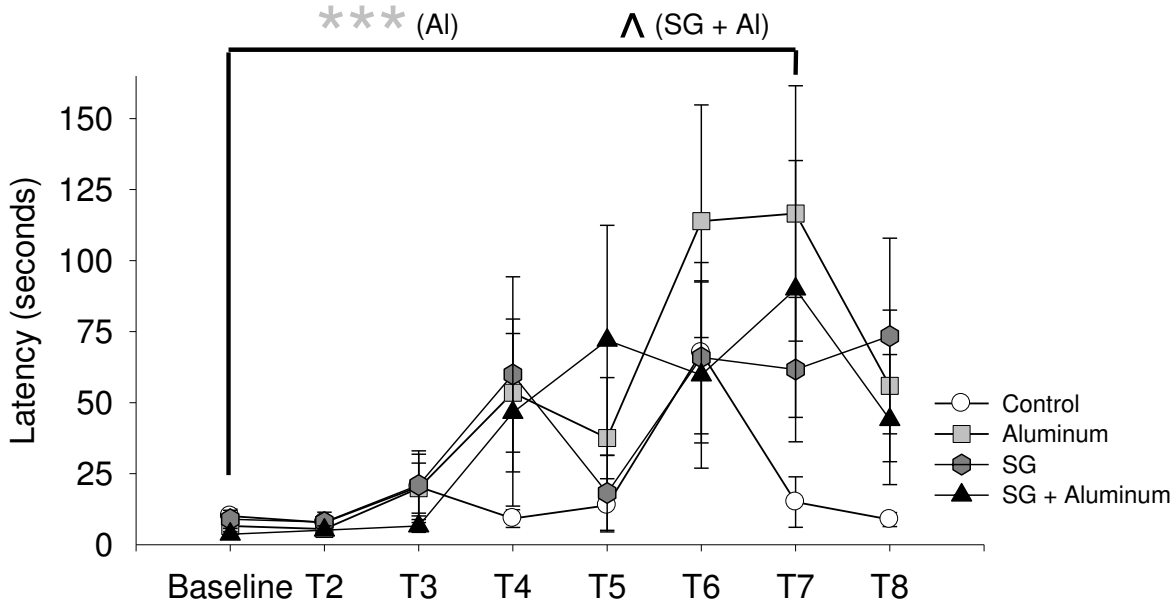


Figure 3-14: Light-Dark Box Latency to Enter Light Area – Aluminum

The SG group and the two aluminum groups showed an increase in latency to enter the light area while the control group did not change from baseline. This longer latency was significant to Testing Session #7 for the aluminum group before becoming non-significantly higher than the control group on the last testing session. [Error bars represent SEM; ^ = .05 ≤ p < .10, *** = p < .001]



3.5 Elevated Plus Maze

3.5.1 Restraint Stress – Anxiety Findings

The EPM, like the light-dark box, is used to assess anxiety by relying on a mouse's aversion to high exposed places. This is quantified by measuring the time the test mouse spends in the open, illuminated portion of the maze. Analysis of the time in the open area of the EPM revealed significant decreases across testing sessions for this measure for the SG group and the SG + stress group while the control group showed a non-significant decrease across testing sessions. The stress group did not exhibit a similar decrease in time spent in the open area of the maze, though this is likely an artifact of a non-significantly lower baseline (Figure 3-15).

Statistical examination of the time in the open area of the EPM showed a significant interaction effect ($F(12, 139) = 4.50$, $p < .001$) and a significant main effect of Testing Session ($F(4, 139) = 13.89$, $p < .001$). Post hoc analysis revealed significant decreases across testing sessions for the SG group ($q(5) = 4.43$, $p < .05$) and the SG + stress group ($q(5) = 3.98$, $p < .05$). There were no between group differences on the last testing session. The main effect of Treatment was not significant ($F(3, 37) = .32$, $p = .81$).

3.5.2 Aluminum – Anxiety Findings

The EPM is a standard behavioural test used within the pharmaceutical industry to assess the anxiogenic or anxiolytic properties of drugs (Dawson & Tricklebank 1995). Under the conditions in this study, all treatment groups with the exception of the control

group displayed decreased time in the open area of the EPM. This decreased time in the open area is suggestive of increased anxiety (Figure 3-16).

The statistical examination of the time spent in the open area uncovered decreases for all groups except the control group. These decreases were significant from baseline to the last testing session for the aluminum group ($q(5) = 4.57$, $p < .05$), the SG group ($q(5) = 4.81$, $p < .01$), and the SG + aluminum group ($q(5) = 4.66$, $p < .01$). These post hoc comparisons followed from a significant main effect of Testing Session ($F(4, 139) = 13.47$, $p < .001$). The main effect of Treatment was not significant ($F(3, 37) = .02$, $p = .99$) nor was the factor interaction ($F(12, 139) = 1.35$, $p = .20$).

Figure 3-15: Elevated Plus Maze Duration Spent in Open – Restraint Stress

The SG group and the SG + stress group displayed significant decreases across testing sessions in the time spent in the open area of the maze while the control group displayed a non-significant decrease. A similar decrease was not seen for the stress group, though this may be a function of a lower initial baseline. [Error bars represent SEM; * = $p < .05$]

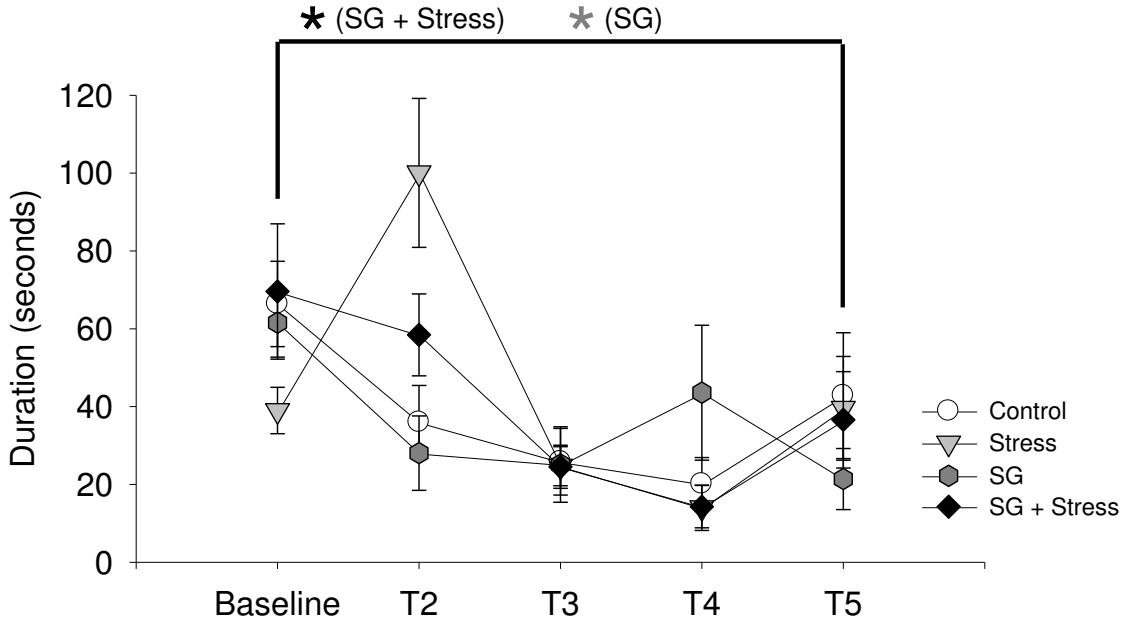
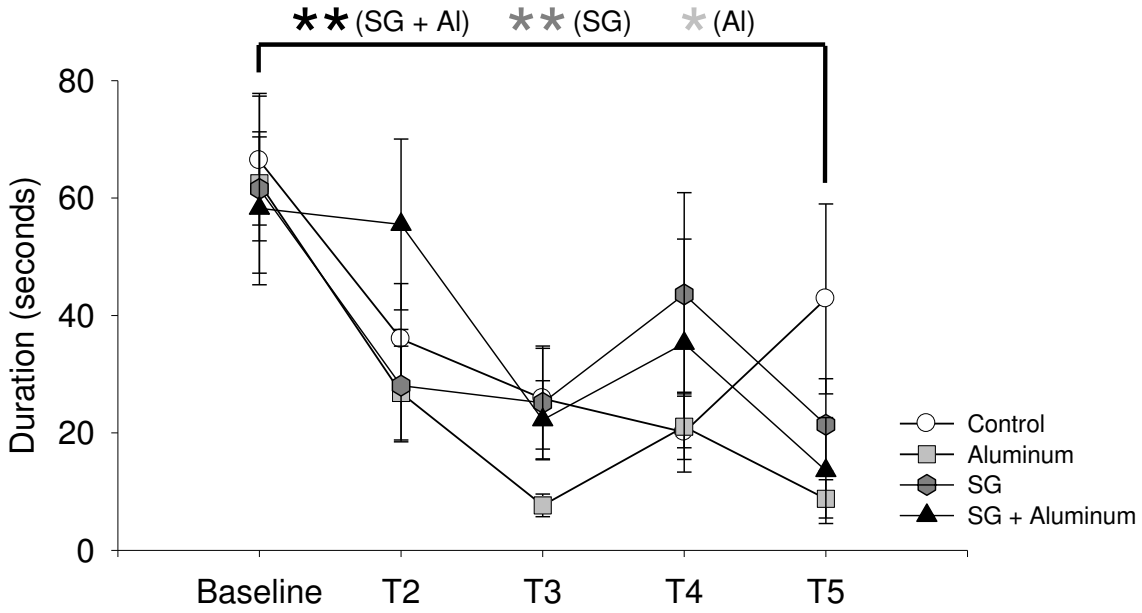


Figure 3-16: Elevated Plus Maze Duration Spent in Open – Aluminum

There was a significant decrease in time spent in the open area across testing sessions for all groups except the control group. [Error bars represent SEM; * = $p < .05$, ** = $p < .01$]



3.6 Gait Pattern Analysis

3.6.1 Restraint Stress – Motor Findings

The DigiGait apparatus was used to assess a number of mouse gait and posture measures. These measures included the ataxia coefficient which quantifies how uncoordinated or irregular mouse muscle movements are. Analysis of the ataxia coefficient revealed that the SG group had a significant increase in ataxia by the last testing session relative to baseline. Further, there was a trend for the SG group to have higher ataxia than the SG + stress group, suggesting a stress ameliorating effect on the SG induced motor impairment. Although the control group had comparable ataxia to the SG group on the last testing session, this was in part due to a higher baseline level of ataxia (Figure 3-17).

