TARGETING GLUTAMATE IN PROSTATE CANCER-INDUCED DEPRESSION

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By

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Lay Abstract

Prostate cancer affects one in every eight Canadian men. Cancer patients are at a much higher risk of developing depression than the rest of the population. Unfortunately, current antidepressants are limited in their ability to improve depressive symptoms in cancer patients. Therefore, this project sets out to identify new options for treating depression in prostate cancer patients. Glutamate is a signalling molecule that is released in abundance by cancer cells and is largely responsible for communication between neurons in the central nervous system. This project showed that limiting the amount of glutamate released by cancer cells and limiting glutamate-based signaling improves depressive-like symptoms in mice with prostate cancer tumours. These results suggest that targeting glutamate could be an effective antidepressant therapy in the cancer population.

Abstract

Affecting one in every eight Canadian men, prostate cancer is the most common type of cancer among males. As with other forms of cancer, men with prostate cancer are much more likely to develop comorbid depression than the general population without cancer diagnoses. Depression negatively affects these men's quality of life and increases mortality rates among cancer patients. Therefore, effective therapies to manage depression in this unique subpopulation are needed. This project sets out to assess the efficacy of glutamate-targeting drugs as antidepressants. The major excitatory neurotransmitter in the central nervous system, glutamate is released in excessive quantities by cancer cells. It is thought that this abundance of glutamate leads to excitotoxicity and neurodegeneration, affecting neurons in important regions of the brain relating to mood and mood regulation. A validated mouse model of depression was established using RM1 murine prostate cancer cells. This model was then used to test the properties of three drugs: sulfasalazine (SSZ), (*S*)-4-carboxyphenylglycine ((*S*)-4-CPG), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo[*f*]quinoxaline-7-sulfonamide (NBQX). Results show that these drugs were able to improve depressive-like behaviours and symptoms to varying degrees, at least partially reversing the negative effects of tumours. This project showed that disrupting glutamate release and/or signaling could be an effective approach for an antidepressant therapy or adjuvant in prostate cancer patients.

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To mom and dad – I'm fairly certain that, to this day, you have no idea what it is that I do for my Master's; but, that is a testament to your unconditional love and support. Your unbounded work ethic, exceptional dedication to your family, and expansive intellect inspire me to ask more of myself every day. The driving force for all that I do in life is the hope that, one day, I may make all the sacrifices you have made for me worthwhile.

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List of Abbreviations

^{1}H-MRS	Proton magnetic resonance spectroscopy
5-HT	5-hydroxytryptamine
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AVP	Arginine vasopressin
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
CRHR	Corticotropin-releasing hormone receptor
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EAAT	Excitatory amino-acid transporter
ECF	Extracellular fluid
ERK	Extracellular signal-regulated kinase
FST	Forced swim test
GABA	γ-aminobutyric acid
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element

GSH	Glutathione
HAT	Heteromeric amino acid transporter
HPA	Hypothalamic-pituitary-adrenal
HPLC	High-performance liquid chromatography
HR	Hazard Ratio
HRP	Horseradish peroxidase
HSP	Heat shock protein
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukin
IP$_3$	Inositol-1,4,5-triphosphate
KYN	Kynurenine
KYNA	Kynurenic acid
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinases
MC2R	Melanocortin type 2 receptor
mGluR	Metabotropic glutamate receptor
MMP	Matrix metalloprotease
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo[*f*]quinoxaline-7-sulfonamide
NMDA	*N*-methyl-D-aspartate
OFT	Open field test
PCaID	Prostate cancer-induced depression
PIP$_2$	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C

PLC	Phospholipase C
PSA	Prostate-specific antigen
PVN	Paraventricular nucleus
QUIN	Quinolinic acid
RCF	Relative centrifugal force
RHT	Retinohypothalamic tract
ROS	Reactive oxygen species
(*S*)-4-CPG	(*S*)-4-carboxyphenylglycine
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptor
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
SSZ	Sulfasalazine
TAM	Tumour-associated macrophage
TCA	Trichloroacetic acid
TNF	Tumour necrosis factor
TST	Tail suspension test
VGLUT	Vesicular glutamate transporter

Declaration of Academic Achievement

I, Kimberly Young, performed all animal handling, testing, sacrificing, and lab-based assays, procedures, and experiments related to this project.

Animal surgeries were assisted by several of my lab colleagues who aided me in anesthetizing and preparing mice – including administering analgesic and shaving. I am also thankful to Robert Ungard and Tanya Miladinovic who stepped in to perform several surgeries for me one day when I had fallen ill. With the exception of that particular subset of animals, I performed all other animal surgeries.

CHAPTER 1: Introduction

Prostate Cancer

Prostate cancer is the fourth most common type of cancer overall, accounting for 11% of all new cancer diagnoses and 21% of male cancer diagnoses (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016). It is estimated that 1 in 8 males in Canada will be diagnosed with prostate cancer in their lifetime, with 1 in 27 dying as a result (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016; Fradet, Klotz, Trachtenberg, & Zlotta, 2009). In 2008, the Canadian Cancer Society estimated an incidence rate of 129 per 100,000 males, as well as a mortality rate of 23.6 per 100,000 males (Fradet et al., 2009). Historical increases in incidence rates of prostate cancer can be largely attributed to improved means of early detection as a result of the prostate-specific antigen (PSA) blood test, which is used as a screening tool (Fradet et al., 2009). Two peaks in incidence rates in 1993 and 2001 corresponded to two periods of intensive screening using the PSA test **(Figure 1.1)** (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016). At present, incidence rates are declining by 1.5% per year (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016). With a sharp spike in incidence past the age of 50, prostate cancer is most common in males between the ages of 60 and 69, who account for 40% of all cases (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016)

Early detection of prostate cancer resulting from widespread use of the PSA test has yielded improvements in early intervention. Yet, still, prostate cancer accounts for 10% of all male cancer-related deaths, the third deadliest form of cancer for males (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016).

Beyond the epidemiological statistics, prostate cancer highly impacts the Canadian healthcare system. Prostate cancer poses a significant economic burden, with direct medical costs estimated to total $9.76 billion (Fradet et al., 2009).
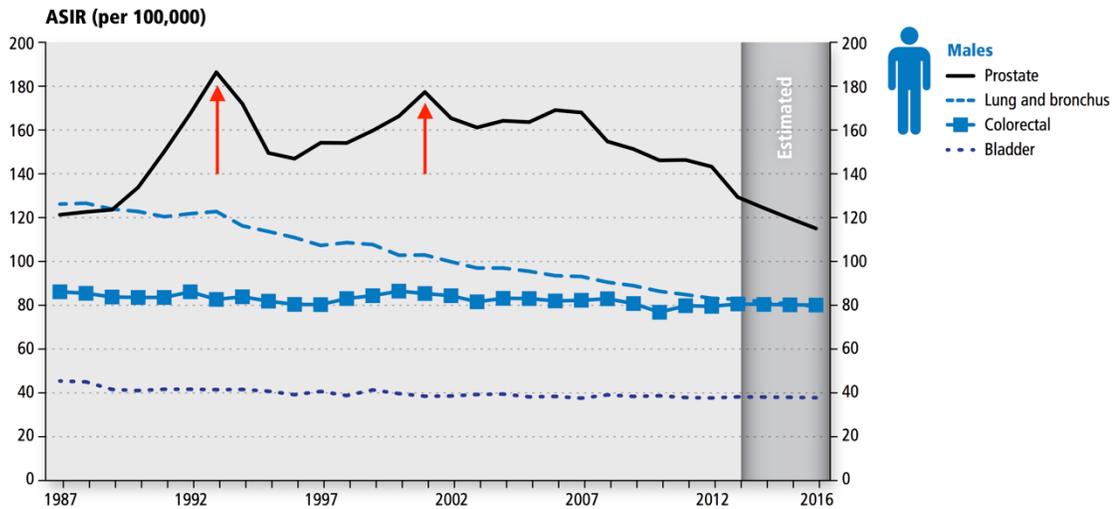
Figure 1.1: Age-standardized incidence rates (ASIRs) for several male cancers in Canada. Prostate cancer is denoted by the solid black line. Two peaks in incidence rates are indicated with red arrows (Image adapted from Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2016).

Prostate Cancer and Depression

It seems rather intuitive that cancer patients will be negatively impacted by their diagnoses; however, one must be cautious to differentiate between a patient who is appropriately sad versus one who is clinically depressed. As it happens, depression is the only psychological disorder that disproportionately affects cancer patients relative to the

general population (Satin, Linden, & Phillips, 2009). The incidence rate for depression is two to three times higher in the cancer population compared to the general population (Currier & Nemeroff, 2014; Watts et al., 2015). It is estimated that a third of cancer patients will find the experience distressing and have a comorbid psychiatric disorder, with the risk of suicide rising to approximately 2.5 times that of the general population in the first year post-diagnosis (Currier & Nemeroff, 2014; Fradet et al., 2009; Prasad et al., 2014). However, interpreting depression within the cancer population as purely a reactionary outcome ignores the biological mechanisms and processes that play a part in its onset, undermining the legitimacy and significance of depression in the context of cancer. It is interesting to note that Prasad et al. (2014) found that 4.6% of men were diagnosed with a depressive disorder in the two years preceding their prostate cancer diagnosis. Similarly, a meta-analysis done by McGee, Williams, & Elwood (1994) reviewed seven prospective longitudinal studies in order to investigate the relationship between depression and cancer. They found evidence of a link between depressive symptoms and a later diagnosis of cancer, though this relationship did not reach statistical significance. Another study also identified depression's robust effect on the incidence of cancer, increasing the Hazard Ratio (HR) by 29% in addition to elevating the mortality HR by 34% (Chida, Hamer, Wardle, & Steptoe, 2008). The temporality of these three studies do not support the idea that being diagnosed with cancer is a necessary antecedent to the development of depression. Though a causal link between depression and cancer cannot be concluded with certainty, this data suggests that the environment that fosters the progression of cancer could beget depression at the molecular level in spite of the

patient being unaware of their disease status. The psychological impact of a concrete

diagnosis could certainly exacerbate depressive symptoms later on. Fears surrounding

treatment or perceived threats to masculinity, like erectile dysfunction, could also

contribute to feelings of depression or anxiety; these fears are especially pervasive among

elderly men (Llorente et al., 2012; Prasad et al., 2014). Further investigations into how

the biology of cancer and the cancer environment may contribute to the development of

depression may inform treatment options for this unique subpopulation.

Cancer patients could highly benefit from effective therapies to manage their

depressive symptoms. Men with prostate cancer who have comorbid depression

experience a poorer quality of life and are less likely to seek or comply with definitive

treatments, a potential contributing factor to the correlation between depression and

mortality in the cancer population (Jehn et al., 2006; Pinquart & Duberstein, 2010; Prasad

et al., 2014; Spiegel & Giese-Davis, 2003). Similar effects in terms of mortality were

found between studies that looked at depression preceding a cancer diagnosis and those

that looked at depression after the patient was diagnosed with cancer (Pinquart &

Duberstein, 2010). Cuijpers et al. (2014) reported a relative risk of excess mortality in

depressed cancer patients of 1.61 (95% CI = 1.56-1.90) and Lloyd-Williams, Shiels,

Taylor, & Dennis (2009) identified depression as an independent predictor of elevated

mortality in cancer. Cancer patients who exhibit depressive symptoms have a 26% higher

mortality rate, while those who receive a clinical diagnosis of major depressive disorder

experience a 39% greater mortality rate (Satin et al., 2009). The difference between those

two statistics suggest that clinical depression may be more likely than the presentation of

depressive symptoms to impact a patient's will to live; not surprisingly, this has negative implications for the desire to pursue or adhere to therapies and may increase suicide risk (Pinquart & Duberstein, 2010). It is important to consider this difference: a) when conducting animal studies, where we can only measure depressive symptoms and are unable to ascertain a medical diagnosis of depression; and b) when making interpretations about studies, as the framework for how depression is defined may affect its ability to be detected. For example, different studies utilize different criteria for depression – while some only look at patients with a clinical diagnosis of depression, others consider patients that present with symptoms without an accompanying medical diagnosis (Pinquart & Duberstein, 2010). In this regard, it is important to note that patients with depressive symptoms that are below criteria threshold for a diagnosis of depression are often overlooked in studies; therefore, available statistics and analyses may not be wholly representative of the scope of problem that depression poses within the cancer population (Pinquart & Duberstein, 2010). For example, the prevalence of depression has been found to be as low as 2% and as high as 58% in cancer patients, depending on how depression was assessed (Chochinov, 2001; Hinz et al., 2016; C. W. Jackson & Jackson, 2007; Jehn et al., 2006; H. Smith, 2015; Snyderman & Wynn, 2009). Criteria for a diagnosis of major depression also include symptoms that may overlap with symptoms of cancer or cancer treatment, making it harder for studies to accurately identify depression in cancer patients (Jehn et al., 2006). Another limitation of many studies that analyze the impact of depression on mortality is that they can only speak to the effects on all-cause mortality,

unable to discern cancer-specific deaths because exact causes are often unknown or not disseminated (Satin et al., 2009).

Contributing to elevated mortality, there exists a high suicide rate among men diagnosed with cancer; specifically, among men over the age of 65 who are 4.24 times more likely to commit suicide than their healthy counterparts. The risk for suicide may even be underestimated, as deaths of patients who commit suicide at home may be misinterpreted as being a result of cancer or natural causes (Llorente et al., 2012). The fact that patients with depression are less likely to have social support compared to patients without depression could be a contributing factor to negative feelings of isolation that drive patients to consider suicide (Lamkin et al., 2011; Pinquart & Duberstein, 2010). Llorente et al. (2012) also found that a quarter of the cancer population they sampled viewed cancer-induced pain as suicidal motivation. This is especially poignant given that prostate cancer is ranked second in terms of painful malignancies and, therefore, pain resulting from prostate cancer is commonplace and significant (Llorente et al., 2012). The presentation and impact of pain is further compounded by societal gender norms, whereby men are thought of as having to be strong and able to cope with pain. These statistics highlight the importance of proactive efforts to monitor mental health statuses of patients for the duration of cancer treatment, as early interventions to ameliorate depressive symptoms have been shown to lead to improved survival (Prasad et al., 2014). Health care providers should also strive to be self-aware of subconscious biases or stereotypes that influence how they interact with and treat cancer patients with depression (Prasad et al., 2014)

Depression

Inflammation

People with major depression have been found to have elevated levels of pro-inflammatory cytokines (Pace & Miller, 2009; Satin et al., 2009). However, the locus of activation of inflammatory pathways has not yet been pinpointed (Miller, Maletic, & Raison, 2009). Cytokines are released both peripherally, by macrophages and lymphocytes, and centrally, by astrocytes and microglia (Reiche, Nunes, & Morimoto, 2004). Psychological and psychosocial stressors are both capable of initiating inflammation and can do so centrally or peripherally (Leonard, 2017; Miller et al., 2009). Chronic stress is correlated with increases in C-reactive protein (CRP) and interleukin (IL)-6, among other inflammatory mediators, and seems to act largely through microglia (Miller et al., 2009). The activation of immune pathways in the periphery – perhaps as a result of an infection – begets decreased neurogenesis in the brain, specifically key areas for behaviour and cognition, and neuroinflammation (Leonard, 2017; Miller et al., 2009). Any prolonged stress response can have negative consequences for the brain and peripheral organs (Leonard, 2017). Regardless of location of origin, cross-talk between the central nervous system (CNS) and periphery plays a large role in the onset of depressive symptoms during inflammation (Bortolato et al., 2017). It is also of interest to note that the relationship between inflammation and depression may be bidirectional: while cytokines may promote depression, the reverse situation where depression upregulates inflammatory signaling could also be true (Aldea, Craciun, Tomuleasa, & Crivii, 2014).