The ataxia coefficient was found to have a significant main effect of Testing Session ($F(5, 165) = 2.39$, $p < .05$). Post hoc analysis revealed that the SG treatment group had a significant increase in ataxia from baseline to the last testing session ($q(6) = 4.34$, $p < .05$), while the other treatment groups remained within their respective baseline levels of ataxia. The increased ataxia in the SG group led to it having trend higher ataxia on the last testing session than the SG + stress group ($t(14) = 2.06$, $p = .06$). There was no main effect of Treatment ($F(3, 37) = 1.99$, $p = .13$), nor was there an interaction effect ($F(15, 165) = 1.37$, $p = .17$).

An examination of the stance width (fore and rear stance widths averaged) revealed that by the last testing session the stress group had a significantly increased stance

width relative to the control and SG groups. This finding was despite no within group differences across testing sessions for any group; rather, slight non-significant within group changes for all groups led to significant between group differences on the last testing session (Figure 3-18).

There was a significant main effect of Testing Session for stance width ($F(5, 164) = 2.67$, $p < .05$), which led to post hoc comparisons which included *a priori* planned t-tests on the last testing session. These revealed that the stress group had significantly wider stance width on the last testing session than the control group ($t(16) = -3.04$, $p < .01$) and the SG group ($t(16) = 2.67$, $p < .05$). Interestingly, the SG + stress group had non-significantly lower stance width than the stress group, suggesting a mediating effect of SG ($T(9) = 44$, $p = .11$). There was no interaction effect ($F(15, 164) = 1.20$, $p = .28$) or main effect of Treatment ($F(3, 37) = 1.50$, $p = .23$).

Statistical examination of the stride length revealed that the SG group had a significantly shortened stride length by the last testing session relative to its baseline value; no other group displayed a comparable motor impairment across testing sessions. This shortened stride length for the SG group was significantly shorter than the stride length for the stress group on the last testing session, but not the control or SG + stress groups. Importantly, the lack of stride length impairment for the SG + stress group comparable to what is seen with SG alone is suggestive of a stress restorative effect on SG induced stride length impairment (Figure 3-19).

The main effect of Testing Session was found to be significant for stride length ($F(5, 165) = 3.21$, $p < .01$). Post hoc comparisons showed that stride length for the SG group was significantly shorter by the last testing session relative to its baseline value ($q(6) = 4.24$, $p < .05$). *A priori* planned t-tests on the last testing session revealed that the SG group stride length was significantly shorter than the stress group ($t(16) = -2.26$, $p < .05$). There was no interaction effect ($F(15, 165) = 1.28$, $p = .22$) or main effect of Treatment ($F(3, 37) = .39$, $p = .76$).

3.6.2 Aluminum – Motor Findings

Aluminum neurotoxicity can produce measurable deficits on behavioural performance and motor coordination (Sahin et al. 1995). In order to establish whether motor coordination deficits were occurring and to quantify them, the DigiGait automated treadmill apparatus was employed to assess mouse gait patterns.

The GPA results reveal that mice receiving aluminum laced water were more prone to motor deficits such as increased stance width. Further, all treatment groups displayed an increase in ataxia across testing sessions, though this increase was not significant for the control group.

Analysis of the ataxia coefficient revealed significant increases across testing sessions (Figure 3-20). These increases were significant from baseline for the SG group ($q(6) = 4.35$, $p < .05$) and from Testing Session #2 for the SG + aluminum group ($q(6) = 5.40$, $p < .01$). There was also a trend increase from Testing Session #2 for the aluminum group

($q(6) = 3.78$, $p = .08$). The decreased ataxia seen from baseline to Testing Session #2 for the two aluminum groups may be reflective of a reduced neophobia effect toward the DigiGait apparatus. The above pairwise comparisons were conducted following the finding of a significant main effect of Testing Session ($F(5, 166) = 5.40$, $p < .001$) and a significant factor interaction ($F(15, 166) = 2.41$, $p < .01$). The main effect of Treatment was not significant ($F(3, 37) = .21$, $p = .89$).

Statistical examination of the stance width (fore and rear paw stance widths averaged) followed from a significant main effect of Testing Session ($F(5, 166) = 2.31$, $p < .05$) and a near significant factor interaction ($F(15, 166) = 1.56$, $p = .09$). This examination revealed that the control, SG and SG + aluminum groups stayed within baseline stance width levels throughout testing (Figure 3-21). By contrast, the stance width for the aluminum group showed a trend increase across testing sessions from baseline to Testing Session #5 ($q(6) = 3.87$, $p = .07$). The progressively wider average stance seen in the aluminum group led to a trend difference on the terminal testing session when compared with the control group ($q(4) = 3.31$, $p < .09$). The main effect of Treatment was not significant ($F(3, 37) = 1.29$, $p = .29$).

Figure 3-17: Gait Pattern Analysis Ataxia Coefficient – Restraint Stress

The SG group displayed a significant increase in the ataxia coefficient across testing sessions which led to it having a trend higher ataxia than the SG + stress and stress alone treatment groups on the last testing session. Note that although the control group displayed comparable ataxia to the SG group on the last testing session, this was in part due to a higher baseline level. [Error bars represent SEM; * = $p < .05$]

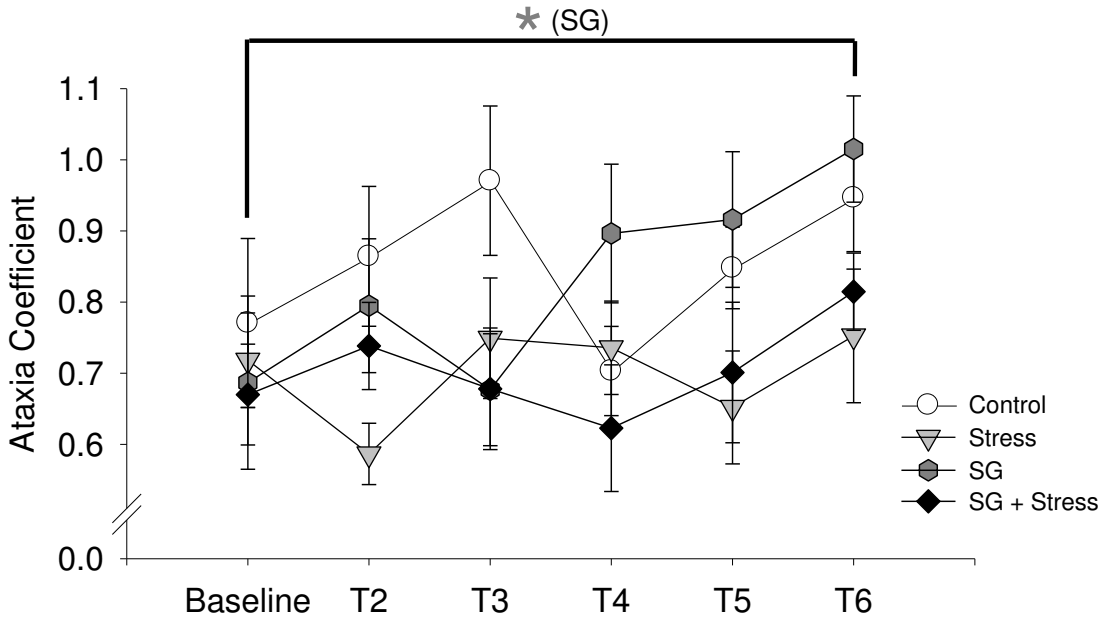


Figure 3-18: Gait Pattern Analysis Stance Width – Restraint Stress

The stress group displayed a significantly wider stance, an indication of motor impairment, on the last testing session as compared to the control and SG groups. Interestingly, the SG + stress group did not display similar stance impairment. The stance widths for all treatment groups were not significantly different from their baseline values; slight changes led to the significant between group differences on the last testing session. [Error bars represent SEM; * = $p < .05$]

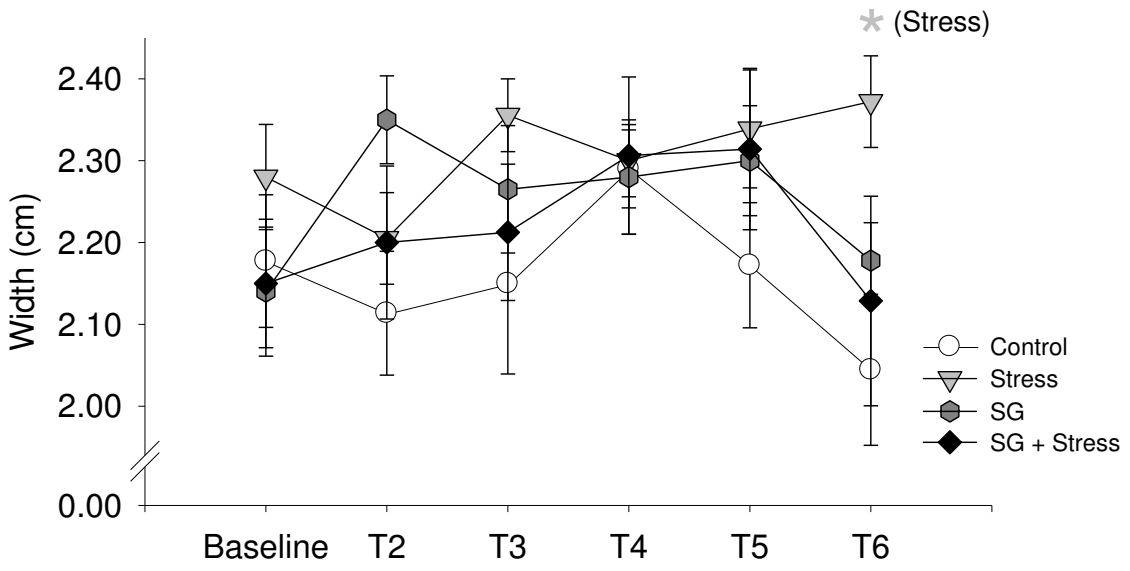


Figure 3-19: Gait Pattern Analysis Stride Length – Restraint Stress

The SG group displayed a significantly shortened stride length by the last testing session. This shorter stride length for the SG group was not significantly different from other treatment groups nor did any other group, including the SG + stress group, display a comparable motor impairment. This finding suggests a stress ameliorating effect on a toxin induced behavioural motor impairment, similar to what was seen with the ataxia coefficient. [Error bars represent SEM; * = $p < .05$]

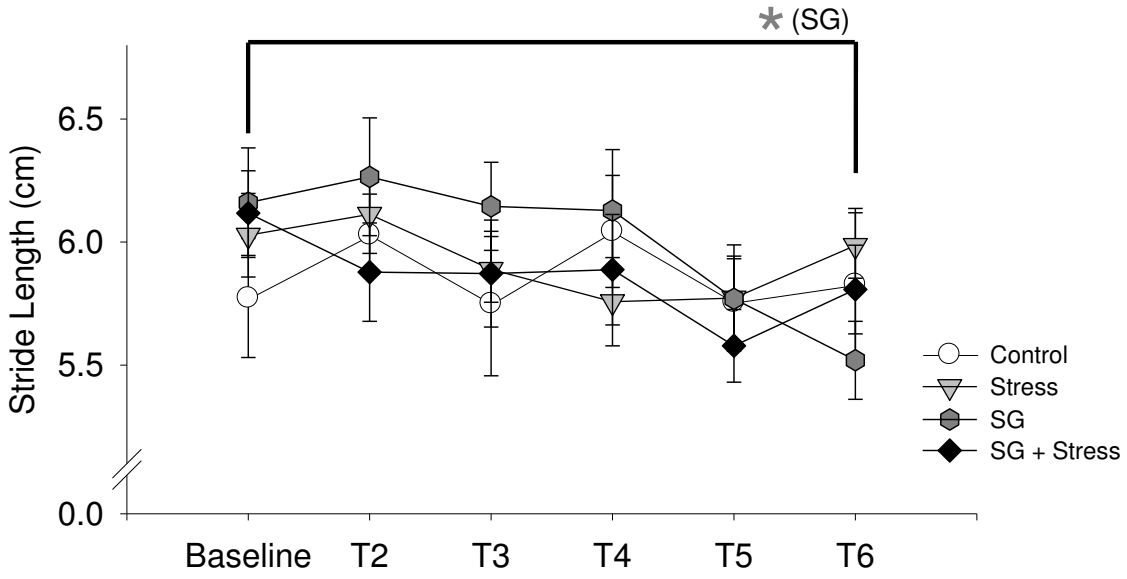


Figure 3-20: Gait Pattern Analysis Ataxia Coefficient – Aluminum

There was a significant increase across testing sessions in the ataxia coefficient from baseline for the SG group and from Testing Session #2 for the SG + aluminum group. There was also a trend increase in the ataxia coefficient from Testing Session #2 for the aluminum group. [Error bars represent SEM; ^ = .05 ≤ p < .10, * = p < .05, ** = p < .01]

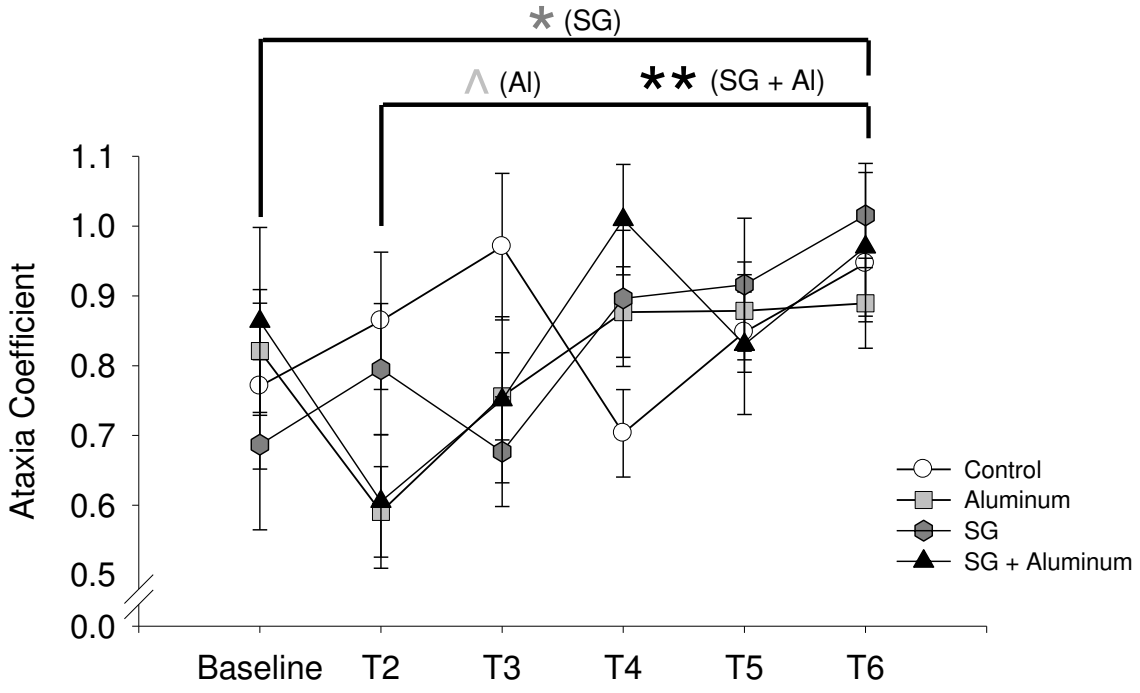
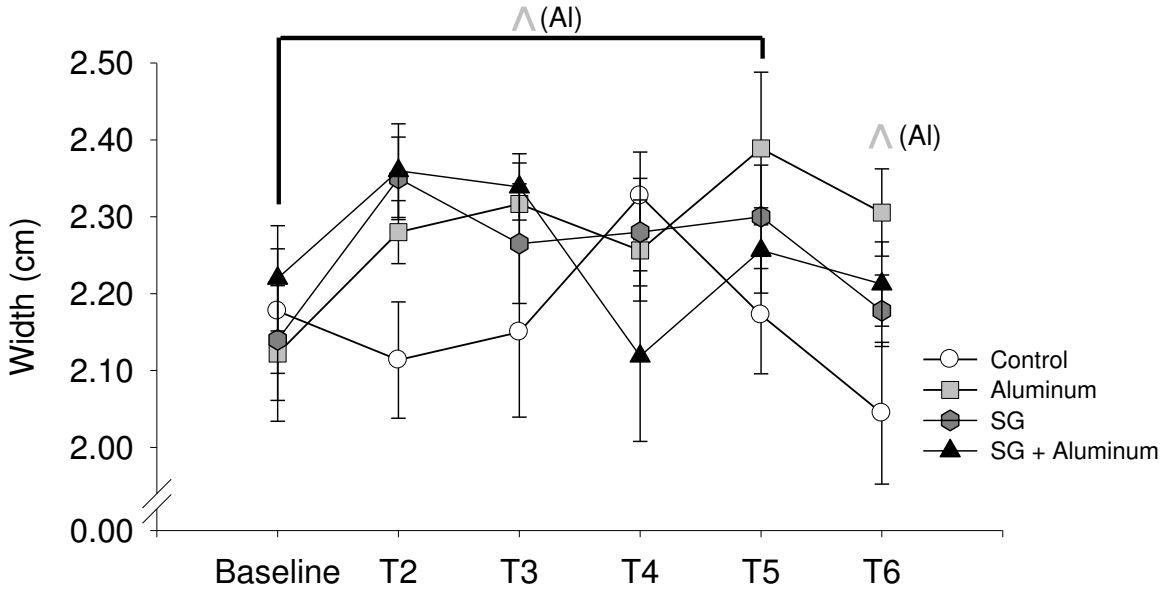


Figure 3-21: Gait Pattern Analysis Stance Width – Aluminum

There was a trend increase in stance width (fore and rear stance widths averaged) for the aluminum group from baseline to Testing Session #5. There was also a trend difference between the aluminum and control groups on the last testing session. [Error bars represent SEM; ^ = .05 ≤ *p* < .10]



3.7 Forced Swim Test

3.7.1 Restraint Stress – Anxiety Findings

The forced swim test (FST) assessed the learned helplessness of test mice through the amount of time they spent immobile. Mouse immobility was defined as less than 13% change in pixels from one time point to the next as computed by the Ethovision 3.1 software.

Analysis of the FST immobility time revealed a significant increase across testing sessions in immobility time for the control group and a trend for the SG group. The stress group did not display a comparable increase in immobility. Interestingly, the combination of SG and stress resulted in a significantly reduced immobility on the last testing session compared to control mice. This is suggestive of an impaired ability respond to uncontrollable stress with learned helplessness in the SG + stress mice (Figure 3-22).

Evaluation of FST immobility showed a significant interaction effect ($F(15, 172) = 5.48$, $p < .001$) and a significant main effect of Testing Session ($F(5, 172) = 5.65$, $p < .001$). Post hoc analysis revealed that the control group had spent significantly more time immobile by the last testing session than it had at baseline ($q(6) = 7.18$, $p < .001$). Further, it was found that the SG group had a trend increase in immobility time across testing sessions ($q(6) = 3.86$, $p = .07$). A comparison of treatment groups on the last testing session also showed that the control group had significantly greater immobility

time than the SG + stress group ($t(15) = 2.26$, $p < .05$). There was no main effect of Treatment ($F(3, 37) = .98$, $p = .41$).

3.7.2 Aluminum – Anxiety Findings

The FST captures depression-correlated locomotor activity in rodents by comparing change in different mobility levels found in the first 5 to 10 minutes of testing (Kitada et al. 1981). An analysis of the FST immobility time data revealed increased learned helplessness across testing sessions, there were no significant differences between treatment groups.

Statistical inspection of the FST immobility time data (<13% movement) revealed significant increases in mouse immobility for all groups across Testing Sessions (Figure 3-23). This mouse tendency to float motionless increased significantly to Testing Session #5 for the SG group ($q(6) = 4.19$, $p < .05$) and the aluminum group ($q(6) = 5.86$, $p < .001$) and was significant to end state testing for the SG + aluminum group ($q(6) = 5.85$, $p < .001$) and the control group $q(6) = 6.69$, $p < .001$). Pairwise assessment of the mouse immobility time followed from a significant main effect of Testing Session ($F(5, 172) = 23.10$, $p < .001$) and a nearly significant factor interaction ($F(15, 172) = 1.63$, $p = .07$). The main effect of Treatment was not significant ($F(3, 37) = 0.43$, $p = .73$).