Pro-inflammatory cytokines are thought to promote abnormalities complicit in the pathophysiology of depression, including neurotransmitter metabolism, neural plasticity, and neuroendocrine function (Currier & Nemeroff, 2014; Miller et al., 2009). Given the role of inflammation in depression, it is not hard to conceptualize how these conditions may manifest themselves in the context of cancer. Even in the early stages of cancer progression, tumours initiate an inflammatory response from the body and produce pro-inflammatory cytokines, including IL-6, CRP, and tumour necrosis factor (TNF)-α (Aldea et al., 2014; Lamkin et al., 2011; Pyter, Pineros, Galang, McClintock, & Prendergast, 2009). Cytokines are also produced as a result of cell death incurred by cancer treatments such as chemotherapy or radiation, which recruits immune cells to the site of injury, as well as inducing the production of cytokines by neighbouring cells and initiating signaling pathways (Aldea et al., 2014; Currier & Nemeroff, 2014).

Characterizing key biomarkers that contribute to depression would enable clinicians to identify patients who are at increased risk of developing depression and/or those for whom conventional therapies may not be as effective. For example, a study by Jehn et al. (2006) found that patients with depression had a significantly higher plasma concentration of IL-6 relative to patients without depression. Produced by both immune and non-immune cells, like tumour cells and tumour-associated macrophages (TAMs), IL-6 is one of the key biomarkers of depression (Jehn et al., 2010; Lutgendorf & Sood, 2011). Maes et al. (1997) noted increased serum IL-6 levels not only in depressed patients, but also patients with treatment resistant depression. As a biomarker, IL-6 has a proposed sensitivity of 79% and a specificity of 87% (Aldea et al., 2014).

Neurotransmitter Metabolism

Monoamine neurotransmitters have long been known to have significant roles in mood regulation in the brain. Of the various monoamines, which include dopamine and norepinephrine, serotonin – also referred to as 5-hydroxytryptamine (5-HT) – has perhaps garnered the most attention, especially with the wide range of selective serotonin reuptake inhibitors (SSRIs) that have emerged as antidepressants. Cytokines are able to dysregulate serotonin synthesis via their ability to activate the enzyme indoleamine 2,3 dioxygenase (IDO), which has been found to be overly expressed in cancer and is widely distributed in the brain, kidneys, lungs, and immune cells (Aldea et al., 2014; Bortolato et al., 2017; Leonard, 2017; Myint & Kim, 2003; Pace & Miller, 2009). IDO converts tryptophan, the primary amino acid precursor of serotonin into kynurenine (KYN). The consequences of IDO activation are two-fold: a) decreased levels of tryptophan, resulting in serotonin deprivation; and b) activation of the KYN pathway, the process whereby KYN is converted into neurotoxic metabolites (Aldea et al., 2014; Bortolato et al., 2017; Miller et al., 2009; Myint & Kim, 2003; Pace & Miller, 2009).

In astrocytes, KYN is converted to kynurenic acid (KYNA) whereas it is preferentially converted into quinolinic acid (QUIN) in microglia (Bortolato et al., 2017; Miller et al., 2009). QUIN is a potent *N*-methyl-D-aspartate (NMDA) receptor agonist, resulting in excess glutamate release, oxidative stress, and astrocyte apoptosis – all of which lead to neurodegeneration and neural excitotoxicity linked to depression (Aldea et al., 2014; Bortolato et al., 2017; Currier & Nemeroff, 2014; Guillemin, Wang, & Brew, 2005; Leonard, 2017; Miller et al., 2009). Stress and inflammation are both capable of

activating microglia, which may be associated with the neurodegenerative pathway that contributes to decreases in brain volume observed in patients suffering from chronic depression (Bremner et al., 1999; Guillemin et al., 2005; Leonard, 2017). KYNA, on the other hand, has suggested neuroprotective properties given its ability to inhibit glutamate release and its antagonistic effect on the NMDA receptor (Lamkin et al., 2011; Leonard, 2017; Miller et al., 2009; Myint & Kim, 2003).

Moreau et al. (2008) developed an animal model of depressive-like symptoms in mice by inducing chronic inflammation using *Bacillus Calmette-Guerin* (BCG), a chronic activator of IDO both in the brain and the lungs. In the immediate aftermath of inoculation, only sickness behaviours were noted; however, these were later replaced with depressive-like behaviours that were sustained for several weeks. This suggests an association between inflammation, IDO, and depression that could be at play in the cancer environment.

Neural Plasticity

Within the brain, cytokines including IL-6 and TNF-α are typically tasked with promoting neurogenesis and offering neural trophic support; however, overactive immune pathways lead to dysregulation of these processes. This leads to a reduction in neural growth, in addition to increases in oxidative stress and glutamate release described in the previous section (Miller et al., 2009). Altogether, these abnormalities result in excitotoxicity that disrupts the plasticity of neural networks (Bortolato et al., 2017).

An increase in glutamate release as a result of the action of cytokines is further coupled with a downregulation of glutamate transporters on glial cells, which means that

the increased synaptic concentrations of glutamate are further exacerbated by reduced reuptake (Miller et al., 2009). Under physiological conditions, astrocytes work to regulate local glutamate concentrations; however, under conditions of prolonged glutamatergic activation, NMDA receptors are over stimulated and neural apoptosis occurs as a result (Leonard, 2017; Miller et al., 2009).

Furthermore, these cytokines may promote the release of reactive oxygen species that contribute to oxidative stress. Glial cells in various brain regions that are significant in mood regulation, like the medial prefrontal cortex (mPFC) may become damaged in these environments and a pathological brain morphology that contributes to the onset of depression may be established (Miller et al., 2009).

Neuroendocrine Function

The activity of the immune system can also be linked to the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Leonard, 2017; Reiche et al., 2004). Cytokines, like TNF-α, IFN-α, and IFN-γ, have been demonstrated to potently stimulate the HPA axis (Raison & Miller, 2003; Reiche et al., 2004). The key players of the HPA axis and its various mechanisms of action will be discussed in detail next.

HPA Axis

One of the theories surrounding the biology of depression that may be especially relevant in cancer is the role of the HPA axis. When various stressors threaten homeostasis, our bodies initiate a coordinated stress response from the immune, endocrine, and nervous systems to mediate the stimuli. The primary actors involved are hypothalamus, pituitary gland, and adrenal gland; therefore, as the name suggests, they

are collectively referred to as the hypothalamic-pituitary-adrenal, or HPA, axis **(Figure 1.2)** (S. M. Smith & Vale, 2006). It is thought that chronic activation of this network leads to dysregulation of the HPA axis, resulting in many negative consequences for the body and homoeostasis (Pinquart & Duberstein, 2010; Satin et al., 2009). Hyperactivity of the HPA axis has been robustly proven to be a hallmark characteristic of major depressive disorder (Pace & Miller, 2009).

The activity of the HPA axis begins in the paraventricular nucleus (PVN) of the hypothalamus, specifically the parvocellular subdivision. Here, hypophysiotropic neurons are responsible for producing and releasing corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) in response to aversive stimuli. Within the median eminence of the hypothalamus, CRH is secreted into the hypophyseal portal system, a network of blood vessels that connect the hypothalamus with the anterior pituitary gland – the next structure that makes up the HPA axis. The anterior pituitary gland contains corticotrophs which express two subtypes of CRH receptors (CRHRs), CRHR1 and CRHR2, though CRH binds to CRHR1 with greater affinity relative to CRHR2. After CRH binds to its receptor, which is a G-protein coupled receptor (GPCR), adenylyl cyclase is activated and initiates a cyclic adenosine monophosphate (cAMP) pathway. Ultimately, the outcome of this cascade is the release of adrenocorticotropic hormone (ACTH) from the pituitary cotricotrophs into the systemic circulation **(Figure 1.2)** (Pariante & Lightman, 2008; S. M. Smith & Vale, 2006).

The receptor for ACTH is known as the melanocortin type 2 receptor (MC2R) and can be found in the adrenal cortex, specifically on parenchymal cells of the adrenocortical

zona fasciculata. The binding of ACTH to its receptor initiates yet another cAMP pathway; this time, the end result is the production and secretion of glucocorticoids, among other steroid hormones including mineralocorticoids. The predominant glucocorticoid in humans is cortisol, while the equivalent in rodents is corticosterone. Both serve to regulate a plethora of metabolic and immune processes. Glucocorticoids bind to glucocorticoid receptors (GRs), which are widely expressed both centrally and peripherally. In the inactive state, the GR resides in the cytoplasm as part of a multimeric complex of chaperon proteins, including several heat shock proteins (HSPs). The binding of glucocorticoids induces a conformational change into its active form, dissociating GR from its complex so that it may translocate to the nucleus. Once inside the nucleus, the GR may bind to glucocorticoid response elements (GREs) or transcription factors to regulate the expression of various target genes (Pariante & Miller, 2001; S. M. Smith & Vale, 2006).

The HPA axis is regulated by negative feedback, whereby glucocorticoids – released as a result of HPA axis activity – binding to their respective GRs subsequently turns it off (Blackburn-Munro, 2004; Pariante & Miller, 2001). Unlike the mineralocorticoid receptor (MR), which has a high affinity for corticosteroids, the GR has a low affinity for endogenous steroid hormones. As a result, during a stress response when concentrations of these substances are higher than their basal levels, the GR is thought to be more important than the MR for regulation (Pariante & Lightman, 2008). Negative feedback is thought to occur at both the hypothalamus, on CRH secretion, and the pituitary, on ACTH release **(Figure 1.2)** (Pariante & Lightman, 2008; S. M. Smith &

Vale, 2006). The hippocampus has also been suggested as a possible regulatory site based on its density of GRs and observations that the stress response is muted following infusion of glucocorticoids into this region (S. M. Smith & Vale, 2006). Yet, although these regulatory mechanisms are in place, a consistently observed pathophysiology in patients with major depression is hyperactivation of the HPA axis (Miller et al., 2009; Pariante & Miller, 2001). This aberrant behaviour is thought to result from faulty feedback inhibition due to desensitized GRs and, therefore, reduced responsiveness to glucocorticoids (Jehn et al., 2006; Leonard, 2017; Miller et al., 2009). Proinflammatory cytokines are thought to contribute to this desensitization by impairing the translocation and/or function of GRs (Jehn et al., 2006). As a result, hypercortisolemia is seen in approximately half of the depressed population, rising to an 80% prevalence among those with severe depressive disorder (Pariante & Miller, 2001). Prolonged exposure to high concentrations of glucocorticoids may have pathological effects on brain morphology; for example, Colla et al. (2007) reported that depressed patients have decreased hippocampal volumes compared to healthy controls, while de Kloet, Joels, and Holsboer (2005) discussed the decrease in dendritic branching resulting from chronic stress. Studies have shown antidepressants to be effective in upregulating GR expression and normalizing GR function, allowing the HPA to respond appropriately to negative feedback mechanisms (Montkowski et al., 1995; Pepin, Pothier, & Barden, 1992).

A frequently discussed side-effect of HPA axis dysregulation is abnormal circadian rhythms of cortisol release. This phenomenon can be attributed to the interaction between the HPA axis and the circadian clock system. Two components

combine to form the ubiquitous internal circadian clock: a central "master" clock and peripheral "slave" clocks located in tissues and organs. The master clock is found in the suprachiasmatic nucleus (SCN) located in the ventral hypothalamus. With the retinohypothalamic tract (RHT) acting as a conduit, the master clock receives light/dark information from the eyes to ascertain the time of day. If environmental cues dictate the release of glucocorticoids, the master clock may orchestrate this using two mechanisms: 1) an HPA-dependent mechanism, whereby SCN neurons communicate with PVN neurons to release CRH or AVP **(Figure 1.2)** or 2) an HPA-independent mechanism, whereby the SCN communicates directly with the adrenal gland via the splanchnic nerve of the autonomic nervous system (ANS); this allows the SCN to modulate the sensitivity of the adrenal cortex to ACTH based on light information, with peak sensitivity corresponding to peak activity.

In humans, the active phase is in the morning while the opposite is true for nocturnal animals, like rodents. Therefore, humans experience an increase in cortisol levels in the morning whilst a decrease is seen in the evening. However, when there is chronic input to the PVN as a result of stressors, a blunting of the typical evening decrease in glucocorticoids is seen (Nader, Chrousos, & Kino, 2010; Son, Chung, & Kim, 2011). For example, Jehn et al. (2010) found that patients with major depression had higher cortisol concentrations relative to non-depressed patients both in the morning, at 8:00 AM, and in the evening, at 8:00 PM. Miller et al. (2009) report a correlation between cortisol blunting and IL-6 levels in patients in the advanced stages of cancer, which corroborates what we know about the role of inflammation in inducing HPA activity.
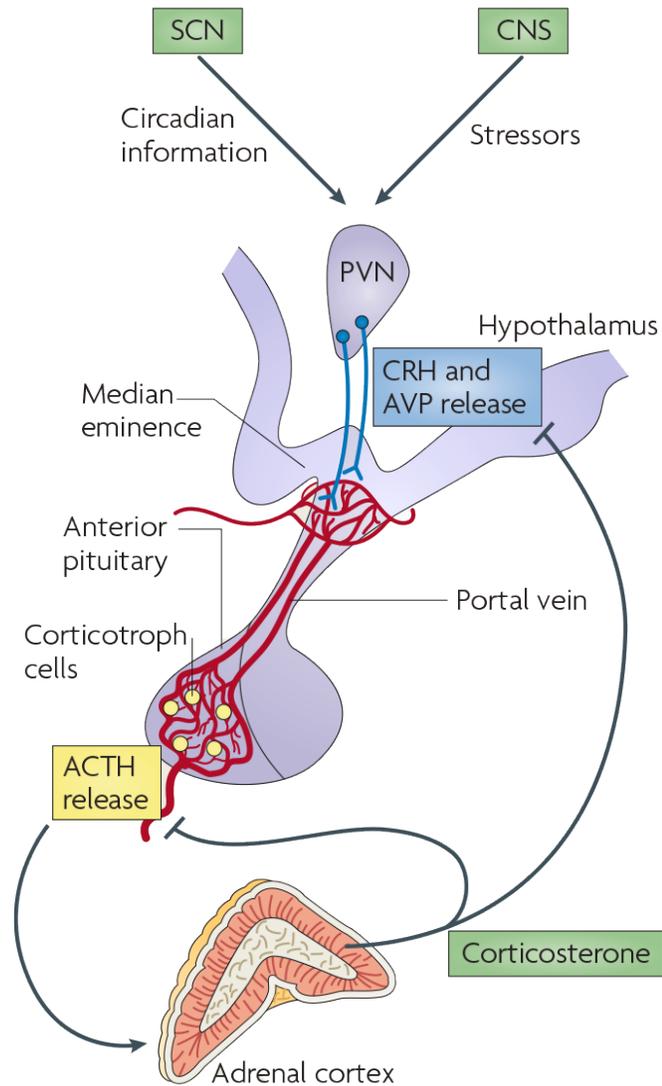
Figure 1.2: the HPA axis. The SCN, relaying light/dark information, and CNS, relaying stress information, both provide input to the PVN of the hypothalamus. CRH and AVP are released into the portal vein system and act on corticotrophs to release ACTH. ACTH acts on the adrenal gland to release glucocorticoids – either cortisterone in rodents or cortisol in humans (Image adapted from Lightman & Conway-Campbell, 2010).

In the previous section, the role of the immune system in HPA axis activation was briefly discussed. Recall that proinflammatory cytokines act to stimulate the HPA axis; therefore, it is not hard to recognize how increased inflammation in depression and/or cancer can induce hyperactivity (Raison & Miller, 2003; Soygur et al., 2007). Under normal conditions, glucocorticoids are known to be potent and robust anti-inflammatory agents: they limit both the production and effectiveness of cytokines, suppress the proliferation of T cells, and inhibit various immune pathways (Lutgendorf & Sood, 2011; Pace & Miller, 2009; Reiche et al., 2004; Sephton et al., 2009). However, despite a high concentration of endogenous glucocorticoids, a surge of proinflammatory immunological actors is characteristic of depression and is thought to be attributable to glucocorticoid resistance developed by the immune system (Leonard, 2017; Miller et al., 2009; Y. Pollak & Yirmiya, 2002; Raison, Capuron, & Miller, 2006). In summary, a paradoxical co-existence of glucocorticoids and inflammatory agents – resulting from delicate and complex altered bilateral communication between the HPA axis and immune system – frequently exists in depression.