Figure 3-22: Forced Swim Immobility Time – Restraint Stress

The control group displayed a significant increase across testing sessions in immobility time relative to its baseline. This increase in immobility time was not seen in the stress or SG + stress groups. Indeed, the combination of SG and stress led to a significantly reduced immobility on the last testing session compared to control mice. As this was not seen with stress or SG alone, this is suggestive of an incremental facilitation of impaired learned helplessness in the SG + stress mice. [Error bars represent SEM; ^ = .05 ≤ p < .10, * = p < .05, *** = p < .001]

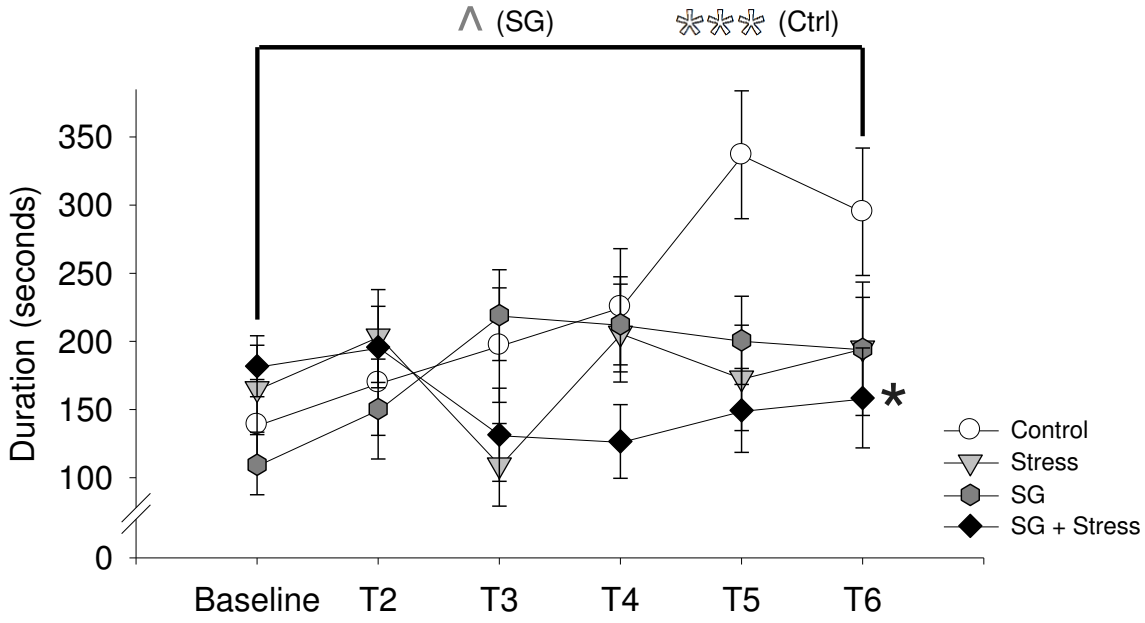
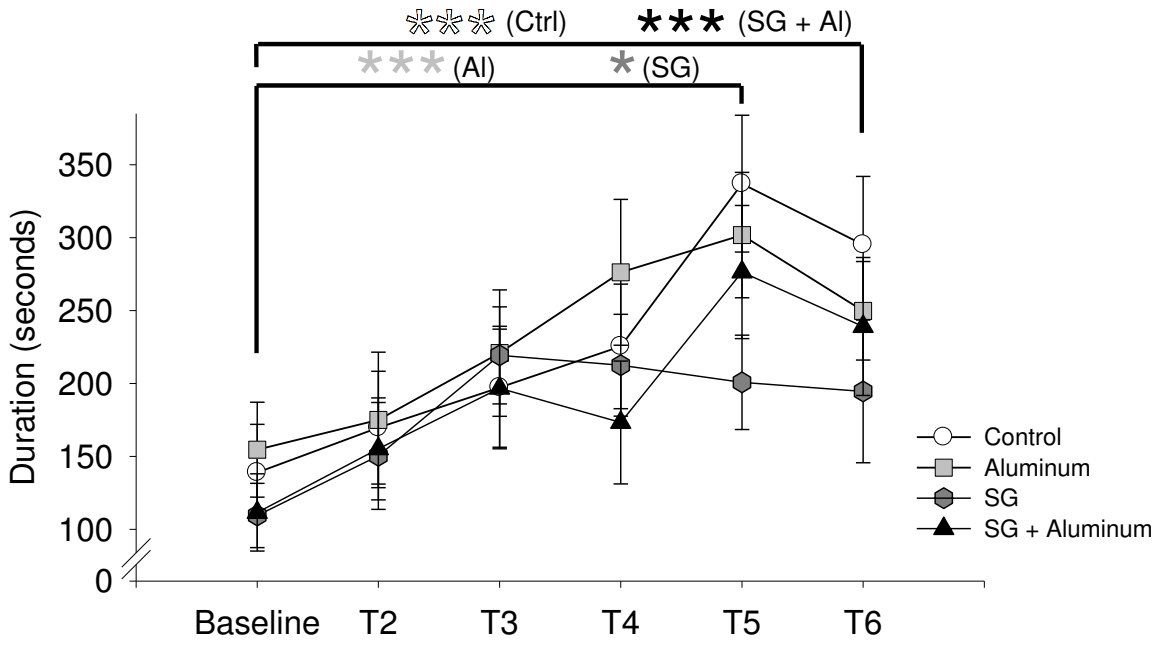


Figure 3-23: Forced Swim Immobility Time – Aluminum

All groups displayed significantly elevated immobility time across testing sessions. This increase was significant to Testing Session #5 for the aluminum and SG groups and to the last testing session for the control and SG + aluminum groups. [Error bars represent SEM; * = $p < .05$, *** = $p < .001$]



Chapter 4 Discussion

This thesis set out to study the interactions between the motor and cognitive impairments produced by a neurotoxin-induced neurodegenerative model of ALS-PDC and two environmental factors, namely aluminum and chronic restraint stress. These two multi-hit experiments entailed using the dietary toxin SG to induce an ALS-PDC phenotype, then concurrently exposing mice to chronic restraint stress or aluminum chloride treated water.

The worsening of ALS-PDC symptomatology was expected to be observed through behavioural alterations and thus a variety of behavioural tests were incorporated to capture as many subtle changes as possible. Indeed, the confirmation or refutation of some subtle behavioural changes is likely to require strong correlation between multiple metrics in order for a definitive conclusion to be made. This need for behavioural parallelism among several metrics in part explains the rationale for incorporating tests which capture overlapping behavioural quantities. This thesis utilized 8 principal behavioural tests; these can be grouped into 3 broad behavioural quantities. These quantities are motor, anxiety and memory. Thus, the findings in this discussion are categorized using these groupings.

4.1 Stress Results

4.1.1 Motor Findings

Finding #1: This experiment has found in male CD-1 mice that chronic restraint stress, administered for 1 hour twice weekly for 18 weeks, does not result in locomotor

changes whether applied in conjunction with the SG toxin or alone, relative to the control group. Further, the SG toxin alone led to a significant decrease in locomotion (Figure 3-3).

The application of chronic restraint stress to rodents has been reported to induce hyperlocomotion. For example, Ito et al. (2010) while examining chronic stress effects on synaptic plasticity, found that mice exposed to chronic restraint stress exhibited elevated levels of locomotion in an OFT. Similarly, Strekalova et al. (2005), in a study assessing whether chronic stress can induce unspecific changes in locomotion, reported that chronic restraint stress applied to mice for 4 weeks led to hyperactivity. Moreover, Roig et al. (2006), using 2 hours per day for 14 days of chronic restraint during the gestation period of rats found that the offspring had non-significantly higher horizontal activity in an OFT. However, in contrast, Pechlivanova et al. (2011) showed reduced locomotor and exploratory activity in the OFT for restraint stressed rats. Additionally, Bowman et al. (2002) discovered that rats chronically restraint stressed for 21 days at 6 hours per day leads to reductions in the number of sector crossings in the OFT, again suggesting lowered locomotor activity. Together these studies paint a mixed picture of the influence of chronic restraint stress on locomotion. However, it is likely that a number of variables, such as sex, species and severity of restraint stress could help parse out some of these apparently contradictory findings. Indeed, chronic restraint stress appears to induce hyperlocomotion preferentially in mice versus rats. This thesis seemingly had more methodological similarity with Ito et al. (2010) and Strekalova et al. (2005) as compared to the study by Bowman et al. (2002) which found lowered

locomotor activity and therefore the prediction for this study was that mice exposed to chronic restraint stress would exhibit increased locomotion in the OFT. Further, as the SG toxin has previously been shown to elevate motor activity (Tabata 2008), the combination of the SG toxin and restraint stress was expected to increase locomotor activity above what was seen with either treatment alone.

The results for this experiment revealed no significant locomotor changes for the restraint stress group as well as the group combining restraint stress and SG. In contrast, the SG toxin group displayed a significant decrease in locomotion.

The lack of a significant increase in hyperlocomotion for the two treatment groups exposed to chronic restraint stress may be in part explained by the light intensity used throughout the experiment. For instance, Strekalova et al. (2005) showed that chronically stressed mice tested under bright or moderate illumination exhibited hyperactivity. Conversely, weak illumination produced normal levels of locomotion (Strekalova et al. 2005). The behaviour room where testing was conducted had had some of the lighting lowered or partially blocked during the OFT in order to improve CCD camera performance. Thus it may be that mouse hyperactivity, which is typically observed following chronic restraint stress, was suppressed by weak room illumination. However, as no measurement of the light intensity was taken, it is unknown whether this met the standard for ‘weak’ illumination as described by Strekalova et al. (2005).

Finding #2: The application of chronic restraint stress to male mice led to a significant increase in stance width relative to non-stressed mice, suggesting gait impairment. Moreover, the combination of the SG toxin and chronic restraint stress did not lead to similar stance impairment (Figure 3-18). Ingestion of the SG toxin alone induced increased ataxia and shortened stride length relative to its baseline, though these differences were not significant from other treatment groups (Figure 3-17; Figure 3-19).

Clinical cases of gait disturbance are frequent in the older population. These gait disturbances can be attributed to a wide variety of causes, though neurodegenerative diseases are foremost among them (Axer et al. 2010). The clinical ALS-PDC phenotype is known to include gait disturbances, and further the SG dietary toxin being used here has been shown to produce increased stride frequency, shorter strides and longer stance durations (Tabata 2008). These points led to the inclusion of a behavioural test for gait in this thesis and further to the prediction that the SG toxin would lead to gait impairments which would be revealed by the GPA.

Chronic restraint stress has been reported to impair skilled motor movement in male and female rats. Jadavji & Metz (2008), using rats restrained daily for 15 days and tested on skilled reaching and walking tasks, found deficits in both skilled motor movements and in recovery following chronic stress. Chronic stress has also been shown to exacerbate gait disturbances in a mouse model of rapid-onset dystonia parkinsonism. Sugimoto et al. (2014) applied chronic stress loading to Atp1a3-deficient heterozygous mice and observed a worsening of gait as quantified by a shortening of

stride length. The evidence from these studies led to the prediction that the application of chronic restraint stress will result in gait impairments. Further, as observed in Sugimoto et al. (2014) where chronic stress facilitated a deterioration of gait in a parkinsonian model, it was predicted that the combination of the SG toxin and chronic restraint stress would lead to an incremental increase in gait deficits beyond what was seen with either treatment alone.

The analysis the GPA results showed that chronic restraint stress increased stance width relative to non-stressed mice. However, the combination of chronic restraint stress with the SG toxin did not lead to a comparable deficit. Further, the SG toxin produced deficits on the ataxia coefficient, which increased, and stride length, which decreased, but not stance width. These observed changes in the ataxia coefficient and stride length are together indicative of gait disturbance induced by the SG toxin, which is consistent with previously reported findings (Banjo 2009; Tabata 2008).

The increased stance width observed in mice subjected to chronic restraint stress is recognized as a measure of gait deterioration (Dorner et al. 1996). However, two questions arise here. Firstly, why were gait impairments not observed on other behavioural metrics in the GPA? And secondly, why did the combination of chronic restraint stress with the SG toxin not promote more severe deficits? The answer for both of these questions may lie in the compensatory strategies adopted by mice exposed to chronic stress.

The application of chronic restraint stress to mice is generally accepted to modify motor behaviour, such as increasing locomotor activity. An aspect of motor coordination, posture, is also changed following chronic stress. For example, stretch attends are typically exhibited by stressed rodents in non-social situations and involve elongating the body while standing still (Kaesermann 1986). Moreover, chronic restraint stress appears to promote task-specific compensatory strategies. Kirkland et al. (2012), exposing rats to daily restraint stress following a focal motor cortex lesion, found that chronic stress limited motor recovery on a food reaching task while promoting recovery on a separate tray reaching task. Kirkland et al. (2012) further suggested that the task-specific improvements were a function of compensatory strategies.

These findings by Kirkland et al. (2012) could explain why the gait disturbances observed with the SG toxin were not exacerbated in the group which combined SG with restraint stress. Indeed, the SG toxin deficits may have been masked by chronic restraint stress induced compensatory mechanisms in the group combining the SG toxin and restraint stress. Further, these compensatory mechanisms are likely merged with postural changes that occur following stress. For example, stretch attends involve the rodent pressing its belly against the ground and stretching forward, which could lengthen the stride and improve the animal's balance, thus constituting a compensatory mechanism. Given this possible deficit masking effect of chronic restraint stress, a future paradigm might include an additional treatment group in order to verify if this is indeed taking place. This additional treatment group would receive both chronic restraint stress and the SG toxin, but restraint stress would be terminated after several months. If

indeed a masking effect is occurring, the termination of the restraint stress should allow for unmasking and thus confirm restraint stress' role in mediating the phenotypic presentation of motor impairment.

The wider stance seen with the chronic restraint stress group may have, similar to the restraint stress and SG toxin group described above, been a function of stress induced postural changes. Indeed, a stretch attend-like behaviour where a mouse maintains an elongated body would alter stance width. Thus it is suggested that the increased stance width observed for the chronic restraint stress group is an adaptive postural change in response to stress.

Finding #3: This experiment found that the administration of chronic restraint stress, whether in combination with SG or alone, does not result in a loss of neuromuscular tone relative to non-stressed mice (Figure 3-1).

This experiment incorporated a behavioural test intended to capture motor neuron dysfunction. This was the WHT, which assessed neuromuscular strength. Previous reports using the SG toxin have observed impaired neuromuscular strength as measured by a decreased latency to fall on the WHT (Tabata 2008). This led to the prediction that ingestion of the SG toxin would lead to neuromuscular impairments.

The administration of chronic restraint stress has been observed to impair motor function. For instance, Smith et al. (2008) using a 20min daily restraint stress paradigm

for 6 weeks (2 weeks pre-lesion and 4 weeks post-lesion) assessed the impact of chronic restraint stress on 6-OHDA induced motor deficits. They found that chronic restraint stress exacerbated motor deficits, specifically skilled reaching and walking tasks and further impaired recovery following the 6-OHDA insult. Moreover, these deficits were shown to be a function of stress-dependent acceleration of midbrain DA-producing neuron loss during the first week post 6-OHDA lesion. In addition, these deficits in motor function have been shown to be linked to HPA axis activity. Indeed, Metz et al. (2005) reported that deficits in skilled movement accuracy in reaching and walking were partially a function of GC levels following chronic restraint stress, confirming the involvement of the HPA axis in coordinated motor movement.

The BG is known to have a substantial density of GRs (Ahima et al. 1991), and the dopaminergic system has been shown to be susceptible to stress (Pani et al. 2000). These findings, along with evidence from Smith et al. (2008) showing that restraint stress led to deficits on skilled motor function and further exacerbated overt behavioural symptoms in a rodent model of PD, led to the prediction that chronic restraint stress alone would impair performance and in combination with SG worsen impairments on the WHT.

This experiment found no chronic restraint stress or SG induced impairments on neuromuscular strength. The loss of neuromuscular strength in the non-stressed control mice is likely a function of age-related muscle loss. Indeed, a progressive loss of skeletal muscle mass and force generating capacity occurs with aging, with mice

reliably demonstrating these aging-associated changes (Russell et al. 2015). Importantly, age-related muscle mass deterioration in mice can be prevented with exercise (McMahon et al. 2014).

4.1.2 Anxiety Findings

Finding #1: This experiment has found that chronic restraint stress leads to increases in anxiogenic behaviour. This was observed for the LDT with increased latency to enter the light area and with lower time in the illuminated area (Figure 3-11; Figure 3-12). Curiously, there was also an increase in anxiolytic behaviour on the OFT for the restraint group (Figure 3-4). The toxin SG was found to have induced increased anxiogenic behaviour on the LDT and EPM (Figure 3-11; Figure 3-15). Perhaps most interestingly, the combination of SG and restraint stress was found to have slightly ameliorated anxiogenic behaviours seen with SG and restraint stress alone on the LDT (Figure 3-11; Figure 3-12).

The application of chronic restraint stress to rodents might, perhaps by definition, be assumed to positively correlate with behavioural measures of anxiety. However, the findings in the literature are inconsistent with some researchers reporting increases on anxiety metrics and others reporting decreases. For example, Zhu et al. (2014) showed that chronic restraint stress applied to mice for 4 weeks led to increased anxiety as assessed by time in the open on the EPM. Similarly, Liu et al. (2013) reported that single housed but not group housed mice exposed to chronic restraint stress displayed significantly decreased time in the open area of an EPM. However, Ito et al. (2010)

found no difference between controls and chronic restraint stressed mice tested for anxiety in a LDT. And further complicating the picture, Huynh et al. (2011) reported that chronic restraint stress increased anxiety in male rats as measured by reduced time spent in the centre of an OFT, but only when rats were tested in their nocturnal phase. When rats were tested during their light phase, no effect of chronic restraint stress on anxiety was found. This apparent light cycle related effect may extend to room illumination, as Strekalova et al. (2005) found that chronic restraint stress produced anxiolytic behaviour under bright light but normal behaviour under dim light.

The prediction for this experiment was that the administration of chronic restraint stress would lead to increases in behavioural measures of anxiety, similar to Zhu et al. (2014). Further, the ingestion of the SG toxin was expected to increase anxiety as previous ethological observations of SG fed mice suggested a more anxious phenotype (Tabata 2008). The combination of the SG toxin and restraint stress was anticipated to exacerbate anxiety seen with either treatment alone. This prediction was based on the notion that any motor deficits which presented following SG ingestion would be inherently stressful and that chronic restraint stress would amplify the animal's already stressful state through a worsening of motor symptoms. Indeed, this is similar to what Smith et al. (2008) found with 6-OHDA lesioned rats, where chronic restraint stress accelerated motor deficits.

This experiment has found an increase in anxiety following the administration of chronic restraint stress or treatment with the SG toxin on the LDT, but not for the combination.

The SG toxin treatment group and the group combining SG and restraint stress displayed significant increases in anxiety on the EPM, but not the restraint stress group. However, the lack of a significant finding of increased anxiety for the restraint stress group on the EPM may be a function of a significantly lower baseline. The observed effects with SG are in line with predictions as it has been shown to induce an anxiogenic phenotype. Similarly, the restraint stress group appears to produce anxiogenic behaviour on the LDT and the EPM, which was expected. The combination of the SG toxin and restraint stress has however slightly ameliorated anxiety on the LDT compared to each treatment alone. This unexpected result may be a function of, like the lack of gait disturbances described above, compensatory mechanisms following the administration of chronic restraint stress.

As Kirkland et al. (2012) note, restraint stress may facilitate adaptive compensatory movement strategies. Indeed, the compensatory strategy which a rodent adopts is likely to be a function of, at a minimum, the nature of the task and the level of stress. Further, in humans there is a well established U-shaped response between stressor exposure and adaptation, such that lower amounts of stress exposure lead to improved physiological and mental function while high levels lead to adverse health outcomes (Ganzel et al. 2010). Therefore, it may be that the anxiogenic behaviours observed with the SG toxin group and with the restraint stress group are the result of adaptive compensatory movement strategies which fall on one side of the U-shaped response. By contrast, the combination of restraint stress and the SG toxin may have led to an elevated level of anxiety, placing the rodent's response on the opposite side of the U-

shaped response curve and resulting in an entirely different behaviour, which in this case was lowered anxiety in the LDT.

The somewhat inconsistent finding that chronic restraint stress leads to increased anxiolytic behaviour in the OFT (ie: decreased thigmotaxis), is possibly a testing artifact which might have disappeared had the experiment been continued for a longer period. Indeed, the group combining the SG toxin and restraint stress did not display a similar anxiolytic behaviour on the OFT to what was seen with the restraint stress alone group. Further support for this line of reasoning comes from the fact that the chronic restraint stress group only had significantly elevated anxiolytic behaviour on the last testing session – there was no similar effect on any of the preceding testing sessions.

Finding #2: The combination of the SG toxin and chronic restraint stress resulted in a decrease in learned helplessness as measured by immobility in the FST. In contrast, both the control and SG groups displayed increased learned helplessness relative to their baseline levels (Figure 3-22).

The FST is typically used in rodents to indirectly assess depressive behaviour. This is achieved through measurement of the amount of time spent floating immobile; this time is termed learned helplessness (Bogdanova et al. 2013). A number of studies have reported that following chronic restraint stress, rodents will exhibit reduced learned helplessness. For example, Dunn & Swiergiel (2008) found that in both rats and mice, chronic restraint stress reduced immobility time in a FST. Indeed, this antidepressant

ability of chronic restraint stress to reduce immobility in the FST has been previously explored by Platt & Stone (1982). The study by Platt & Stone (1982) found that the reduced immobility in the FST induced by chronic restraint stress was comparable to that produced by the antidepressant desmethylimipramine. Moreover, they noted that a single acute application of restraint stress had no effect on FST behaviour. However, others have found no effect at all for chronic restraint stress on FST learned helplessness. Zhu et al. (2014) when comparing depression and anxiety paradigms in relation to chronic restraint stress and unpredictable chronic mild stress, found that while both chronic stress models induced anxiety in mice, only unpredictable chronic mild stress could induce depression as measured in a FST. Chronic restraint stress was found to have no effect on immobility in the FST. In contrast, Kompagne et al. (2008) reported elevated immobility by rats in a FST using a 3 week chronic mild stress regime. Lastly, and perhaps most interestingly, Liu et al. (2013) attempted to parse apart some of the experimental variables which would alter FST immobility time in the chronic restraint paradigm. In particular, following chronic restraint stress, they noted that immobility time in the FST was increased for single housed mice, but that there was no effect for group housed mice. Furthermore, chronic restraint upregulated levels of serum CORT and reduced hippocampal GR in single but not group housed mice (Liu et al. 2013).