Glutamate

Glutamate is widely acknowledged as the major excitatory neurotransmitter in the CNS, functioning antagonistically to γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter (Institute of Medicine, 2011; Mathews, Henter, & Zarate, 2012). As the major actor in the brain, glutamate plays an integral role in key processes like learning and memory by inducing long-term potentiation (LTP) (Rudy, Hunsberger, Weitzner, & Reed, 2015; Sanacora, Treccani, & Popoli, 2012). LTP and its counterpart long-term

depression (LTD) are two mechanisms of prolonged synaptic plasticity, strengthening and weakening the excitatory synapse, respectively (Luscher & Malenka, 2012).

The glutamatergic synapse is often referred to as the tripartite synapse due to the existence of three structural components: 1) a presynaptic neuron, 2) a postsynaptic neuron, and 3) glia **(Figure 1.3)** (Mathews et al., 2012; Rudy et al., 2015). Altogether, these structures work in concert to achieve glutamate release, uptake, and clearance of glutamate. Within the CNS, glutamate can be produced using one of two processes: 1) *de novo* synthesis using glucose as a precursor or 2) via the glutamate-glutamine cycle (Niciu, Kelmendi, & Sanacora, 2012; Rudy et al., 2015). In the latter pathway, glutamate in a synapse is taken up by astrocytes wherein glutamine synthetase converts it to glutamine; glutamine is then released and taken up by neurons that convert it back to glutamate using the enzyme glutaminase (Haroon, Miller, & Sanacora, 2016; Niciu et al., 2012). In the presynaptic neuron, glutamate is then packed into vesicles, a process aided by vesicular glutamate transporters (VGLUTs). Following depolarization of the presynaptic neuron and subsequent calcium influx, soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complexes aid in the exocytosis of these vesicles so that glutamate may be released into the synapse (Mathews et al., 2012; Rudy et al., 2015). After glutamate reaches the synapse, it is free to bind to its various receptors, which can be classified into two broad categories: the ionotropic glutamate receptors and the metabotropic glutamate receptors; both classes will be discussed in more detail in the next section.
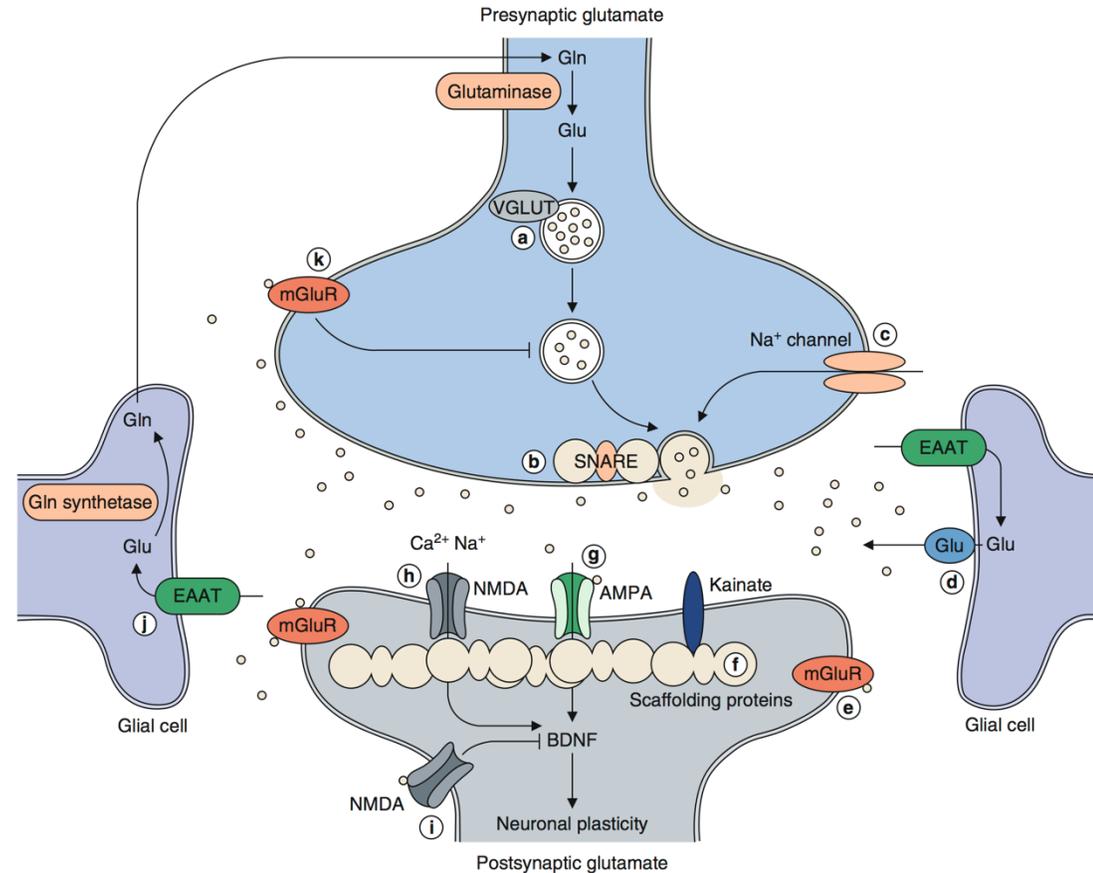
Figure 1.3: the glutamate synapse. VGLUT aids in the packaging of glutamate into vesicles, which undergo exocytosis with the aid of SNARE complexes. Once released into the synapse, glutamate can bind its various receptors (the ionotropic NMDA, AMPA, and kainate receptors or the metabotropic glutamate receptors). Activation of the NMDA and AMPA receptors increases BDNF, which offers trophic support. Activation of extrasynaptic NMDA receptors has the opposite effects on BDNF. EAATs, primarily expressed on glial cells, are responsible for reuptake of glutamate from the synapse. Glumatine synthetase converts glutamate to glutamine (Image from Sanacora, Zarate, Krystal, & Manji, 2008).

Once glutamate has been released into the extracellular space, regulation and clearance become highly important so as to avoid overabundant concentrations that impair both synaptic and extrasynaptic processes. Glutamate transporters found on all three elements of the tripartite synapse help in this process, specifically the excitatory amino-acid transporters (EAATs). To date, five EAATs have been characterized – EAAT1 through EAAT5. EAAT1 is primarily found on oligodendrocytes; EAAT2 is localized to astrocytes; EAAT3, EAAT4, and EAAT5 are mainly found on neurons, with EAAT5 being specific to retina (Haroon et al., 2016; Haroon & Miller, 2016; Institute of Medicine, 2011; Niciu et al., 2012). As previously described, glutamate taken up by EAATs on astrocytes can then be funneled into the glutamate-glutamine cycle **(Figure 1.3)**.

By this point, it should be evident that the regulation and coordination of glutamate release and clearance is tightly regulated. Yet, in depression – and a variety of other CNS disorders, neurodegenerative disorders, and neuropsychiatric disorders – glutamate has been measured in high concentrations in the brain, plasma, and cerebrospinal fluid (CSF) (Haroon & Miller, 2016; Murrough, Abdallah, & Mathew, 2017; Sanacora et al., 2012; J. Wang, Jing, Toledo-Salas, & Xu, 2014). This leads to the excessive glutamatergic signaling that has become recognized as a hallmark of depression (Currier & Nemeroff, 2014). These conditions result in excitotoxicity, which describes cell/neuronal death as a result of sustained activation beyond regular levels (Murrough et al., 2017). Recall from the previous section detailing the HPA axis that a robust relationship between chronic stress and depression has been observed. Many animal

models studying the link between chronic stress and depression have noted elevated

extracellular glutamate levels in stress-induced depressed animals due to impaired

clearance by EAATs and hampering of the glutamate-glutamine cycle (Haroon & Miller,

2016; Murrough et al., 2017; Sanacora et al., 2012). Numerous studies of glutamate in

mood disorders have utilized proton magnetic resonance spectroscopy (^{1}H-MRS) to

measure Glx which, put simply, reflects total glutamatergic availability, encompassing

both glutamate and glutamine. ^{1}H-MRS studies have found decreased Glx levels in the

mPFC in patients with depression, coupled with increased Glx levels in the occipital

cortex (Hashimoto, Sawa, & Iyo, 2007; Mechawar & Savitz, 2016; Yüksel & Öngür,

2010). One may infer a correlation between those findings and others that note neuronal

atrophy and dysfunctional synaptic plasticity in the same brain regions (Murrough et al.,

2017; J. Wang et al., 2014). In summary, excessive glutamate and glutamatergic signaling

may induce neuronal degeneration and interfere with synaptogenesis, resulting in

important cognitive and behavioural impairments.

It is also possible that peripheral tumours may play a role in elevated glutamate

levels in the brain. Recall that tumours release various proinflammatory cytokines.

Byproducts of inflammation are capable of activating matrix metalloproteases (MMPs)

which, in turn, disrupt the basement membrane and tight junction proteins of the blood-

brain barrier (BBB) (Cardoso, Brites, & Brito, 2010; Danielski et al., 2017; Hendriksen,

van Bergeijk, Oosting, & Redegeld, 2017; Zlokovic, 2008). Other pathologies like

chronic stress have also been implicated in disrupting the BBB. The BBB is tasked with

regulating solute transport between the blood and brain, maintaining CNS homeostasis

(Skultetyova, Tokarev, & Jezova, 1998). When the integrity of the BBB is impaired, solutes are able to move with greater ease from the periphery to the brain. For example, lipopolysaccharide (LPS)-activated peripheral inflammation has been shown to lead to elevated cytokine levels and inflammation in the brain (Banks & Erickson, 2010; de Timary, Starkel, Delzenne, & Leclercq, 2017; Gatti & Bartfai, 1993; Laye, Parnet, Goujon, & Dantzer, 1994; Quan, Stern, Whiteside, & Herkenham, 1999; Wardill et al., 2016). Therefore, a disrupted BBB could be a route through which peripherally released glutamate can directly cause aberrant signaling centrally, in addition to acting from the periphery via signal transduction. This exact phenomenon has been demonstrated both in animal models and clinical research involving pathological states, including neurodegenerative disorders and gliomas (Leibowitz, Boyko, Shapira, & Zlotnik, 2012).

Glutamate Receptors

Ionotropic Glutamate Receptors

It was previously mentioned that the neurotransmitter glutamate acts on a variety of different glutamate receptors that can be broadly categorized as either ionotropic or metabotropic **(Figure 1.4)**. Ionotropic glutamate receptors are termed as such because they are ion channels; these receptors propagate current and allow for neurotransmission via the flux of cations. In other words, ionotropic receptors allow glutamate to exert its effects on synaptic plasticity through the passage of ions in and out of neurons. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors work though the movement of Na^+ ions; the NMDA receptor, though also permeable to Na^+, functions primarily via Ca^{2+} (Murrough et al., 2017; Niciu et al., 2012).
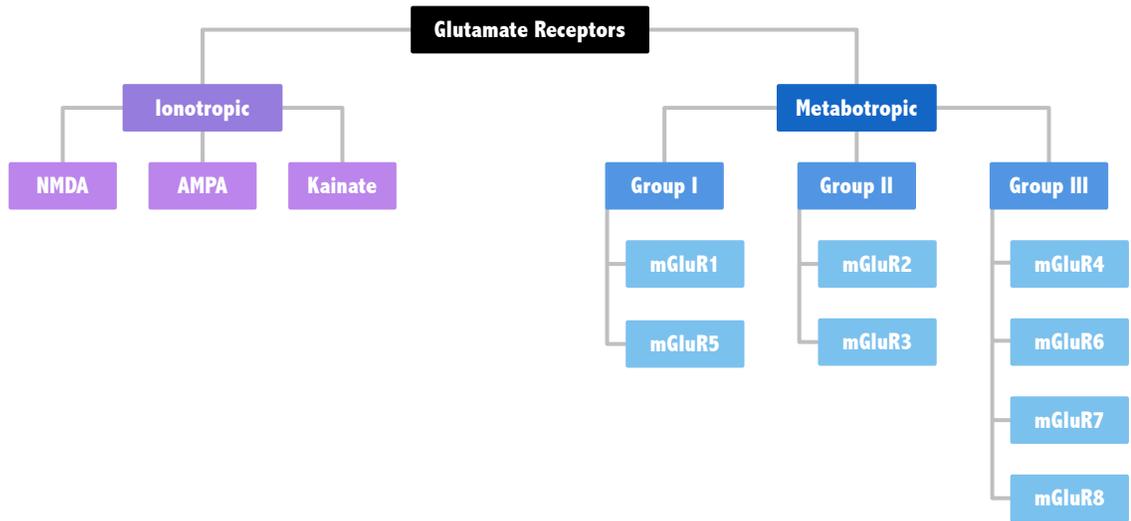
Figure 1.4: the different subtypes of glutamate receptors.

All ionotropic glutamate receptors are tetrameric complexes comprised of various subunits. The AMPA receptor subunits include GluR1-GluR4; kainate receptor subunits include GluR5-GluR7 and KA1-KA2; and NMDA receptor subunits include NR1, NR2A-NR2D, and NR3A-NR3B, with two NR1 and two NR2 subunits forming the typical composition. All ionotropic glutamate receptors are ligand-gated, requiring the binding of glutamate to rapidly open their ion channels. However, NMDA receptors are more tightly regulated than AMPA and kainate receptors, with additional mechanisms beyond glutamate binding required for activation. NMDA receptors also require a co-agonist, glycine, that binds concurrently with glutamate; glutamate binds NR2 subunits whilst glycine binds NR1 subunits. The ion channel pore in NMDA receptors is also blocked with Mg^{2+} to impede the flux of cations **(Figure 1.5)**. The removal of Mg^{2+} from the pore is voltage-dependent, meaning that the neuron must be depolarized via another

receptor to allow for NMDA receptor activity (Murrough et al., 2017; Niciu et al., 2012).

Given the rapid neurotransmission achieved by AMPA receptors and its co-localization

with the NMDA receptor, the removal of the Mg^{2+} occlusion typically follows AMPA-

mediated depolarization between -30 and 0 mV (Bigge, 1999; Sprengel, 2013). Following

the opening of the ion channel, Ca^{2+} influx-mediated LTP activates several kinases,

including PKC, MAPK, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).