The mice used in this study were single housed, and thus the study by Liu et al. (2013) would suggest that FST immobility time would increase; however as bulk of studies in the literature appear to report reduce immobility in rodents following chronic restraint

stress, it was similarly predicted for this study that chronic restraint stress would lead to a lowering of immobility time. Further, as anecdotal evidence suggests that SG fed mice display an anxiogenic phenotype (Tabata 2008), it was predicted that ingestion of the SG toxin would lead to increased immobility, given the relationship between anxiety and depression (Paul 1988). Perplexingly, this meant that the SG toxin was expected to increase immobility time while the application of restraint stress was expected to reduce it; thus the outcome resulting from the combination was unclear. However, several variables such as the single housing of the mice and the slightly unpredictable nature of the stress (only twice a week rather than daily) suggested that increased learned helplessness was the more likely outcome. Thus, the prediction for the combination of restraint stress and the SG toxin was an increase in immobility time.

The results of this experiment revealed that the combination of the SG toxin and chronic restraint stress led to a decrease in immobility time relative to the control treatment group. Further, the control group and the SG toxin group displayed elevated immobility time relative to their baselines. The finding of increased immobility for the SG toxin group is confirmatory and expected, however the reduced immobility for the combination of SG and restraint stress is not.

These results may in part be explained by the locomotion results for the OFT. As noted by Brotto et al. (2000), the OFT is often run in conjunction with the FST in order to rule out the possibility that significant changes in general locomotor activity are confounding results. Indeed, an animal which displays hyperlocomotor activity in the OFT will likely

continue to do so in the FST. Recall that for the OFT, there were no significant treatment group differences, however it was noted that the combination of the SG toxin and restraint stress was suggestive of an increase in locomotion (relative to the SG group), while the SG toxin alone led to a decrease. Further, the control group findings were suggestive of lower locomotion on the last three testing sessions in the OFT. Together these locomotor findings seem to in part explain the FST immobility findings, given the apparent inverse mirroring relationship between the OFT locomotion results and the FST immobility results. Thus, using this paradigm of chronic mild restraint stress in mice, the measures of learned helplessness appear secondary to alterations in motor behaviour.

4.1.3 Memory Findings

Finding #1: This experiment has found that chronic restraint stress and the SG toxin, whether alone or in combination, do not result in spatial working or spatial reference memory deficits (Figure 3-7; Figure 3-8).

One of the most common adverse outcomes associated with chronic stress is cognitive impairment. In order to assess this impairment mice are normally tested in a behavioural apparatus such as the RAM. The RAM measures spatial working and spatial reference memory respectively through counting the number of repeat entries to baited arms or the number of erroneous entries into unbaited arms (Wenk 2004). Typically, impairments for spatial reference but not spatial working memory are reported for rodents in the RAM following the administration of chronic restraint stress. For

instance, deficits have been reported in both acquisition and spatial reference memory but not on spatial working memory in a partially baited RAM using adult male rats subjected to restraint stress for 21 days (6 hours per day) (Srikumar el al. 2006; Srikumar et al. 2007). Indeed, nearly all studies assessing spatial memory in chronically stressed rodents which use appetitively motivated tests report spatial reference memory deficits (for review: Conrad 2010). Given this, the prediction was made that chronic restraint stress would lead to spatial reference but not spatial working memory deficits. Further, as SG induces ALS-PDC, a syndrome known to have a distinct dementia component (Lee 2011), it was expected that restraint stress would exacerbate ALS-PDC-related cognitive deficits and that these impairments would be captured by spatial memory tasks in the RAM. However, statistical analyses of the results for this paradigm found no deficits for restraint stress, SG or the combination.

The neurochemical underpinnings of the effects of restraint stress on cognitive function have been associated with monoaminergic disfunction and the sex of the animal under study. For example, Srikumar et al. (2007) reported that chronic restraint stress induced decreases in dopamine levels in the hippocampus and frontal cortex. Further, Luine (2002) found that chronic restraint stress is differentially mediated by sex with stressed male but not female rats displaying decreased dopaminergic activity in the frontal cortex and amygdala. Moreover, in the CA3 region of the hippocampus, Luine (2002) reported increased levels of 5-HT and NE in female but not male stressed rats and increased GABA in males but not females. Offering some insight into this apparent chronic restraint sex difference, Bowman et al. (2003), using ovariectomized females with and

without estradiol, showed that estrogen exerts both organizational and activational influences in response to chronic stress.

These sex-specific neurochemical responses suggest that estradiol, through its actions on the HPA axis, may be responsible for the protective or sometimes enhancing effect seen with stressed female rodents in spatial memory tasks (Bowman et al. 2003). Further, the memory impairing effects of chronic restraint stress on male rodents may be a function of low or depleted estradiol and/or estrone. This is pointed to by the findings of Andersen et al. (2004) who found reduced estrone and estradiol in restraint stressed male rats relative to controls. Moreover, the administration of estradiol to male rats has been shown to enhance performance in a spatial memory task in a testosterone-independent manner (Gibbs 2005), suggesting its involvement in male spatial memory.

The experimental results for the RAM, which found no effect for chronic restraint stress on spatial reference memory performance, are in strong disagreement with the literature. This is particularly true since there is near general consensus in the literature for spatial reference memory impairing effects in male (but not female) rodents in the RAM following the application of chronic restraint stress. The lack of identifiable memory deficits in the RAM may be a function of the restraint stress protocol used. This protocol involved male mice being stressed twice per week for 1 hour over a period of 18 weeks. Though this resulted in over 30 restraint stress administrations, which is in agreement with paradigms used by other research groups, the spacing between these sessions

may have led to a decreased stress effect. For example, it is typical to apply restraint stress once per day on continuously sequential days (Jeong et al. 2013; Kim & Leem 2014), which would mean that sessions are separated by 24 hours or less, rather than 48 or 72 hours as was used herein. In this thesis, restraint stress was only administered twice a week in an effort to minimize the HPA suppression which occurs with more frequent homotypic exposures (Grissom et al. 2007). However, it may be that this spacing between restraint sessions excessively reduced the stressful experience, leading to only modestly stressed mice. Therefore, in the context of spatial memory performance in the RAM, these relatively sparse restraint stress applications may not have depressed estradiol or estone levels sufficiently so as to lead to a clinically measurable impairment.

The design of the RAM necessitates the test animal to utilize extra-maze cues in order to find the food rewards in the least amount of time. This use of spatial navigation, whether assessed through working or reference memory, requires hippocampal function (Floresco et al. 1997). Chronic stress is known to impair hippocampal function through elevated GC levels which promote dendritic retraction in the hippocampus (Conrad 2008). Indeed, chronic stress paradigms in Sprague Dawley rats have shown that restraint for 6 hours per day for 21 days results in CA3 dendritic retraction (Kleen et al. 2006) which is directly linked to impaired spatial ability (Conrad 2010) and which recovers to pre-stress conditions 5 to 10 days following stress termination (Conrad et al. 1999). Further, McLaughlin et al. (2007) argues that reductions in the number of restraint sessions or the number of hours of restraint results in a lack of dendritic

retraction and fails to impair spatial ability. However, others have reported that shorter restraint durations (2 hours per day for 10 days) do result in dendritic atrophy and debranching in CA3 pyramidal neurons (Vyas et al. 2002). Regardless, given the lack of spatial reference memory deficits seen in this experiment, it is worth considering that the use of 1 hour of restraint per session may have been insufficient and that future experimental iterations should adopt a longer restraint period.

4.2 Aluminum Results

4.2.1 Motor Findings

Finding #1: This experiment has found in male CD-1 mice that the chronic oral ingestion of aluminum chloride at intermediate levels leads to decreased locomotor activity as measured by horizontal movement. Further, chronic dietary intake of the SG toxin led to a similar decrease in locomotion. Unexpectedly, no cumulative facilitation of locomotor impairment was found for the combination of aluminum chloride and the SG toxin. Indeed, there was a possible ameliorating effect for the combination (Figure 3-5).

Locomotor performance is typically decreased following the chronic administration of aluminum chloride to rodents (Abdel-Aal et al. 2011b; Erazi et al. 2010; Hu et al. 2005). This experiment, which chronically administered aluminum chloride to mice, similarly led to decreased locomotor activity. The prediction that the multi-hit combination of the SG toxin and oral aluminum intake would lead to an incremental decrease of motor performance was not observed. This prediction was based on observations made by others that aluminum toxicity: leads to decreased TH labeling in the rat SN (Erazi et al.

2011), dose-dependently decreases densities of D1 and D2 receptors in the mouse striatum and cortex (Kim et al. 2007), enhances 6-OHDA induced striatal neurodegeneration and oxidative stress in rats (Sánchez-Iglesias et al. 2009) and *in vitro* impairs mitochondrial function in neuronal cells (Niu et al. 2005). These findings suggest that aluminum can act to impair neuronal function and further appears to specifically target dopaminergic neurons in the striatum and SN, which are known to be affected in SG-induced ALS-PDC (Banjo 2009; Tabata 2008).

The lack of a facilitated impairment effect on locomotor activity for the combination of aluminum chloride and SG may be a function of the poor ALS-PDC phenotype produced following 14 weeks of SG administration at 3mg per day, 5 times a week. Indeed, the SG toxin group did not display any locomotor disturbances such as decreased overall movement, in contrast to what has been reported previously following 15 weeks of SG administration at 1mg per day to CD-1 male mice (Tabata 2010). No confirmatory chemical analysis was conducted on the SG toxin supplied by Neurodyn Inc.; thus the possibility of chemical contamination cannot be excluded.

Of course, aluminum may simply not interact with this mouse model of ALS-PDC in a manner that leads to motor deficits. For instance, the observations which supported this thesis' prediction of a summative toxic effect between aluminum and the SG toxin, that aluminum leads to decreased TH labeling in the SN and that aluminum enhances 6-OHDA induced striatal neurodegeneration, were made in rats not mice (Erazi et al. 2011; Sánchez-Iglesias et al. 2009). Further, Golub et al. (2000) has noted that some

mouse strains such as the Swiss Webster and C57/BL/6J mice do not appear to be good models for studying Al-induced neurodegeneration; the SG toxin induced CD-1 male mouse model of ALS-PDC may similarly be a poor choice for examining the neurodegenerative effects of aluminum.

Finding #2: The chronic ingestion of oral aluminum chloride in male CD-1 mice resulted in gait disturbances as measured by stance width. Surprisingly, this stance width impairment was not seen for the SG toxin or the aluminum-SG combination (Figure 3-21). Further, despite significant increases in ataxia for some treatment groups, all groups had comparable levels of ataxia on the last testing session. The lack of treatment differences despite significant increases was in part due to differing baseline levels of ataxia (Figure 3-20).