CaMKII phosphorylates the GluR1 subunit in AMPA receptors, promoting AMPA

receptor trafficking and incorporation into the synaptic membrane. On the other hand,

LTD mediates internalization of the AMPA receptor from the synaptic membrane (Lau &

Zukin, 2007).
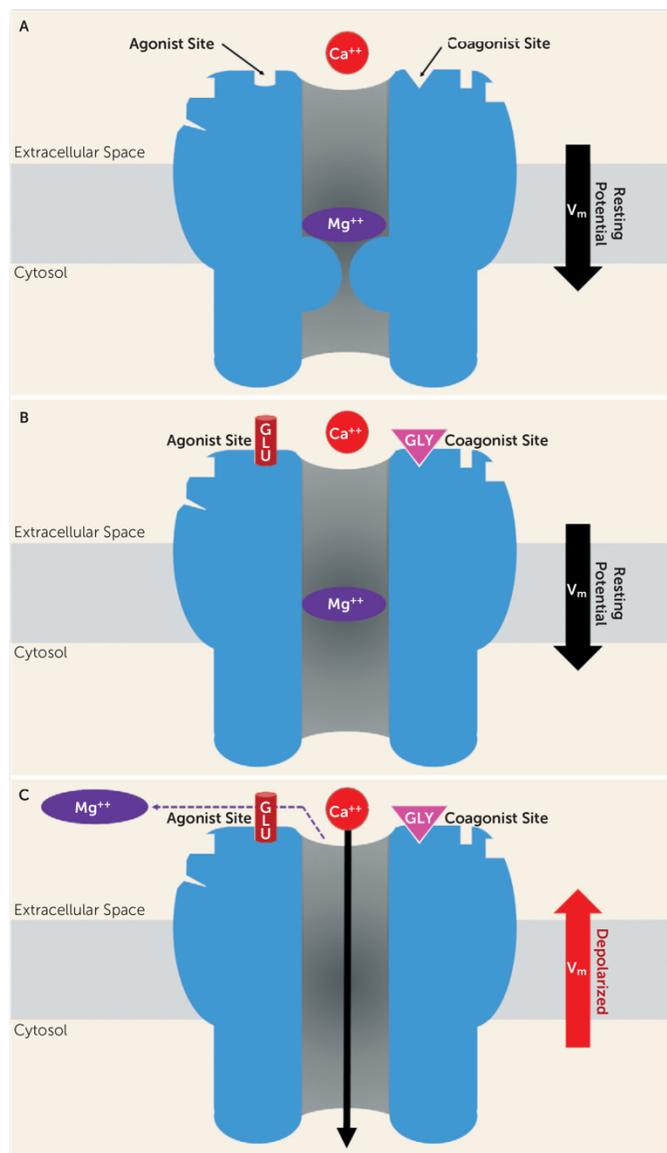
Figure 1.5: the NMDA receptor's Mg^{2+} block. (A) the NMDA receptor is in its resting state. A voltage-gated Mg^{2+} ion is occluding the ion channel and the ligand gate is also closed. (B) Binding of the agonist, glutamate, and the co-agonist, glycine, brings the NMDA receptor into its activated state. (C) Depolarization of the membrane removes the Mg^{2+} ion block, opening the channel to allow Ca^{2+} influx. (Image adapted from Newport et al., 2015)

Much attention has been focused on the NMDA receptor, specifically, when investigating the role of ionotropic glutamate receptors in excitotoxicity. After Olney (1969) first observed the deleterious effects of glutamate on CNS neurons, many investigations were done looking into glutamatergic signaling and neuronal death. Rothman and Olney (1987) later suspected the role of Ca^{2+} influx in glutamate-mediated neurotoxicity. Around the same time, others were already demonstrating that Ca^{2+} influx via the NMDA receptor played a key role in excitotoxicity (Berdichevsky, Riveros, Sanchez-Armass, & Orrego, 1983; Choi, 1987; Coyle, 1983; Tymianski, Charlton, Carlen, & Tator, 1993). Interestingly, it is also widely known that neuronal development and survival are reliant on the activity of NMDA receptors (Bowie, 2008; Hardingham & Bading, 2010). It has long been suspected that the switch between beneficial and harmful glutamatergic activity happens upon escalation of NMDA receptor activity from moderate to excessive (Hardingham & Bading, 2010; Sprengel, 2013). However, as of late, new theories have emerged into how the NMDA receptor may be implicated in excitotoxicity. Contrary to previous suppositions that these receptors were largely immobile, especially relative to AMPA receptors, the NMDA receptor has been found to be capable of lateral movement from synaptic to extrasynaptic sites (Lau & Zukin, 2007). It is thought that NMDA receptors located at the synapse proper are linked to neuroprotective pathways, conferring neuronal survival, whereas extrasynaptic NMDA receptors trigger apoptotic pathways (Hardingham & Bading, 2010). Ca^{2+} entry via synaptic NMDA receptors potently activates cAMP response element binding protein (CREB), a transcription factor that is widely touted for its involvement in neuronal plasticity and overall survival

(Hardingham, Fukunaga, & Bading, 2002; Mantamadiotis et al., 2002; Mayr &

Montminy, 2001; Shaywitz & Greenberg, 1999). CREB then increases the expression of

the *Bdnf* gene, which encodes the neuroprotective brain-derived neurotrophic factor

(BDNF) (Hardingham et al., 2002; Hardingham & Bading, 2010). BDNF also promotes

synaptic plasticity and, as the name suggests, neuronal growth (J. Wang et al., 2014).

However, the same degree of Ca^{2+} influx that confers neuroprotection via synaptic

NMDA receptors induces cellular damage through extrasynaptic NMDA receptor

activity; this would seem to support the theory that it is the location of the receptor with

reference to the postsynaptic density – and not the absolute amount of Ca^{2+} influx – that

distinguishes excitotoxic signaling from regular signaling (Hardingham et al., 2002).

Notions surrounding the role of the NMDA receptor in excitotoxicity were further

supported after ketamine, an NMDA receptor antagonist, garnered widespread attention

as a fast-acting and long-lasting antidepressant (Autry et al., 2011; Berman et al., 2000;

Drewniany et al., 2014; Hasselmann, 2014; Li et al., 2011; Maeng et al., 2008; Mitani et

al., 2006; Murrough, 2011; Strasburger et al., 2017; Zarate et al., 2006). Ketamine, or

(*RS*)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone, originally emerged in the 1960s

as an anesthetic (Drewniany et al., 2014). It is characterized as a non-competitive, non-

selective, high-affinity antagonist of the NMDA receptor (DeWilde, Levitch, Murrough,

Mathew, & Iosifescu, 2015; Hasselmann, 2014). Although its rapid effect makes it an

attractive candidate as an antidepressant, especially when compared to the slow-acting

monoamine-targeting pharmacotherapies (e.g. SSRIs), it does come with some

unfavourable side effects. Even at subanesthetic doses, patients have experienced

dissociative symptoms, altered perception, cognitive impairment, and even schizophrenia-like behaviours (Andrade, 2017; Krystal et al., 1994). Yet, it is still a widely popular and efficacious antidepressant, ameliorating many depressive symptoms such as anhedonia and suicidal ideation (Murrough et al., 2017). Given the success of ketamine as an antidepressant agent, glutamate has garnered a lot of interest as a potential therapeutic target for major depressive disorder.

Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) differ from ionotropic glutamate receptors in various ways. As opposed to the fast-acting ionotropic receptors, mGluRs are class C GPCRs that rely on slow-acting second messengers and downstream signal transduction pathways in order to modulate presynaptic release of glutamate and postsynaptic excitability in response. To date, eight mGluRs (mGluR1-mGluR8) have been characterized and further subdivided into three groups on the basis of sequence homology and shared signal transduction pathways: group I mGluRs consist of mGluR1 and mGluR5; group II mGluRs include mGluR2 and mGluR3; the remaining mGluR4, mGluR6, mGluR7, and mGluR8 make up group III (Murrough et al., 2017; Niciu et al., 2012; Ribeiro, Vieira, Pires, Olmo, & Ferguson, 2017; Swanson et al., 2005).

Group I mGluRs are largely postsynaptic, located in the periphery of the postsynaptic densities, and promote excitatory glutamate signaling. Coupled to G_q/G_{11}, mGluR1 and mGluR5 activate phospholipase C (PLC) which, in turn, hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce the end products inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates its receptors on the

endoplasmic reticulum in order to release stored Ca^{2+}. The released Ca^{2+}, in addition to Ca^{2+} derived from other sources and locales, subsequently works in concert with DAG to activate protein kinase C (PKC). PKC has been posited to stimulate a host of other actors, including mitogen activated protein kinases (MAPKs), as well as NMDA receptors. Beyond this main signaling pathway, group I mGluRs can activate Akt and extracellular signal-regulated kinase (ERK) pathways via interactions with Homer proteins; these pathways are neuroprotective and ERK, especially, promotes cell proliferation and growth (Baude et al., 1993; Golubeva, Moloney, O'Connor, Dinan, & Cryan, 2016; Jones, 2017; Ménard & Quirion, 2012; Niswender & Conn, 2010; Ribeiro et al., 2017; Witkin, Marek, Johnson, & Schoepp, 2007). The distribution of mGluR1 and mGluR5 in the brain overlaps with regions that have been posited to play a role in depression. mGluR1 has been found in the thalamus, hypothalamus, cerebellum, and olfactory bulb. Meanwhile, mGluR5 has been localized to the telencephalon, hippocampus, amygdala, and basal ganglia. Though most mGluRs are expressed on neurons mGluR5, as well as mGluR3, has also been found to be expressed on glial cells (Bhattacharyya, 2016; Pilc, Chaki, Nowak, & Witkin, 2008; Ribeiro et al., 2017).

Group II and III mGluRs are primarily localized presynaptically and are both coupled to inhibitory G_i/G_o proteins which inhibit adenylyl cyclase and decrease cAMP levels, as well as inhibit Ca^{2+} channels via $G_{\beta\gamma}$. As a result, the activation of group II and III mGluRs negatively modulates glutamate release and excitation of the postsynaptic membrane upon glutamate binding (Golubeva et al., 2016; Niciu et al., 2012; Niswender & Conn, 2010; Pilc et al., 2008; Ribeiro et al., 2017; Darryle D Schoepp, 2001).

Abundant mGluR2 expression has been found in the olfactory bulb and cerebellar cortex, whilst mGluR3 has been expressed in a wider range of locales within the CNS, including the cerebral cortex, dentate gyrus, and – as previously discussed – glia. Though mGluR2s are in keeping with the broad statement that group II and III metabotropic receptors are largely presynaptic, mGluR3 is predominantly found on postsynaptic membranes in addition to glial surfaces. Within group III mGluRs, mGluR6 is unique in its very limited expression to the retina. mGluR7 is expressed in numerous structures spanning the CNS, while mGluR4 and mGluR8 are slightly more concentrated to regions like the cerebral cortex and olfactory bulb, for instance (Cartmell & Schoepp, 2000; Ferraguti & Shigemoto, 2006; Pilc et al., 2008; Ribeiro et al., 2017).

The participation of mGluRs in glutamatergic signaling in cancer cells has garnered a lot of speculation about its potential as a therapeutic target. In fact, many studies have been done looking at the effect of inhibiting mGluRs using a variety of different cancer types and cell lines. It has been repeatedly observed that mGluR inhibition hampers cancer cell proliferation and induces apoptosis (Martino et al., 2012; Speyer et al., 2011; Yu, Wall, Wangari-Talbot, & Chen, 2016; C. Zhang et al., 2015). Koochekpour (2013) observed this phenomenon specifically in prostate cancer cells and found overexpression of mGluR1 in prostate cancer tissues.

System x_c^-

Vesicular release of glutamate from presynaptic neurons was previously described. As discussed, glutamate is packaged into synaptic vesicles and released into the synapse via exocytosis. There are also, however, non-vesicular mechanisms of

glutamate release; one in particular that has attracted a lot of interest is the system x_c^- antiporter.

A member of the heteromeric amino acid transporter (HAT) family, system x_c^- is comprised of a heavy chain, 4F2hc, and a light chain, xCT, linked by a disulfide bond (Bridges, Natale, & Patel, 2011; Lewerenz et al., 2013). xCT is credited with allowing system x_c^- to transport amino acids and is therefore known as the functional subunit. 4F2hc supports local trafficking and is required for expression of the transporter on the cell surface (Bridges et al., 2011). The system x_c^- antiporter's structure has been studied and it is predicted to contain 12 transmembrane domains, intracellular N- and C-termini, and a re-entrant loop between loops 2 and 3. It is chloride-dependent, sodium-independent, and electroneutral (Bridges et al., 2011; Lewerenz et al., 2013).

System x_c^- is known as an antiporter because of its bilateral exchange of amino acids; namely, intracellular L-glutamate for extracellular L-cystine at a 1:1 ratio **(Figure 1.6)** (Bridges, Lutgen, Lobner, & Baker, 2012; Massie, Boillée, Hewett, Knackstedt, & Lewerenz, 2015). Though the antiporter can move either amino acid in either direction, it essentially exclusively imports cystine and exports glutamate (Bridges et al., 2011). This is dictated by the concentration gradients of both cystine and glutamate established across the membrane: intracellular concentrations of cystine are negligible whilst extracellular glutamate levels are lower than intracellular levels (Bridges et al., 2012).

System x_c^- is the primary source for intracellular cystine and, therefore, plays a large role in protecting the cell against oxidative stress. When a cell experiences high levels of metabolic activity, it produces free radicals – usually reactive oxygen species

(ROS). It is the job of antioxidants to protect our cells – specifically, DNA and proteins – from the harmful effects of ROS. Glutathione (GSH) is one such small-molecule antioxidant. A tripeptide composed of glutamate, glycine, and cysteine, the synthesis of GSH is largely dependent on the intracellular availability of cysteine. Cystine imported via system x_c^- is rapidly reduced to cysteine and incorporated into GSH production **(Figure 1.6)** (Lewerenz et al., 2013; Maurya et al., 2016). This process is what keeps cystine at such low concentrations intracellularly.
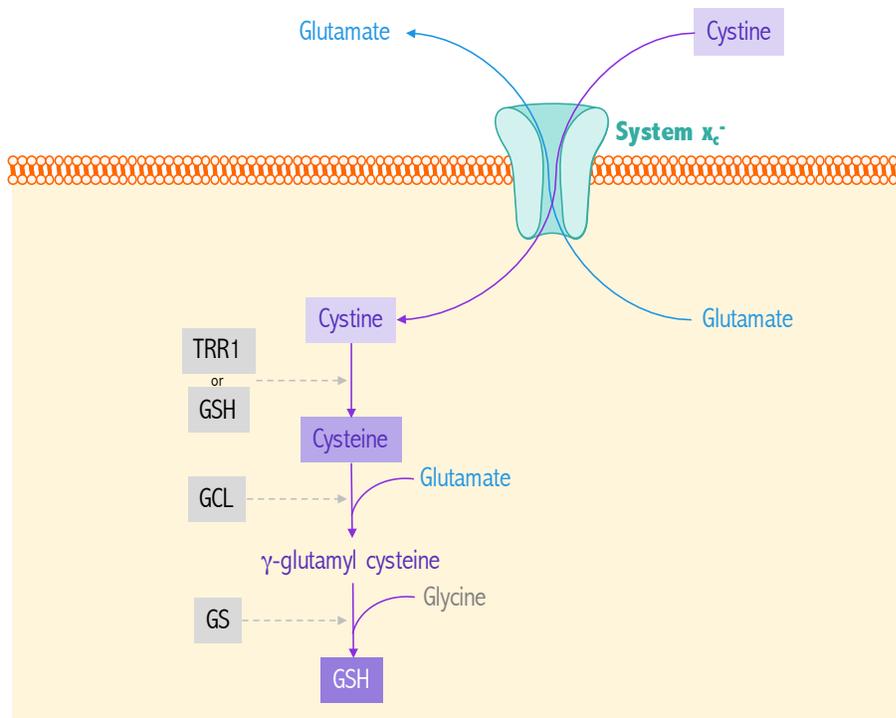
Figure 1.6: system x_c^- exports glutamate and imports cystine. Cystine is reduced to cysteine by either thioredoxin reductase 1 (TRR1) or glutathione (GSH). Glutamate cysteine ligase (GCL) catalyzes the addition of glutamate to cysteine to produce γ-

glutamyl cysteine. Glutathione synthase (GS) catalyzes the final step: the addition of

glycine to produce GSH (Image adapted from Lewerenz et al., 2013).

Glutamate released from system x_c^- can go on to participate in glutamatergic

signaling. However, under pathological conditions, system x_c^- may act as another route

through which excessive amounts of glutamate may be released into the extracellular

environment. Ye and Sontheimer (1999) found that human glioma cells secrete excessive

amounts of glutamate and were able to limit it using (*S*)-4-carboxyphenylglycine (((*S*)-4-

CPG), an mGluR antagonist. We now know that glioma cells highly express the system

x_c^- antiporter, using it for 50% of their glutamate transport across the cell membrane, and

that (*S*)-4-CPG is also a potent inhibitor of system x_c^- (Cho & Bannai, 1990; Hu, Lim,

Donaldson, & Kalloniatis, 2008; Seidlitz, Sharma, & Singh, 2010; Ye, Rothstein, &

Sontheimer, 1999). Exacerbating the harmful effects of system x_c^- upregulation in

gliomas, EAAT2 expression has been found to be concurrently downregulated meaning

that the excessive release of glutamate is not being cleared appropriately (Shukla et al.,

2011). Besides excitotoxicity, glutamate released via system x_c^- may also elicit another

phenomenon known as oxidative glutamate toxicity. See, glutamate is also a competitive

inhibitor of system x_c^-; therefore, it can inhibit the import of cystine and interrupt GSH

synthesis, depleting GSH supply in the cell and inducing oxidative stress (Lewerenz et al.,

2013).

System x_c^- is not expressed exclusively by neuronal cells; in fact, its expression

has been noted in immune tissues, the spleen, hepatocytes, and fibroblasts (Bridges et al.,

2011; Lewerenz et al., 2013). xCT expression has also been seen in a variety of cancer cell lines, including MAT-LyLu, a prostate cancer cell line derived from rats (Sharma, Seidlitz, & Singh, 2010).

Studies have been conducted to look at the effects of system x_c^- inhibition on behaviour. Lutgen et al. (2014) administered sulfasalazine (SSZ), a system x_c^- inhibitor, to rats and measured their effects on various behavioural tests. They did not see a significant effect of SSZ in improving depressive-like behaviours; however, this could be due to their acute drug administration. SSZ was delivered via intraperitoneal injection two hours prior to behavioural testing. On the other hand, Bentea et al. (2015) studied the role of system x_c^- in depressive-like behaviours using xCT –/– mice. They observed improved depressive-like behaviours in these xCT-deficient mice. The difference in the outcomes between these two studies could be due to the difference between acute and chronic system x_c^- inhibition. Perhaps extended treatment of mice with SSZ is necessary in order to see the benefits noted using an xCT knockdown model. This possibility will be explored in this project.

Conclusion

There are numerous mechanisms that are proposed to play a role in the onset of depression; inflammation, the HPA axis, and glutamate excitotoxicity were described here. The cancer environment may serve as an impetus for these pathophysiological phenomena and may explain the high incidence of depression in this specific subpopulation. Of these three theories, glutamate has emerged most recently and has attracted a lot of attention as of late, especially given the therapeutic success of ketamine.

Our lab has found that cancer cells express system x_c^- and secrete large amounts of glutamate into the extracellular environment (Sharma et al., 2010). However, more research into the role of glutamate in cancer-induced depression is needed. The aim of this project is to examine the therapeutic effects of inhibiting glutamatergic signaling in a model of prostate cancer.

Hypothesis

Cancer cells import cystine and release glutamate via the system x_c^- antiporter. This excessive glutamate release leads to aberrant glutamate signalling that may have a role in eliciting a depressive state. Therefore, disrupting glutamate release and/or signalling with pharmacological inhibitors may abrogate symptoms of prostate cancer-induced depression (PCaID).

This hypothesis was investigated through two broad objectives, which are described in more detail below and in the subsequent chapters.

Objective 1: Development of an Animal Model for Prostate Cancer-Induced Depression

The first objective of this project was to develop a validated animal model of PCaID. The animal model would serve two purposes: 1) show that a tumour could induce a depressive state and 2) be used in the second phase of the project to test pharmacological interventions.

A battery of behavioural tests was used to measure depressive-like symptoms. The first was the sucrose preference test (SPT), used to measure anhedonia. Anhedonia is characterized by decreased pleasure or interest derived from activities that one would

normally find enjoyable (D. D. Pollak, Rey, & Monje, 2010). In rodent research, anhedonia is most commonly measured using a two bottle choice test wherein one bottle contains typically 1-2% sucrose solution and the other contains regular water (Eagle, Mazei-Robison, & Robison, 2016; D. D. Pollak et al., 2010). Preference for sucrose over normal drinking water is expected and a reduced preference is characteristic of anhedonia (Samuels et al., 2011; Strekalova, Spanagel, Bartsch, Henn, & Gass, 2004). Rodent models of chronic stress and cancer have shown a decreased preference for sucrose solution and suggests an anhedonic state (Katz, 1982; Lamkin et al., 2011; Stepanichev et al., 2016; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). Sharpley, Bitsika, and Christie (2013) found that anhedonia was highly prevalent in a sample of patients with prostate cancer, specifically. Furthermore, perhaps not surprisingly, social housing has been found to have an interaction effect, as Lamkin et al. (2011) found that individually-housed mice had a lower sucrose preference relative to matched group-housed mice.

A second test used was the forced swim test (FST). It was first designed by Porsolt, Bertin, and Jaifre (1977) as a model of behavioural despair that could be used to test the efficacy of antidepressants and is still widely used today. Mice are placed in an inescapable cylinder of water and, at least initially, actively swim in an effort to leave the confines of the apparatus. They inevitably become relatively immobile, essentially moving only to stay afloat, and this is said to be representative of despair (Kalueff & Tuohimaa, 2004; Kedzierska & Wach, 2016). Antidepressants have been shown to reduce

the amount of time a mouse is rendered immobile in the FST (Petit-Demouliere, Chenu, & Bourin, 2004).

Based off of Porsolt's FST, Steru et al. (1985) developed the tail suspension test (TST), the third behavioural test used in this project. In the TST, mice are suspended upside-down by their tails. Analogous to the FST, mice initially made active efforts to escape the device but became more immobile later on in the test. Therefore, measures of immobility from the TST reflect despair behaviours similar to the FST (Kalueff & Tuohimaa, 2004; D. D. Pollak et al., 2010). Antidepressants have also been shown to improve the duration of immobility on the TST (Castagné, Moser, Roux, & Porsolt, 2010; Cryan, Mombereau, & Vassout, 2005). An advantage of the TST over the FST is that animals do not have to be submerged in water, avoiding hypothermia and the recovery period that follows the FST (Castagné et al., 2010).
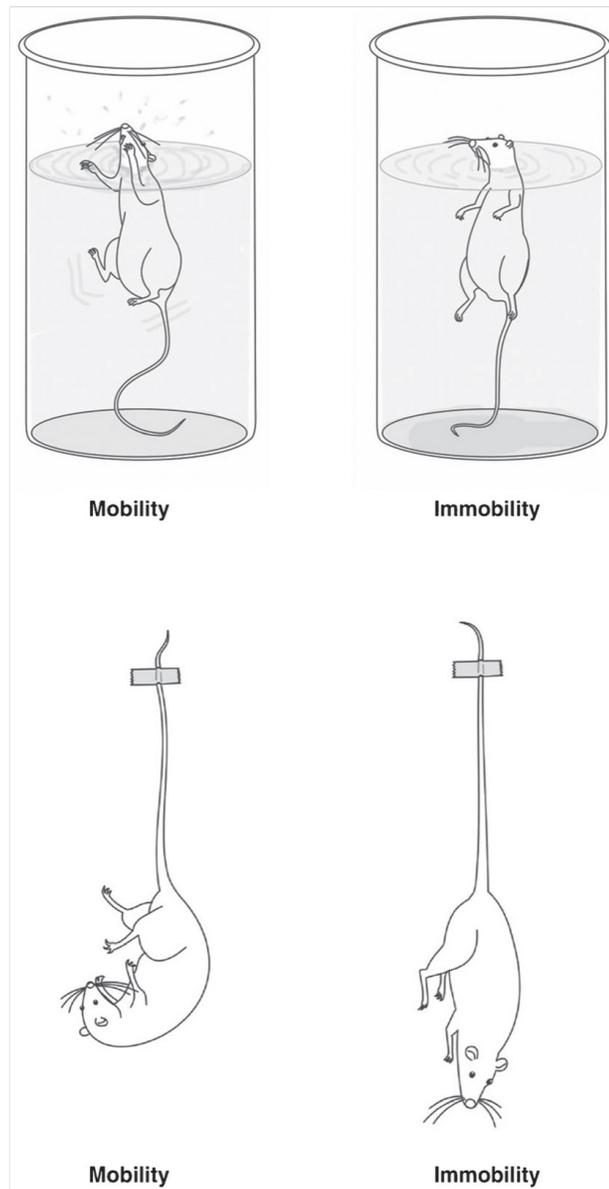
Figure 1.7: behavioural test schematics. Top row: mobility vs. immobility on the FST. Bottom row: mobility vs. immobility on the TST (Image from Abelaira, Reus, & Quevedo, 2013).

Lastly, an open field test (OFT) was developed to measure depressive-like symptoms. According to a review by Kalueff and Tuohimaa (2004), the following parameters that can be measured in an OFT reflect depressive-like behaviours: a) total number of squares crossed in the apparatus, b) squares crossed in the inner area of the apparatus, and c) squares crossed in the outer area of the apparatus. It is thought that depressed mice will spend more time in the outer perimeter of the open field whilst non-depressed mice will be more willing to explore the unshielded inner area. However, it is also important to note that increased exploration of the inner area is also characteristic of anxiolytic-like behaviour (Bailey & Crawley, 2009). Therefore, it may be hard to tease apart anxiety- and depressive-like behaviours, which are closely related and may present similarly in the OFT. With regards to the total number of squares crossed, which may also be indicative of locomotive activity, Lamkin et al. (2011) describe a functional dissociation between the factors that influence immobility times on the TST versus those that determine locomotive activity, whereby less locomotion is independent of outcomes on the TST. This is an important consideration in the context of this project as there may be a concern that tumour burden affects activity. This dissociation means that even if an animal were to be less active in the OFT due to an interaction effect with their tumour, it does not have any bearing on their performance on the TST.

Objective 2: Testing the Effectiveness of Pharmacological Inhibitors of Glutamate Signalling in Treating Prostate Cancer-Induced Depression

Three known pharmacological inhibitors of glutamatergic signaling were used in the second phase of this project in order to gauge their antidepressant properties. The first

drug SSZ, as previously described, is a system x_c^- inhibitor. It has been widely used across numerous studies to block the activity of system x_c^-, either in an effort to reduce glutamate release or limit cystine import (R. Chen et al., 2008; W. J. Chung & Sontheimer, 2009; Evonuk et al., 2015; Guan et al., 2009; Shukla et al., 2011). Our lab has previously noted the ability of SSZ to improve anhedonia-like behaviours on the SPT and despair-like behaviours on the FST, perhaps as a result of limiting glutamate release (Nashed et al., 2017). Furthermore, by blocking cystine uptake and GSH synthesis as a result, SSZ has also demonstrated anti-tumour properties limiting the proliferation and survival of tumour cells (W. J. Chung et al., 2005; Narang, Pauletti, Gout, Buckley, & Buckley, 2007; Sontheimer & Bridges, 2012). Doxsee et al. (2006) tested SSZ on human prostate cancer cells, DU-145 and PC-3, both *in vitro* and *in vivo* and observed growth inhibition in both cases, as well as intracellular GSH depletion.

The second inhibitor, also previously mentioned, is (*S*)-4-CPG. Though originally known as a group I mGluR antagonist, its potency as a system x_c^- inhibitor has been observed in the literature (Evonuk et al., 2015; Shukla et al., 2011; Venè et al., 2011). With its seemingly dual effects limiting glutamate release and subsequent signaling, it seemed like an attractive drug to include in this project. Zhang et al. (2017) induced depressive-like behaviours in rats using prenatal restraint stress and noted an increase in mGluR1 and mGluR5 mRNA and protein levels. They subsequently found that the drug icariin was able to reverse the effects of stress on mGluR1 and mGluR5 expression, which was followed by improvements in depressive-like behaviours from the SPT and

FST. We seek to determine whether or not inhibiting mGluR1 and mGluR5 will have the same functional properties as modulating expression.

The last pharmacological regulator of glutamate used was 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo[ƒ]quinoxaline-7-sulfonamide (NBQX). A preponderance of the literature discussing the role of NBQX in depression focuses on its ability to hinder the antidepressant properties of ketamine. It has been observed in several studies that the prior administration of NBQX attenuates the effect of subsequent ketamine treatment (Fukumoto, Iijima, & Chaki, 2016; Koike, Iijima, & Chaki, 2011; Nguyen & Matsumoto, 2015; Zhou et al., 2014). The same studies showed NBQX on its own had slight improvements in depressive-like symptoms, though not statistically significant and not to the extent of ketamine without NBQX pre-treatment. However, it is of interest to note that all studies used NBQX acutely – a single administration often as little as 30 minutes prior to behavioural testing, with only one study (Koike et al., 2011) treating 72 hours prior to testing. It would be interesting to see whether or not the antidepressant effects of NBQX could be amplified if chronically administered. Shimizu, Kurosawa, and Seki (2016) found that NBQX significantly improved FST immobility and sucrose preference in social isolation-induced depressed mice. Though they also administered NBQX shortly before behavioural testing (35 minutes), it's possible they saw more drastic antidepressant effects due to their direct infusion into the brain.
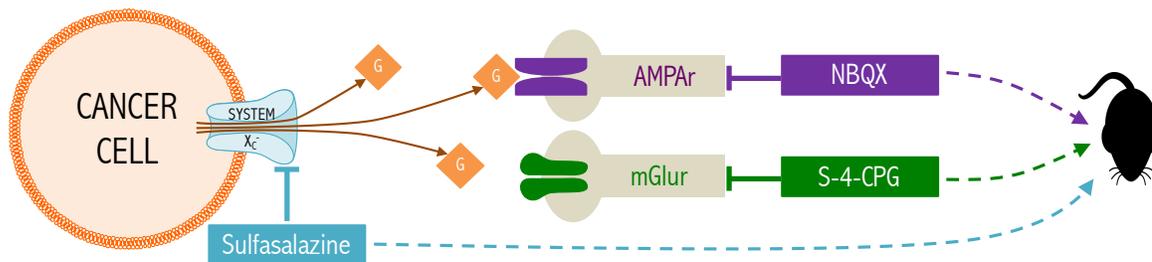
Figure 1.8: overview of glutamatergic inhibitors used in this project. Sulfasalazine is a system x_c^- inhibitor. NBQX is an AMPA receptor antagonist. S-4-CPG is an mGluR1 and mGluR5 antagonist, but has also been demonstrated to be a potent system x_c^- inhibitor.

CHAPTER 2: Methods

Objective 1:

Mice

36 male C57BL/6 mice aged 4-6 weeks were ordered from Charles River Laboratories (St. Constant, QC, Canada) and housed in a 12/12 hour light/dark cycle, at a constant temperature of 24˚C with access to autoclaved water and food *ad libitum*. All mice were singly-housed for logistical and theoretical purposes. For the SPT, animals need to be in individual cages to monitor water intake per mouse. If mice were group-housed and only moved to individual cages for the duration of the SPT, a possible confounding factor could be introduced whereby differences in sucrose preference may be attributed to being separated. Furthermore, group-housing is thought to incur protective effects. As mentioned previously, a study by Lamkin et al. (2011) showed that singly-housed mice with tumours demonstrated a lower sucrose preference compared to their group-housed counterparts. This mirrors the effect of social factors on the relationship between cancer and depression that we see clinically with cancer patients. Upon initial arrival to the animal facility, mice were assigned an identification number and left to acclimate for one week prior to the start of the experiment.

All experiments were performed in accordance with national guidelines as per the Canadian Council on Animal Care and approved by the Animal Research Ethics Board at McMaster University.

Experimental Groups

Mice were randomly divided into three groups such that mean baseline sucrose preferences were equal across groups. Groups were then randomly assigned to an

experimental condition: negative control (n=12), positive control (n=12), and tumour (n=12).

Corticosterone Administration

In order to establish a positive control for depression, a subset of mice (n = 12) was administered exogenous corticosterone (CORT), the major stress hormone in mice analogous to cortisol in humans. CORT has been shown to induce a depressive-like state in rodents in various animal models, verified using behavioural tests like the FST (Demuyser et al., 2016; S. A. Johnson, Fournier, & Kalynchuk, 2006; Zanos et al., 2016; Zhao et al., 2008). CORT was prepared according to a procedure described by Gourley and Taylor (2009). CORT hemisuccinate was weighed out and added to Milli-Q water, with volumes based on a daily fluid intake of ~5 mL per mouse and accounting for ~15 mL of dead space in the 50 mL drinking tubes. The solution was raised to a pH of 12-13 using 10 N NaOH and subsequently left to stir overnight while maintained at 4°C in a cold room. The next day, the pH of the solution was neutralized between a pH of 7-7.4 using 10 N HCl. The final solution achieved was a concentration of 25 µg/mL and was administered *ad libitum* to mice, substituting their regular drinking bottles, for a period of 14 days. However, CORT begins to degrade after it is in solution; thus, CORT had to be changed 72 hours post-dissolution. Given that solutions had to be stirred overnight prior to administration, this meant that CORT bottles were switched after two days. After the two week chronic administration of CORT a six day weaning period ensued, broken down into three days of CORT administration at 50% of the original concentration (12.5 µg/mL) and the last three days at 25% of the original concentration (6.25 µg/mL).