Gait disturbances have been reported following the chronic intracisternal administration of aluminum chloride to white rabbits (Strong et al. 1991; Wakayama et al. 1996) and thus it was anticipated that chronic oral aluminum would similarly lead to gait anomalies. Indeed, as measured by increased stance width, chronic oral aluminum treatment led to gait impairment. However, others have reported no stride length or width deficits in rats chronically exposed to oral aluminum chloride (Walton 2009b).

An animal's gait pattern consists of a number of components of which motor coordination is one; a deficit in motor coordination would thus be expected to lead to a gait disturbance. One mechanism for assessing motor coordination is the rotarod

treadmill and this has been used to examine the effects of aluminum toxicity. Sahin et al. (1995) found that the ingestion of aluminum chloride led to deficits in motor performance on the rotarod test in mice. In contrast, Abdel-Aal et al. (2011b) reported no effect of chronic aluminum chloride on rotarod performance in rats. Therefore, as with direct measures of gait patterns noted above, there are mixed findings with respect to whether motor coordination is impaired by aluminum chloride in rodents. In this thesis it was found that aluminum chloride led to exaggerated stance width.

Of perhaps more interest is the lack of effect for the combination of aluminum and the SG toxin for stance width. Indeed, the addition of the SG toxin to aluminum negated the gait impairment observed with aluminum alone. As noted above, aluminum specifically targets the SN and the striatum and promotes toxin induced neurodegeneration in the BG (Erazi et al. 2011; Sánchez-Iglesias et al. 2009). These regions within the BG have been shown to be correlated with gait impairments, as the destruction of nigrostriatal neurons in the SNc using MPTP in mice leads to gait disturbances (Goldberg et al. 2011; Kurz et al. 2007). The SG toxin is further known to target the dopaminergic neurons in the striatum and the SN (Tabata 2008). Thus, aluminum and SG have overlapping sites of toxic action, and these neuroanatomical sites also act to mediate gait. This in turn led to the expectation of an additive impairing effect on gait.

The lack of such an effect may be due to an ineffective or partially effective SG toxin as already described. Another consideration is that gait disturbances in the mouse model of ALS-PDC do not arise in the striatum or SN. For instance, in PDC there is a severe

reduction in the number of DA neurons in the lateral and medial portions of the SN (Goto et al. 1990); however, no such pathology is described for gALS (Gibbs Jr. & Gajdusek 1972). Further, while the SG toxin does induce dopaminergic degeneration in the BG, the SG phenotype more closely resembles human gALS; by contrast, the BSSG toxin induced rat model is more characteristic of the PDC phenotype (Van Kampen 2014). This might suggest that SG induced gait impairment is not the result of toxic action at the level of the BG, but is secondary to another deficit. For example, in clinical ALS, gait performance strongly correlates with lower extremity muscle strength (Goldfarb & Simon 1984). As no deficit in neuromuscular strength was found for the SG toxin (see below), it may be that the severity of the symptomatology had not reached the threshold for clinically identifiable gait disturbances for the SG toxin, let alone the combination of the SG toxin and aluminum.

Finding #3: The administration of chronic oral aluminum chloride, the dietary SG toxin or the combination did not lead to treatment group differences in neuromuscular strength (Figure 3-2).

Chronic aluminum toxicity has been shown to dose-dependently reduce neuromuscular strength, as measured by grip strength, when given as aluminum chloride in mice (Hu et al. 2005) and when given as aluminum citrate from in-utero, in rats (Poirier et al. 2011). This led to the prediction that chronic water-borne aluminum chloride would produce neuromuscular impairments in mice. Further, as SG has been previously shown to impair both motor strength and spinal reflexes (Tabata 2008), it was predicted that the

combination of the SG toxin and aluminum would lead to a more severe impairment. In this experiment, no deficits were observed for neuromuscular strength for aluminum treated mice. Similarly, no impairments were seen for mice treated with the combination of SG and aluminum.

The lack of observable deficits in neuromuscular strength for the group receiving aluminum chloride may be a function of dose size. For instance, Hu et al.'s (2005) high and middle doses of aluminum chloride, at which grip strength deficits were observed, were 300mg/L and 50mg/L respectively; the low dose of 10mg/L used in their study did not result in neuromuscular deficits relative to control mice. In this experiment, aluminum chloride was administered in drinking water at 40mg/L, a dose which falls below Hu et al.'s (2005) middle dose. As the middle dose was the lowest dose at which a deficit was observed for Hu et al. (2005), the 40mg/L amount used in this thesis may not have been sufficient to produce a measurable deficit. This comparison is strengthened by methodological similarities between this experiment and Hu et al. (2005). For example, both studies used outbred adult male mice and the period of aluminum administration was roughly commensurate at 100 days for Hu et al. (2005) and at 138 days for this study.

A separate consideration is that the behavioural test used to assess neuromuscular strength, the WHT, may not have been sufficiently difficult to separate poor performers from normal performers. This is suggested by performance on the last WHT testing session, where the majority of mice, regardless of treatment group, continued to attain

the maximum score of 60s. Indeed, the inability of the WHT to distinguish between treatment groups is suggested by previous work using aluminum hydroxide injections (s.c.) in mice, which while showing *ex-vivo* pathology including increased motor neuron apoptosis, increases in reactive astrocytes and microglial proliferation within the spinal cord and cortex, found no motor strength or endurance deficits despite using near identical tests to those in this thesis (Shaw & Petrik 2009). This suggests that the lack of a significant effect on motor strength for the combination of aluminum and SG may be a function of the limitations of the test paradigm, rather than a lack of effect per se.

4.2.2 Anxiety Findings

Finding #1: Male mice exposed to chronic aluminum chloride, the SG toxin and the combination had increased levels of anxiety relative to control mice; these elevated levels of anxiety for the three non-control groups were comparable to one another (Figure 3-14; Figure 3-16).

The chronic application of aluminum to rodents has been shown to produce both anxiogenic (Sharma et al. 2013) and anxiolytic phenotypes (Rebai & Djebli 2008). For example, Erazi et al. (2010) found using a LDT that rats exposed since intra-uterine age to aluminum chloride in drinking water spent more time in the light portion of the box, a behaviour typically viewed as anxiolytic. Similarly, Rebai & Djebli's (2008) study using chronic aluminum chloride revealed a non-significantly higher preference for the light area of a LDT for aluminum treated mice. In contrast, Sharma et al. (2013), using an EPM, found that adult male rats treated intragastrically with aluminum lactate for 12

weeks spent significantly more time in the closed arms of the maze. In the context of the EPM, the time the test animal spends in the closed arms is considered to positively correlate with anxiogenic behaviour.

The experimental methodology used to assess anxiety in this thesis more closely parallels the studies by Rebai & Djebli (2008) and Erazi et al. (2010), which found anxiolytic phenotypes following aluminum toxicity, rather than the study by Sharma et al. (2013). For instance, the method of administration (oral) and the aluminum salt (aluminum chloride) used to produce toxicity in this thesis are identical with what was used in Rebai & Djebli (2008) and Erazi et al. (2010). As such, the prediction was that aluminum treated mice would display greater anxiety than controls. Further, it has been suggested based on anecdotal observations of motor behaviour that mice fed the SG toxin display increased anxiety (Tabata 2008), and thus it was predicted that SG fed mice would display increased anxiety relative to controls. The combination of aluminum and the SG toxin was not predicted to result in an incremental increase in anxiety.

The prediction for the combination of SG and aluminum is based on the concept that overt behavioural anxiety is secondary to the underlying disease state, and that a doubling of pathological toxicity (assuming a direct additive effect) is unlikely to be reflected in a doubling of behavioural anxiety measures. The inate phobic-like responses of mice to light, open spaces or height are likely to be a function by the mouse's perception of vulnerability. In other words, if the test mouse perceives itself to be sick or otherwise vulnerable, it will exhibit increased cautiousness. This self-

perception of vulnerability could arise from subtle motor impairments or a general feeling of malaise; however, while there are degrees of sickness, it may be artificial to think that mice behaviourally distinguish between slightly sick and slightly sicker.

In this experiment, the aluminum treated, the SG toxin treated and the mice treated with the combination all displayed increased anxiety relative to controls as measured by an increased latency to enter the light area of the LDT and by a decreased time in the open area of the EPM. These increases in anxiety were comparable for all three groups suggesting that no cumulative anxiety effect occurred for the combination of SG and aluminum. The aluminum group was also observed to have a trend decrease in the time in the centre of the OFT, though this is presumed to be an artifact of a trend higher baseline.

The observed preference for the dark areas of the LDT and the EPM is typically described as anxiogenic behaviour since both can be reduced through established anxiolytic drugs (Hascoët et al. 2001; Sánchez & Meier 1997). This increase in anxiety is a possible overt reflection of underlying neurodegenerative disease. While as already noted behavioural anxiety in rodents is likely secondary to a self-perception of vulnerability, increased oxidative stress has been reported in neuronal and glial cells in the cerebellum and hippocampus, in neurons of the cerebral cortex and in peripheral leucocytes of anxious but not non-anxious mice (Rammal et al. 2008). Oxidative stress has been implicated in many processes including neurodegenerative diseases and

Rammal et al.'s (2008) work suggests that the anxiogenic behaviours captured in the LDT and the EPM are reflective of an underlying disease state.

An interesting question is whether the disease state which underlies the observed anxiety is a consequence of the anxiety or a cause. Indeed, in the former case, it is possible that the aluminum or SG toxicity would lead to subtle impairments such as those described in Section 4.2.1, which in turn result in increased anxiety due to their mildly debilitating effects. This elevated anxiety would then *cause* oxidative stress in new or overlapping brain regions which may not have been affected by aluminum or SG toxicity. As this mechanism of anxiety could introduce ROS pathology into new brain regions, it would potentially create a feed forward loop, accelerating neurodegeneration. In the latter case, where the disease state is the direct cause of the anxiety, aluminum or SG toxicity would need to mediate the generation of ROS, through direct or indirect mechanisms, in the neurons and glia of the brain regions described by Rammal et al. (2008). In any event, whether the anxiogenic phenotypes observed in the LDT and the EPM are a cause or consequence of oxidative stress, they are suggestive of an underlying disease state.

Finding #2: The administration of chronic oral aluminum chloride, the SG toxin or the combination did not alter learned helplessness, as assessed by FST mobility, relative to controls (Figure 3-23).

Learned helplessness has been defined as an escape or avoidance deficit following uncontrollable stress; this is regarded as a depression-like coping deficit in aversive but avoidable situations (Vollmayr & Gass 2013). Further, learned helplessness has a suggested causal relationship with increased anxiety (Paul 1988) and therefore its quantification can provide insights into both anxiety and depression. The FST measures learned helplessness through the time the experimental mouse spends in immobility. Indeed, using immobility time in the FST as a proxy measure for depression has been validated by its reduction following the administration of anti-depressants (Yan et al. 2010).