Including the weaning period, total exposure time was 20 days. The mean dose per mouse was calculated to be 5.39 ± 0.8 mg/kg/day.

Cell Culture

Murine-derived RM1 prostate cancer cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic, and maintained in a sterile incubator at 37˚C and 5% CO_2.

Tumour Cell Inoculation

A subset of mice (n = 12) assigned to be the tumour group was injected with 20,000 RM1 cells in 50 μL of sterile phosphate buffered serum (PBS) subcutaneously over their right flank. Both the negative (n = 12) and CORT (positive control) mice were given sham inoculations with 20,000 inactivated RM1 cells in 50 μL of PBS. Cells were inactivated with alternating cycles of five minutes of boiling at 100˚C and freezing in liquid nitrogen, totaling three rounds of boiling and two rounds of freezing. A sample of inactivated cells were removed and stained to ensure lysis occurred and no active cells remained. Once tumours became visible around one week post-inoculation, endpoint monitoring commenced. Animals were weighed and physical appearances and behaviours were observed. Tumours were measured using a digital caliper.

Behavioural Assessments:

Sucrose Preference Test

Anhedonia is defined as the inability to experience pleasure and is operationalized in mice as a diminished preference for sucrose water over regular water (Machado et al., 2012; D. D. Pollak et al., 2010). At baseline, after mice were acclimatized to the animal

facilities for one week, they were habituated to 3% sucrose solution for 72 hours in place of regular drinking water. Habituation was done using two bottles – both filled with sucrose solution – in order to familiarize the mice with the two-bottle set-up used for the SPT. After habituation, two baseline SPTs were done in order to establish baseline preferences. The second baseline SPT was used for baseline preference values. Mice were presented with two bottles in place of regular drinking water: one bottle with Milli-Q water and another with 3% sucrose solution. Each testing period lasted 48 hours and the positions of the two bottles were switched after 24 hours in order to mitigate any effects of location bias (e.g. preference for the bottle inside vs. outside). Consumption was measured by weighing the bottles at the start of the test, at the 24-hour mark when bottles were switched, and at the end of the test. Preference was calculated by dividing the amount of sucrose solution consumed over the total fluid intake (i.e. sucrose intake/(sucrose intake + water intake)). A preference of 50% would indicate that the animal did not have a preference for either sucrose solution or regular water, whereas any value over 50% would indicate a preference for one solution over the other. Another SPT was conducted at endpoint using the same two-bottle system but without sucrose habituation and differences among group preferences were noted.

Tail Suspension Test

The TST is used to measure despair, operationalized as a lack of active escape behaviours (Kalueff & Tuohimaa, 2004). An automated TST system from BioSeb (Vitrolles, France) was used. Animals were brought into the testing room one hour prior to the start of behavioural testing in order to get acclimated to testing room conditions.

All three chambers of the apparatus were calibrated to 0 grams and 20 grams prior to beginning the experiment. A piece of tape was folded like a flag around the tail of each mouse, approximately 2 cm from the end. The tape was pierced onto a hook connected to a strain gauge within the TST apparatus, suspending the mice upside-down by their tails. In order to avoid mice grabbing their tails during the test, which would confound their performance, a plastic tube was placed around the base of the tails. The total testing time was 6 minutes, though the first minute was designated as a latency period to stabilize behaviours and was not included in analysis. Movement, as determined according to a threshold of six for the instrument, was detected by the strain gauge and a primary measure of immobility time, as well as secondary measures of power of movement (PM) and energy, was given.

Forced Swim Test

The FST is another behavioural assessment of despair (Porsolt et al., 1977). An automated FST system from BioSeb (Vitrolles, France) was used that measured immobility time using two inputs: 1) video input from cameras suspended above each beaker, tracking the movement of each mouse; and 2) sensors attached to the sides of each beaker that detected vibrations in the water. The combination of these inputs, visual and mechanical, was used to distinguish an active state of swimming (which represents escape behaviours) from an inactive state of floating (which is indicative of despair). Baseline sensor checks were performed for each of the four beakers in the FST apparatus and visual parameters were set, including the capture of reference images of empty beakers and thresholds for visual tracking. Once again, animals were acclimatized to the

testing room one hour prior to the commencement of testing. Mice were lowered into

separate beakers of autoclaved water, to maintain sterility, at room temperature. The test

duration was a total of six minutes, again with the first minute designated a latency period

for behavioural stabilization. Once the tests were completed, mice were removed from the

beakers and dried off with a towel on a heating pad to avoid hypothermia. Control

animals were used to create calibrations for each beaker (one mouse per beaker). Their

tests were recorded and later manually scored for mobility/immobility. Manual scores

were used to inform the program what thresholds of activity – a combination of visual

and mechanical – constituted active swimming for each beaker. These thresholds would

then be applied to the subsequent animals tested in each beaker to calculate immobility

times and secondary outcomes.

Open Field Test

The OFT assesses depressive-like symptoms. A large 25 x 25 cm box with an

opaque base and 4 opaque walls comprised the main apparatus. A 5 x 5 grid was outlined

on the base, resulting in 5 cm^2 squares. The outer perimeter of squares was designated the

"outer area" whilst the 9 squares comprising the centre was designated the "inner area"

(Figure 2.1). A camera was suspended above the apparatus and connected to a laptop to

allow for live viewing of the OFT.
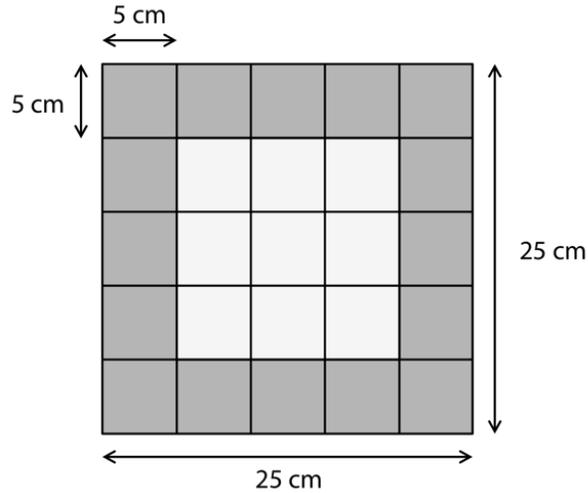
Figure 2.1: OFT apparatus set-up. Grey squares represent the "outer area". White squares represent the "inner area".

The primary measure of this test was number of squares crossed in the inner area vs. outer area, which also gave rise to the total number of squares crossed. Again, animals were acclimatized to the testing room one hour prior to the commencement of testing. The test duration was 5 minutes in total, which is the minimum suggested time in order to assess the critical behaviours involved in exploratory locomotion (Bailey & Crawley, 2009; Kalueff & Tuohimaa, 2004). Two manual counters, one for the "inner area" and one for the "outer area", were used. Each time the animal crossed from one square into another, it was recorded on the appropriate counter. Values from both counters were summed in order to determine the total number of squares crossed.

Euthanasia and Harvesting

At endpoint, animals were sacrificed using gaseous isofluorane anesthesia followed by an intracardial exsanguination using a 1 mL syringe and a 25 gauge needle.

This method was used instead of using CO_2 gas to avoid compromising the chemistry of blood samples obtained. After approximately 0.7-1 mL of blood was obtained, a cervical dislocation was performed. Subcutaneous tumours were excised from under the skin and stored at -80˚C.

After blood samples were collected at the time of euthanasia, they were left to sit at room temperature to allow for coagulation. When all samples had at least 15 minutes of coagulation time, samples were spun down in a tabletop centrifuge at 3600 relative centrifugal force (RCF) at 4˚C for 15 minutes. The resulting samples had a distinct plasma serum layer, which was collected and saved for future glutamate analysis to determine glutamate content in the blood.

Statistical Analysis

To analyze behavioural data from the SPT, TST, FST, and OFT, one-way analysis of variance (ANOVA) was used, followed with a Tukey post hoc test. The significance level was set at $\alpha = 0.05$ *a priori* for all analysis tests. Outliers were identified and removed using the Grubb's test (GraphPad Software, Inc., La Jolla, CA, USA). The D'Agostino-Pearson omnibus normality test was used to analyze data for normality. All data are presented as mean ± the standard error of the mean (SEM). All data analyses and graphing were performed using GraphPad Prism 5 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA).

Objective 2:

Mice

Five separate experiments were run, with a total of 100 mice used. Experiments 1 to 4 had n = 16 mice each and experiment 5 had n = 36 mice. All housing, food, and water conditions are analogous to those of Objective 1.

Experimental Groups

Animals were divided into four groups and each group was assigned to a treatment: SSZ, (S)-4-CPG, NBQX, or the vehicle control group. These groups were also compared to the tumour and negative control groups from Objective 1 for behavioural references. A CORT group was not included as the animal model had already been validated and a positive control group was no longer needed for subsequent experiments.

Cell Culture

Cell culturing was performed in the same manner as in Objective 1.

Tumour Cell Inoculation

As opposed to the 20,000 cells injected in Objective 1, mice in Objective 2 experiments were injected with 15,000 cells. This was done in an effort to delay endpoint for desired experimental timelines. However, tumours were measured for the duration of all experiments and tumour growth for animals injected with 15,000 cells were compared to that of animals who received 20,000 cells. It was found that this difference in initial cell number did not impact tumour growth, allowing us to draw comparisons between animals across Objective 1 and Objective 2 experiments.

In Objective 1, one group was injected with active cells while two groups were sham-injected. In Objective 2, all animals were subcutaneously inoculated with 15,000 active RM1 prostate cancer cells.

Drug Administration

ALZET micro-osmotic pumps, Model 1002, (DURECT Corporation, Cupertino, CA, USA) were used for drug delivery in order to achieve reliable and steady doses. More information about the mechanism of action of these osmotic pumps can be found in **Appendix 1**. Drugs were loaded into 1 mL syringes and blunt-tipped 27 gauge filling tubes were attached to the syringes. The filling tubes were carefully inserted into the pump and the syringe was slowly depressed to fill the pump. Each pump has a reservoir volume of 100 µL. Pumps have a mean pumping rate of 0.25 ± 0.05 µL/hr for 14 days *in vivo*. After all pumps were filled with their respective solutions, they were subsequently incubated in sterile PBS overnight at 37°C to let the pumping rate reach a steady state prior to implantation into the intraperitoneal cavity. Prior to the commencement of surgery, animals were anesthetized with gaseous isofluorane and subcutaneously administered 0.05 mL of the analgesic temgesic buprenorphine, which was mixed 1:10 in 0.9% sterile saline (Schering-Plough, Welwyn Garden City, Hertfordshire, UK). A small incision was made through the skin and peritoneum to gain access to the intraperitoneal cavity. Following implantation of the pump, the peritoneum and skin were separately sutured. Animals and surgical wounds were monitored closely daily for five days following surgery. The use of an osmotic pump for drug delivery avoided the need to administer daily intraperitoneal injections, which are painful and require repeated

handling. It was thought that these repeated stressful interventions could confound behavioural testing as we are interested in observing depressive-like symptoms.

SSZ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 M NH_4OH to a concentration of 67 mM, in order to achieve a target dose of 8 mg/kg/day. (*S*)-4-CPG (Tocris Bioscience, Minneapolis, MN, USA) was dissolved in 1 mM NaOH to a concentration of 87 mM, in order to achieve a target dose of 5 mg/kg/day. NBQX (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in water to a concentration of 87 mM, in order to achieve a target dose of 10 mg/kg/day. Control pumps were loaded with NH_4OH. All desired concentrations to achieve target doses were calculated based off a mouse weighing 20 grams, a pump rate of 0.25 µL/hour, and a reservoir volume of 100 µL.

In Vitro Drug Treatments

The various drugs used in this experiment were tested on RM1 cells *in vitro*. Four concentrations of each drug were used. For SSZ and S-4-CPG, the following concentrations were used: 100 µM, 150 µM, 200 µM, and 250 µM. Since the literature showed that NBQX was usually administered in higher concentrations compared to SSZ and (*S*)-4-CPG, the following concentrations of NBQX were tested: 150 µM, 200 µM, 250 µM, and 300 µM. The vehicles for SSZ and (*S*)-4-CPG, NH_4OH and NaOH, respectively, were also tested in triplicate. Six control wells that contained regular culture media were also tested.

Glutamate Assay

Media from all drug-treated cell cultures was collected after 24 hours and glutamate assays were performed. Glutamate release from cells treated *in vitro* was measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay kit (Invitrogen, Eugene, OR, USA) and quantified using a CytoFluor Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems, Framingham, MA, USA). This assay is able to quantify glutamate via a fluorescent product, resorufin, that is detected by the CytoFluor plate reader. Glutamate samples are added in triplicate to a plate and mixed with an assay mixture containing reaction buffer, 0.25 U/mL horseradish peroxidase (HRP), 0.08 U/mL L-glutamate oxidase, and 0.026 μg/μL Amplex Red reagent diluted in dimethyl sulfoxide (DMSO). L-glutamate oxidase oxidizes L-glutamate to produce α-ketoglutarate, NH_3, and hydrogen peroxide. Hydrogen peroxide reacts in a 1:1 ratio with the Amplex Red reagent, catalyzed by HRP to produce resorufin. Plates were incubated at 37˚C for 30 minutes prior to reading. Further, hydrogen peroxide was added in triplicate to the plate to act as a positive control. A standard curve for glutamate was also prepared using a range of concentrations from 0-25 μM in triplicate. Fluorescence was measured using an excitation wavelength of 530/25 nm and an emission wavelength of 580/50 nm, with the gain at 40. Fluorescence values from the standard curve were plotted against their known glutamate concentrations. A linear equation was generated so that fluorescence values from animal serum samples could be used to calculate glutamate concentrations.

Crystal Violet

Cell numbers for drug-treated RM1 cells were calculated using a crystal violet assay. In short, after media was collected, cells were fixed to the plate using 10% phosphate buffered formalin. After cells were fixed, they were stained with 0.1% (w/v) crystal violet stain in 25% (v/v) methanol. Crystal violet stains cell nuclei and, therefore, the degree to which staining occurs is directly proportional to the number of cells present. After cells were stained for at least 10 minutes, excess stain was removed via repeated rinsing of wells with water. Following successful washing, plates were left to dry overnight. The following day, the crystal violet stain that remained absorbed to cells was brought back into solution using a solubilizer of 0.05 M NaH_2PO_4 in 50% ethanol. The optical absorbance values for all wells were quantified using a BioTek PowerWave XL Plate Reader at 570 nm (BioTek Instruments, Inc., Winooski, VT, USA). Raw optical absorbance values were used to calculate cell numbers using a y=mx+b equation generated from a crystal violet standard curve. Glutamate concentrations were then corrected to cell number for all samples.

Behavioural Assessments:

Sucrose Preference Test

Recall that a 3% sucrose solution was used in Objective 1. It was discovered that a 3% solution was too high, as animals displayed such a strong preference that differences between groups could not be detected. In other words, their preference for 3% sucrose solution was so strong that it could not be overcome and the noise was too high to detect

the signal. In all Objective 2 experiments, 1% sucrose solutions were used and group differences were much more detectable.

Tail Suspension Test

The TST was conducted using the same procedures and parameters as in Objective 1.

Forced Swim Test

Due to technical malfunctions with the FST software, this behavioural test regrettably could not be performed on animals in the five Objective 2 experiments.

Open Field Test

The OFT was conducted using the same procedures and parameters as in Objective 1.

Euthanasia and Harvesting

Endpoint sacrificing and tissue collection was performed in the same manner as in Objective 1. Whole blood samples for two of the five experiments were mistakenly frozen down before being spun down and removing serum. Therefore, serum samples could not be collected and stored for those animals for future processing.

Statistical Analysis

Statistical analyses were performed for behavioural tests in the same manner as described for Objective 1.

CHAPTER 3: Results

Objective 1

Sucrose Preference Test

A decreased sucrose preference is considered characteristic of anhedonia. There were no statistically significant differences in sucrose preference across the three groups ($P > 0.05$) **(Figure 3.1)**. It was thought that this result could be attributed to the fact that a 3% sucrose solution may be too concentrated, whereby mice are unable to overcome their preferences even if differences did exist.

SPT
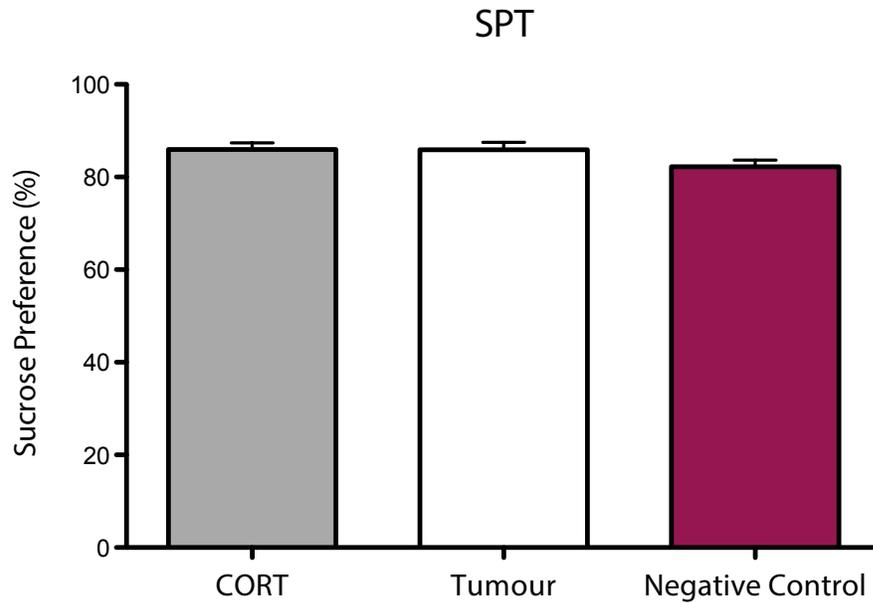
Figure 3.1: mean SPT results. 3 experimental groups: CORT (n = 11), tumour (n = 10), and negative control (n = 11). A 50% preference reflects no preference between sucrose solution and water. There were no significant differences among groups.

Tail Suspension Test

Higher immobility times on the TST are reflective of despair, which is characteristic of a depressive state. The tumour group demonstrated a statistically significant increase in immobility time compared to the negative controls ($P = 0.040$). Mice in the CORT group demonstrated a trend towards an increase in immobility time relative to negative controls ($P = 0.469$) **(Figure 3.2)**.
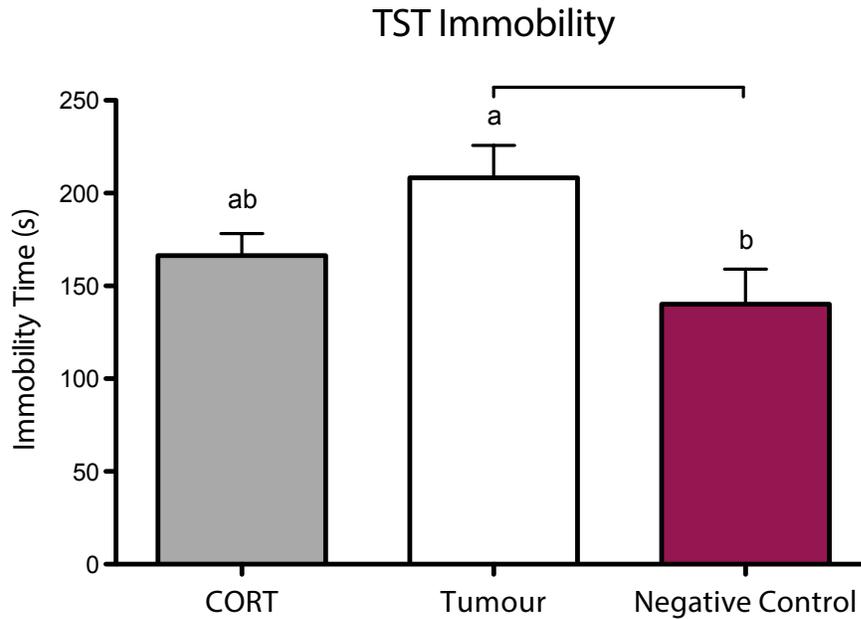
Figure 3.2: comparison of mean TST immobility times. 3 experimental groups: CORT (n = 11), tumour (n = 6), and negative control (n = 12). Tumour mice had a higher immobility time compared to negative control mice ($P = 0.04$). CORT had a trend towards immobility time compared to negative control mice ($P = 0.47$).

Forced Swim Test

Analogous to the TST, higher immobility times on the FST represent despair-like behaviours. There were no statistically significant differences between groups, but both CORT and tumour mice displayed trends toward lower immobility times compared to negative control mice (P = 0.110, P = 0.382, respectively) **(Figure 3.3)**. The large error bar for the tumour group could be due to the small group size (n = 2), that resulted from early attrition prior to the FST and animals being removed from analysis due to drowning during the test.
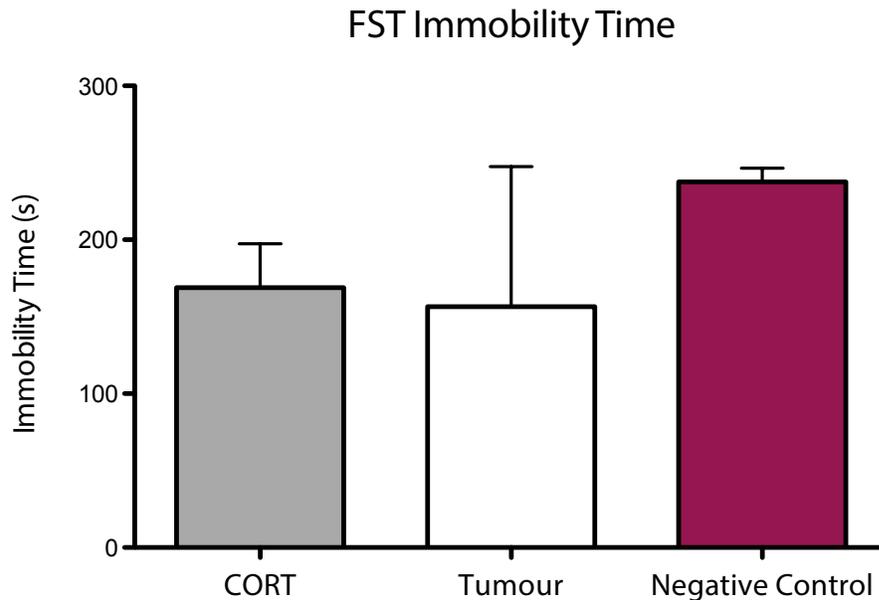
Figure 3.3: comparison of mean FST immobility times. 3 experimental groups: CORT (n = 12), tumour (n = 2), and negative control (n = 11). CORT and tumour mice demonstrated trends toward lower immobility times compared to negative control mice, though these results were not statistically significant.

Open Field Test

Several different parameters can be analyzed via the OFT. The first outcome studied was the total number of squares crossed within the apparatus. Tumour mice demonstrated a statistically significant decrease in total number of squares crossed compared to both CORT mice ($P = 0.009$) and negative control mice ($P = 0.002$). CORT mice were not significantly different compared to negative control mice with regards to total number of squares crossed in the OFT ($P = 0.501$) **(Figure 3.4)**.

OFT Total Squares
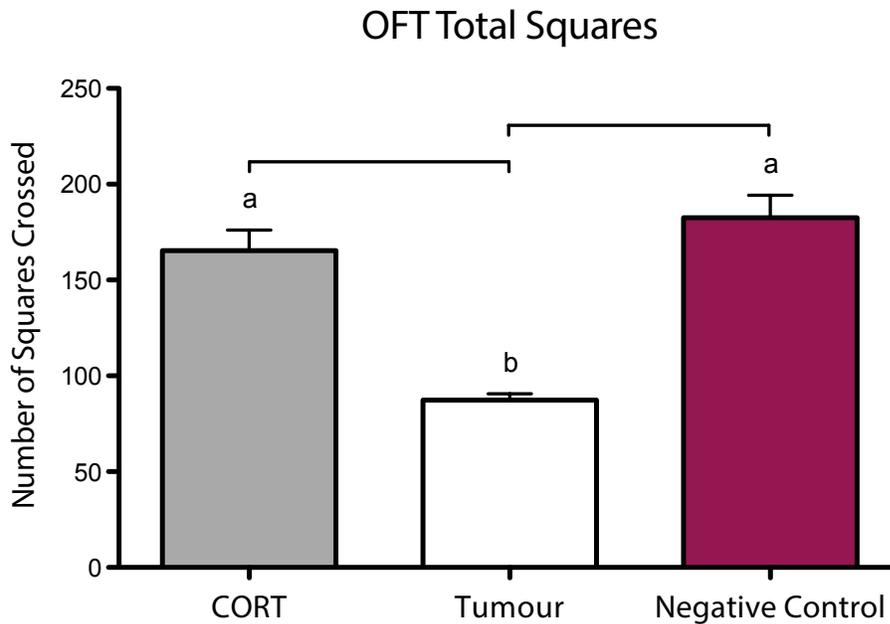
Figure 3.4: comparison of mean squares crossed in the OFT. 3 experimental groups: CORT (n = 12), tumour (n = 3), and negative control (n = 12). Tumour mice crossed significantly less squares than CORT ($P = 0.009$) and negative control ($P = 0.002$) mice.

The other outcome analyzed in the OFT is a preference for the inner or outer area of the field. A preference for the outer area is thought to represent depressive-like behaviours. Both CORT and tumour mice demonstrated a statistically significant stronger preference for the outside area of the apparatus compared to negative control mice (P = 0.004), whilst negative control mice had a significantly stronger preference for the inner area relative to both CORT and tumour animals (P = 0.004, P = 0.005, respectively) **(Figure 3.5)**.

OFT Area Preference
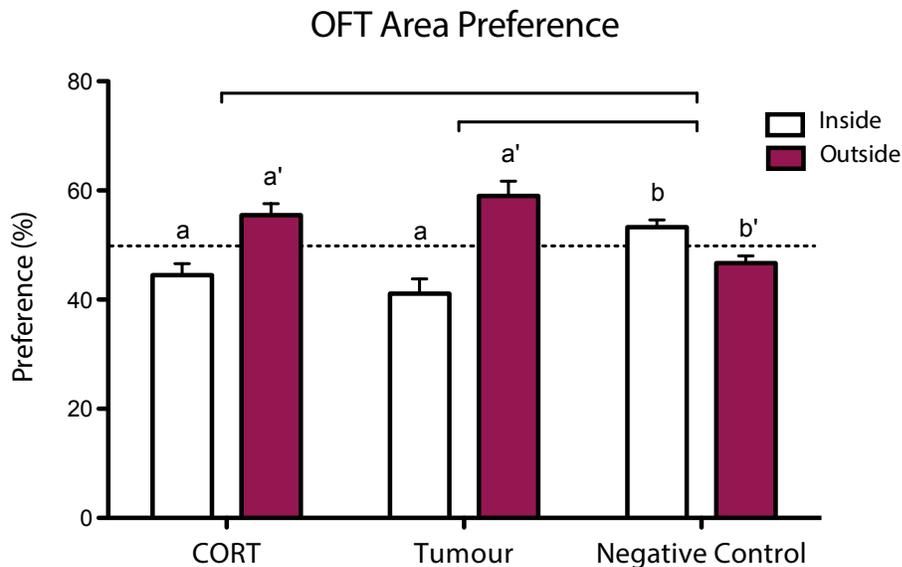
Figure 3.5: comparison of area preferences on the OFT. 3 experimental groups: CORT (n = 12), tumour (n = 4), and negative control (n = 12). Both CORT and tumour mice displayed a higher preference for the outer area, suggestive of depressive-like symptoms, while negative control animals displayed a preference for the inner area.