Learned helplessness has been reported to be elevated in rodents following chronic exposure to aluminum compounds. For instance, Rebai & Djebli (2008) found, following the administration of oral aluminum chloride for 3 months, increased immobility time for aluminum treated mice. However, others have reported that stress tolerance, as measured by mobility in a swim test, was unaffected in rat pups chronically exposed to aluminum lactate (Gonda et al. 1996). Despite these differences on swimming tests, aluminum exposure is known to induce behavioural anxiety (Erazi et al. 2010). Thus, given the suspected reciprocal causal relationship between anxiety and learned helplessness (Paul 1988) it was anticipated that learned helplessness would be elevated in aluminum treated mice, as shown by Rebai & Djebli (2008). Moreover, the combination of aluminum and SG was expected to produce no increase in learned helplessness beyond what was observed for the SG toxin or aluminum alone. In this experiment, no significant differences were identified between treatment groups for

learned helplessness as measured by immobility time. Indeed, all groups displayed increased learned helplessness across testing sessions.

The lack of significant different findings for immobility time in the FST may be attributable to test stress itself. The FST involves water immersion where mice are forced to swim or float for an extended period. This stress is extremely anxiogenic for rodents (Moore et al. 2012) and could have obscured subtle mobility differences. Indeed, the increased immobility observed for all groups over the course of the experiment suggests a treatment-independent increase in anxiety. Further, the argument for a FST-induced stress which obscured treatment differences is strengthened by the observation that anxiogenic behaviours were elevated on the LDT and the EPM for all groups except the control group. This latter point suggests that had test induced stress been lower, the control group may have displayed significantly lower learned helplessness.

4.2.3 Memory Findings

Finding #1: This experiment has found that chronic oral ingestion of aluminum chloride, whether alone or in combination with the neurotoxin SG, does not result in cognitive or memory impairments (Figure 3-9; Figure 3-10).

The RAM is frequently used to assess the neurodegenerative effects of aluminum on cognition and memory. Indeed, oral aluminum chloride is reported to produce memory deficits in the RAM in rats (Abdel-Aal et al. 2011a; Abdel-Aal et al. 2011b). A common

feature of these aluminum-induced impairments on cognitive performance is that they are typically restricted to working and not reference memory. For example, Rebai & Djebli (2008), testing chronic exposure to aluminum chloride in mice, found spatial working memory but not reference memory deficits in a Morris water maze (MWM) paradigm. Ribes et al. (2008), also using a MWM, found that chronic dietary aluminum lactate led to acquisition but not retention (reference) memory impairment. It should be noted that spatial acquisition in the MWM is, while analogous, not identical to spatial working memory in the RAM as it involves place learning across a series of testing sessions with short inter-testing session intervals in the range of 15s (Vorhees & Williams 2006).

In the RAM, chronic oral aluminum chloride administration to aged rats has been shown to result in spatial working memory deficits in most rats (7 out of 10) in the high intake group (1.7mg Al/(kg bw day)), while few rats displayed spatial reference memory deficits (Walton 2009b). Importantly, *ex vivo* histology of rodents exposed to chronic aluminum treatment confirms its involvement in hippocampal neurodegeneration. For instance, Jovanović et al. (2014) found that intrahippocamal injections of aluminum chloride in rats resulted in strong amyloid β and tau staining in the hippocampus. Further, Çabuş et al. (2015) showed that aluminum sulfate, through apoptotic mechanisms, induced neuronal death in the stratum pyramidale of the hippocampus. Lastly, Sreekumaran et al. (2003) found that aluminum chloride injected in the cerebral spinal fluid of rats led to reductions in both axonal length and dendritic branching in the hippocampus. These findings led to the prediction that chronic aluminum chloride, delivered orally to male

mice, would lead to spatial working memory but not spatial reference memory deficits through aluminum's toxic actions on the hippocampus. Further, as the clinical ALS-PDC disease phenotype has a dementia component (Lee 2011), it was hypothesized that the combination of the SG toxin and chronic aluminum chloride would lead to an increase in the severity of the memory impairments seen with aluminum alone. An examination of the RAM results in this thesis showed that neither chronic aluminum chloride alone or in combination with the SG toxin led to measurable behavioural deficits in memory performance on the RAM.

The lack of a memory deficit in the RAM for the combination of the SG toxin and aluminum chloride may be explained by the lack of findings for either treatment alone. The reason for this is that the hypothesis for a combined effect on working memory was predicated on the presumed overlapping regions of toxic action in the brain. This neurodegenerative effect was anticipated to be additive as there was no evidence to suggest a synergistic effect between aluminum and SG toxicity. As no effect was observed for either aluminum or SG alone, a behaviourally measurable effect for the combination would have required subclinical effects for each treatment. The data for memory performance in the RAM does not suggest that subclinical effects exist for aluminum or SG alone (this might be marked by a non-significant difference between treatments), which would explain the lack of an observable effect for the aluminum-SG combination.

The lack of working memory impairment in the RAM following aluminum exposure to rodents has been observed by others. Santucci et al. (1994) examining early exposure to aluminum sulphate which was given i.p. to pregnant females, found no deficits in RAM performance at 70 days in male progeny, though they did report significantly elevated nerve growth factor in the hippocampus, suggesting the occurrence neuroprotective action. Moreover, Struys-Ponsar et al. (1997) treating adult rats with i.p. injections of aluminum gluconate, saw no working or reference memory impairments. However, Struys-Ponsar et al. (1997) did identify increased aluminum concentration in the hippocampus and neocortex. Despite the conflicting findings on memory performance for the RAM following aluminum toxicity, nearly all studies which have conducted *ex-vivo* histology or immunochemistry indicate that the hippocampus is selectively altered in one manner or another by aluminum.

That no RAM impairments were found in this thesis may be a function of the age of the test animals. Walton's (2009) study examining RAM performance in rats following chronic oral aluminum chloride focused on mid- to late-life (rats aged 12-28 months). The mice in this study were only followed until mid-life (12 months) before experimental termination. Of note, aluminum is known to gradually build up in the human brain with age (Roider & Drasch 1999) and thus animal age would need to be accounted for when comparing aluminum studies involving different parts of the lifespan. There are numerous other experimental considerations such as sex, strain, and species which could possibly explain the different findings, however, two further points are worth mentioning. The first is the route of administration which varies widely between studies.

As Exley (2008) points out, the delivery potential of aluminum is route-dependent. Indeed, the majority of aluminum that normally enters the brain is rapidly effluxed, meaning that an administration of aluminum which necessitates the crossing of the BBB would require a much higher dose than a comparable administration directly into the brain extracellular fluid. In order to compare behavioural effects between studies in a meaningful manner, one would need to ascertain how much aluminum is actually reaching the brain. The second point relates to the aluminum salt that is used. As noted in the introduction (see Section 1.3.2), not all aluminum salts act in the same way. In particular, aluminofluoride compounds have their own unique toxic actions (Strunecká et al. 2002). Further, water-borne aluminum salts are likely to result in higher brain tissue levels, as compared to more insoluble aluminum species (Amstrong et al. 1992). Taken together these points illustrate the difficulties of direct comparisons between animal studies of aluminum toxicity and the care that must be taken in assessing whether a finding in one model can be applied to another.

4.3 Conclusion

This thesis has found that the administration of chronic restraint stress leads to, relative to controls, an increase in behavioural measures of anxiety and the appearance of gait impairments as measured by stance width. The combination of the SG toxin and chronic restraint stress ameliorated both the gait impairments and the anxiogenic behaviours seen with stress alone. No cognitive effects were observed for the SG toxin, chronic restraint stress or the combination.

This thesis has also shown that mice given aluminum treated water display, relative to controls, reduced locomotion, some gait impairments and an increase in behavioural measures of anxiety. The combination of aluminum with the SG toxin led to similarly suppressed levels of locomotion and increased anxiety relative to controls. No significant cognitive effects were captured for the SG toxin group, chronic restraint stress group or the combination.

The evidence in this thesis suggests a role for both chronic stress and water-borne aluminum in mediating behavioural outcomes of age-related neurodegenerative disease. These behavioural changes have been observed to be progressive and test-dependent. Further, though not tested here, animal sex would likely strongly influence the impact of these environmental factors on the ALS-PDC murine model, as sex has been previously observed to influence pathologies in this model (Banjo 2009).

Importantly it was worth noting that the high degree of within group variability observed on most of the behavioural tests conducted herein may well be a reflection of varying degrees of individual susceptibility. Indeed, clinical susceptibility to neurodegenerative disease risk is linked to environmental exposures which occur years prior to insidious onset and pathological phenotypes (Tanner et al. 2014). Thus early-life exposure to aluminum or chronic stress might lead to increased risk of a late-life neurodegenerative disorder; this could readily be experimentally examined through aluminum or chronic restraint stress exposure during rodent adolescence and continued behavioural observation into senescence.

There a small but growing community that recognizes that aside from some rare genetic exceptions, the clinical development of age-related neurodegenerative disease is the product of some combination of genetic- and gestation-mediated susceptibility, age, lifestyle and environmental factors. This understanding gives rise to the multi-hit hypothesis and the need to scientifically examine potential disease triggers and their consequences. This was achieved in this thesis and the findings give rise to several provocative lines of future research.

Firstly, the implications of a possible chronic stress mediated protection of age-related muscular wasting are worth considering. For instance, while this effect could be a function of increased exercise, there is also the possibility that HPA controlled GCs are acting in a preventative manner on selective portions of the motor system. Indeed, the results of this thesis do not reveal any motor deficits resulting from chronic restraint stress; rather they are suggestive of a protective role. This could be investigated through the administration of GCs to the ALS-PDC in an age and dose-dependent manner.

Secondly, it is intriguing that while aluminum appears to induce gait disturbances, these are not accentuated, and indeed somewhat ameliorated, when aluminum is combined with the SG toxin. There was reason to expect an incremental worsening of symptoms when aluminum was combined with the ALS-PDC; both induce overlapping fields of degeneration within BG structures and destruction of neurons in these areas has been

correlated with gait disturbances. The possibility that aluminum toxicity has been masked or mitigated through the insidious pathogenesis of ALS-PDC is an interesting hypothesis. This could be both behaviourally and histopathologically investigated through treatment groups combining high and low doses of both the SG toxin and high and low doses of the aluminum treatment. If indeed there is an ALS-PDC masking effect, the expectation would be that the high dose of the SG toxin and the high dose of aluminum would lead to a lower presentation of gait disturbances than the low dose of SG and the high dose of aluminum.

In summary, this thesis sought to expand upon our current academic understanding of how chronic stress and aluminum influence age-related neurodegenerative disease. While some answers have been provided, the results also generate further research questions to be probed. Indeed, these research questions point to the need for the continued investigation of such multi-hit hypotheses, for only through the aggregated sum of increasingly complex gene-environment factor analysis will the causes underlying ‘idiopathic’ disease be revealed.

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