Objective 2

In Vitro Drug Treatments

All three drugs were found to be able to have an effect on glutamate release from RM1 prostate cancer cells in a dose-dependent manner. For SSZ, increasingly higher doses further reduced glutamate release, though lower concentrations were still effective relative to control **(Figure 3.6)**. This was expected as SSZ is a known inhibitor of system x_c^-. (*S*)-4-CPG seemed to be more effective at lower concentrations, whereas it actually increased glutamate release relative to controls at higher concentrations (250 µM) **(Figure 3.7)**. This likely means that higher concentrations of (*S*)-4-CPG are cytotoxic, increasing glutamate release as a result of cell death and apoptosis. NBQX was found to increase glutamate release relative to control at lower concentrations but effectively decrease glutamate release at higher concentrations **(Figure 3.8)**. These results are not too surprising given that NBQX is not known to inhibit system x_c^- and, therefore, glutamate release directly. Altogether, (*S*)-4-CPG at 100 µM proved to be more effective than either SSZ and NBQX at their peak concentrations of 250 µM and 300 µM, respectively. This is not surprising considering the fact that (*S*)-4-CPG is a known potent system x_c^- inhibitor, thought to be more effective than SSZ. SSZ and NBQX were more effective at higher concentrations **(Figure 3.9)**.
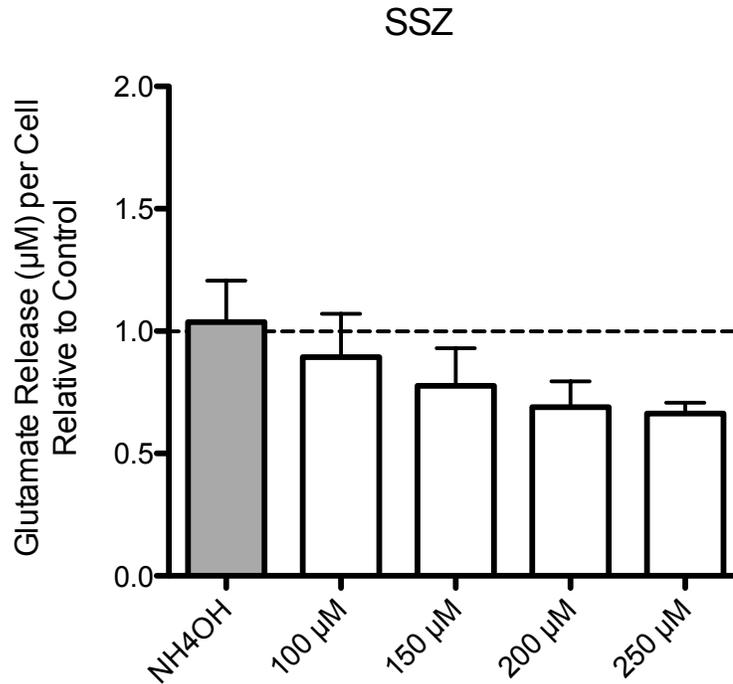
Figure 3.6: Amplex Red glutamate assay on RM1 prostate cancer cells treated with various concentrations of SSZ, or its vehicle NH_4OH. NH_4OH had a negligible effect on glutamate release, increasing it to 1.04-fold (P = 1.00). 100 µM SSZ caused a decrease in glutamate release to 0.89-fold (P = 0.99). 150 µM SSZ caused a decrease in glutamate release to 0.78-fold (P = 0.81). 200 µM SSZ caused a decrease in glutamate release to 0.69-fold (P = 0.54). 250 µM SSZ caused a decrease in glutamate release to 0.66-fold (P = 0.46).
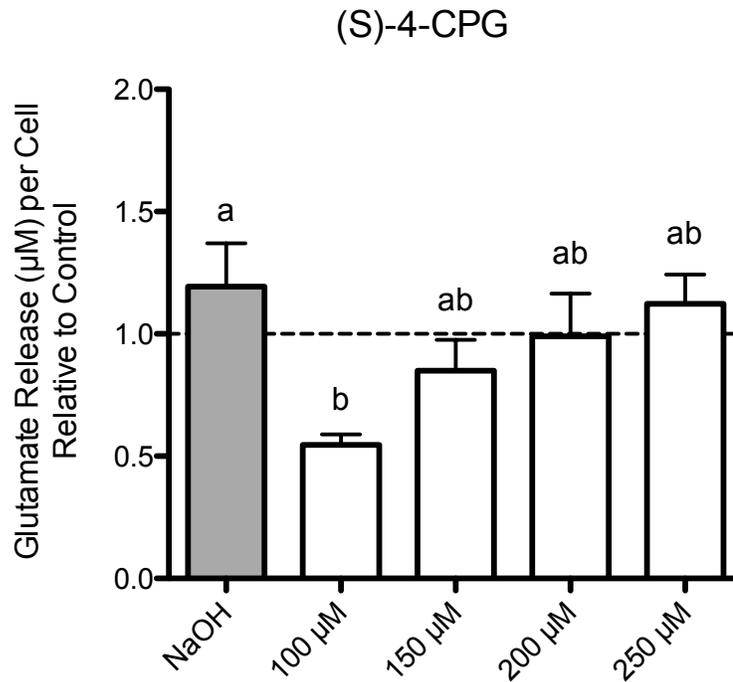
Figure 3.7: Amplex Red glutamate assay on RM1 prostate cancer cells treated with various concentrations of (*S*)-4-CPG, or its vehicle NaOH. NaOH increased glutamate release to 1.20-fold (P = 0.87). 100 µM (*S*)-4-CPG decreased glutamate release to 0.55-fold (P = 0.18). 150 µM (*S*)-4-CPG decreased glutamate release to 0.85-fold (P = 0.95). 200 µM (*S*)-4-CPG had a negligible effect on glutamate release, decreasing it to 0.99-fold (P = 1.00). 250 µM (*S*)-4-CPG increased glutamate release to 1.12-fold (P = 0.98). Further, 100 µM (*S*)-4-CPG was found to significantly reduce glutamate release relative to the vehicle NaOH (P = 0.046).
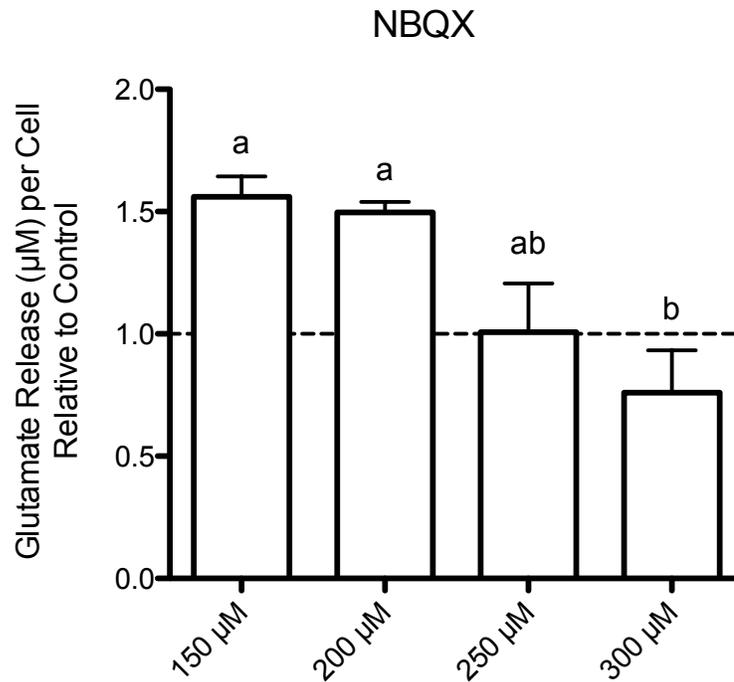
Figure 3.8: Amplex Red glutamate assay on RM1 prostate cancer cells treated with various concentrations of NBQX. 150 µM NBQX increased glutamate release to 1.56-fold (P = 0.061). 200 µM NBQX increased glutamate release to 1.50-fold (P = 0.11). 250 µM NBQX kept glutamate release at 1.00-fold relative to controls (P = 1.00). 300 µM NBQX decreased glutamate release to 0.76-fold (P = 0.67). Moreover, 300 µM NBQX was found to significantly reduce glutamate release relative to 150 µM and 200 µM NBQX (P = 0.015, P = 0.024, respectively). A vehicle was not included for NBQX as it is dissolved in Milli-Q water.
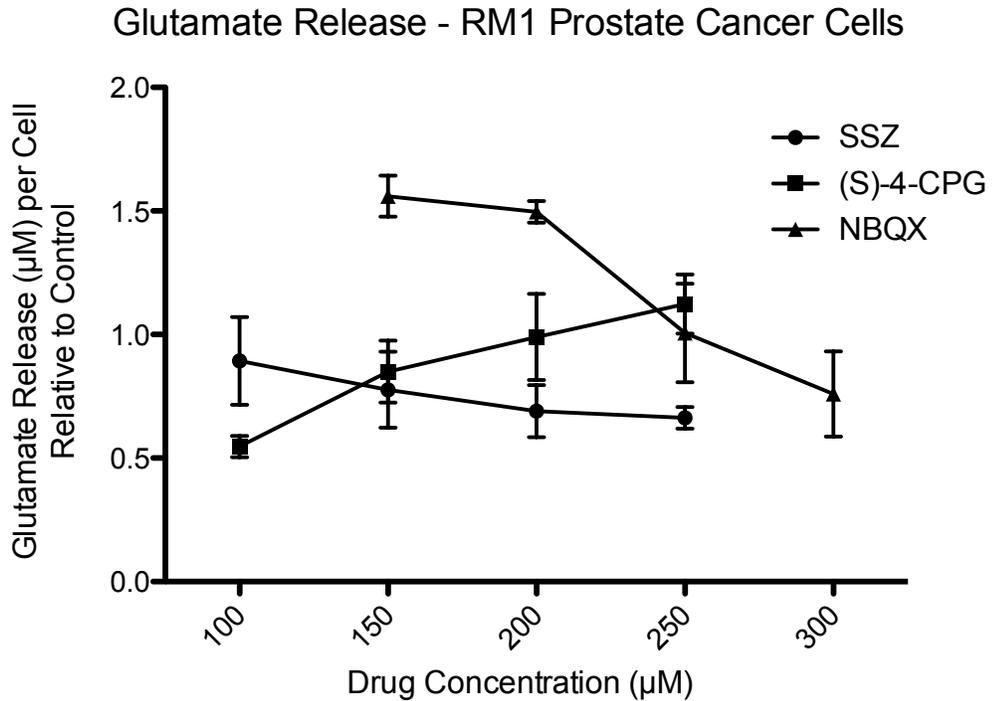
Figure 3.9: the effects of all three glutamatergic inhibitors on RM1 prostate cancer cell glutamate release *in vitro*. (*S*)-4-CPG is shown to be the most effective inhibitor of glutamate release, but only at a low concentration of 100 µM. At higher drug concentrations, SSZ becomes more effective at decreasing glutamate release. Both SSZ and (*S*)-4-CPG, which are known inhibitors of system x_c^-, are more effective than NBQX at concentrations of 150 µM and 200 µM.

Sucrose Preference Test

Recall that a lower preference for sucrose is indicative of anhedonia-like behaviours. In this experiment, the change in sucrose preference from baseline to endpoint was analyzed. In doing so, the ability of each drug to mitigate the effects of

depression on sucrose preference was studied, whilst accounting for differences in baseline preferences across groups. Tumour mice experienced a large – though not statistically significant – decrease in sucrose preference between the baseline (67.5 ± 3.5%) and endpoint (55.4 ± 3.9%). Vehicle mice experienced a similar large decline in sucrose preference, from 72.9 ± 4.1% to 59.9 ± 2.9%. SSZ mice had a lower mean sucrose preference at endpoint (63.6 ± 4.3%) compared to baseline (71.0 ± 2.1%), but the decrease was less than in tumour mice. (*S*)-4-CPG mice experienced a relatively small decline in sucrose preference, from 71.0 ± 3.3% to 69.3 ± 2.9%, as did those receiving NBQX who dropped from 68.5 ± 2.0% at baseline to 67.7 ± 3.9% at endpoint **(Figure 3.10, Table 1.1)**. Though differences between baseline and endpoint values are not statistically significant for any treatment group, we can see a trend whereby glutamatergic inhibitors lessen the degree to which sucrose preference is affected by prostate cancer tumours. A negative control group was not included as this group was comprised of animals from Objective 1, who were administered 3% sucrose solution instead of 1% sucrose solution. The tumour mice from Objective 1 were also removed from analysis.
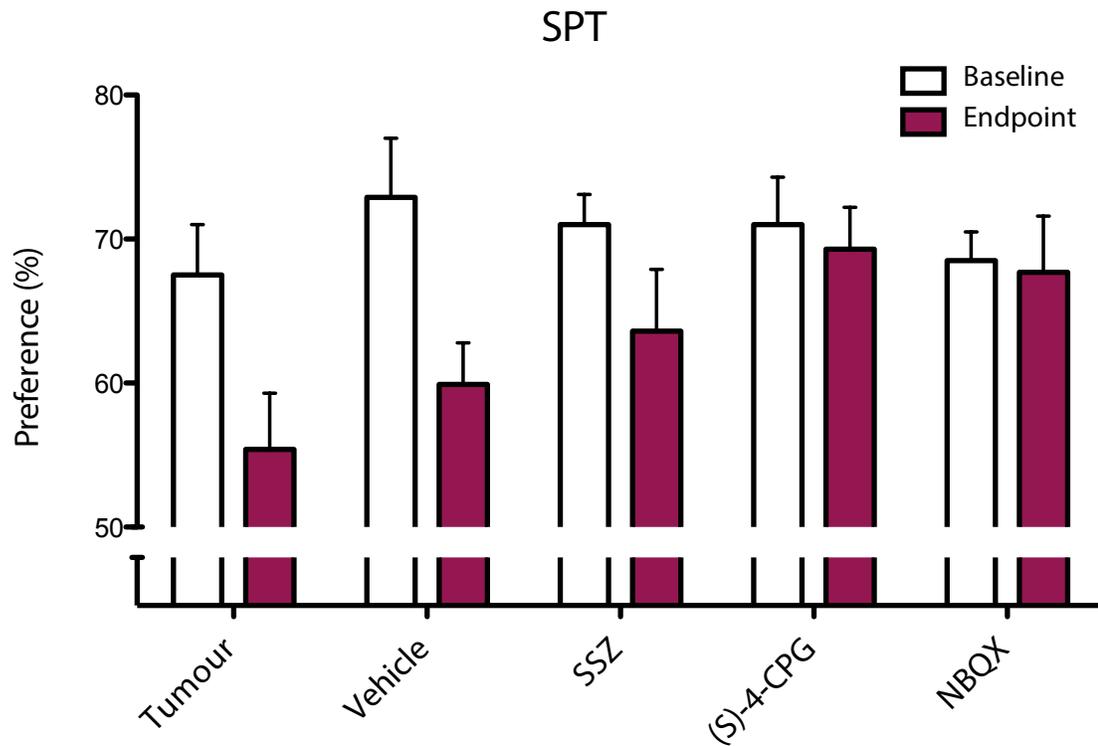
Figure 3.10: comparison of baseline and endpoint sucrose preferences for the SPT. 6 experimental groups: tumour (n = 5), vehicle (n = 10), SSZ (n = 11), (*S*)-4-CPG (n = 8), NBQX (n = 13). Tumour mice experienced a large decrease in sucrose preference from baseline to endpoint. This decrease was mitigated by the various glutamate inhibitors, though not to statistical significance.

Treatment Group	Baseline (%, Mean ± SEM)	Endpoint (%, Mean ± SEM)	Difference between baseline and endpoint (%)
Tumour	67.5 ± 3.5	55.4 ± 3.9	−12.1
Vehicle	72.9 ± 4.1	59.9 ± 2.9	−13.0
SSZ	71.0 ± 2.1	63.6 ± 4.3	−7.4
(S)-4-CPG	71.0 ± 3.3	69.3 ± 2.9	−1.7
NBQX	68.5 ± 2.0	67.7 ± 3.9	−0.8

Table 3.1: baseline and endpoint sucrose preference values, as well as the change in sucrose preference from baseline to endpoint.

Tail Suspension Test

Recall that higher immobility times on the TST represents despair-like behaviours, as it means that animals spent less time actively trying to escape from the apparatus. Negative control mice spent much less time immobile than tumour mice, though this difference was not statistically significant ($P = 0.095$). The various pharmacological inhibitors were able to decrease the immobility time relative to tumour mice, though not to statistical significance (SSZ $P = 0.526$, (S)-4-CPG $P = 0.339$, NBQX $P = 0.079$) **(Figure 3.11)**. Again, (S)-4-CPG and NBQX seem to have more pronounced effects on alleviating depressive-like symptoms than SSZ. In fact, NBQX was able to improve immobility times to essentially restore it to that of negative control mice ($P \approx 1.00$). In summary, glutamate inhibitors were able to improve immobility times on the TST, but not necessarily to the level of negative control mice.
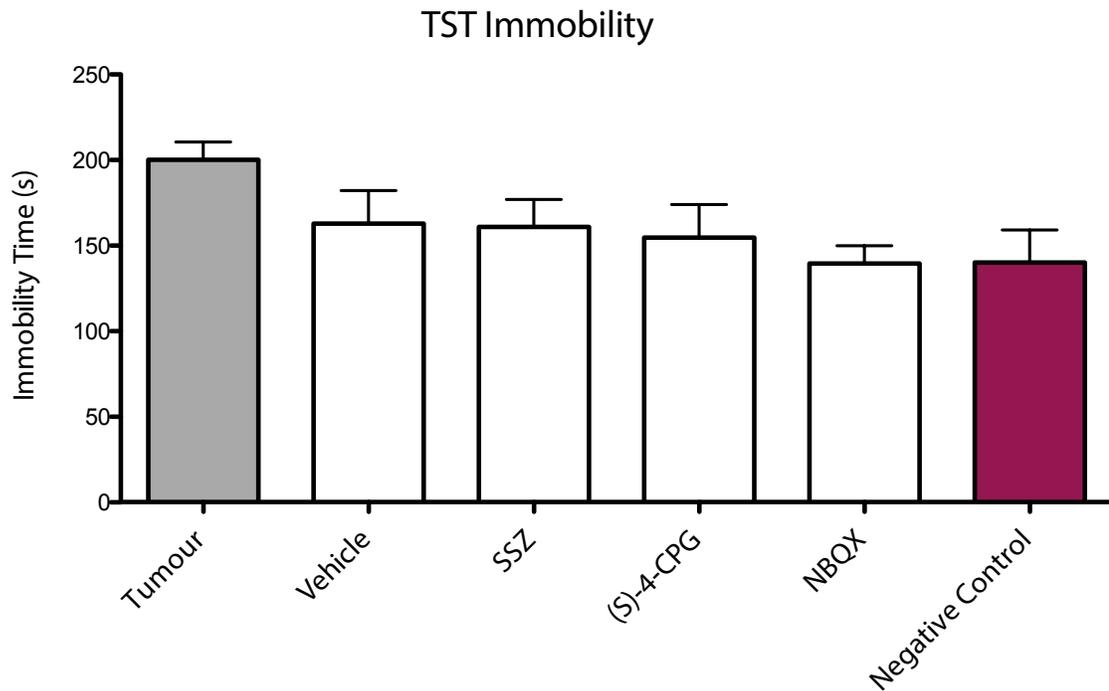
Figure 3.11: comparison of mean immobility times on the TST. 6 experimental groups: tumour (n = 12), vehicle (n = 11), SSZ (n = 11), (*S*)-4-CPG (n = 12), NBQX (n = 13), negative control (n = 12). Negative control mice had the lowest mean immobility time while tumour mice had the highest. Drug-treated mice experienced lower immobility times than tumour mice, but not as low as negative control mice.

Open Field Test

The first parameter studied in the OFT is the total number of squares crossed in the apparatus, thought to be indicative of depressive-like behaviours. All groups were statistically significantly different from negative control mice, having crossed less squares. However, we do note improvements in the number of squares crossed in the three treatment groups relative to the tumour mice, though not to statistical significance.

For instance, while P ≤ 0.0001 between the negative control group and both the tumour and vehicle groups, P ≤ 0.001 for SSZ and NBQX groups. (*S*)-4-CPG seemed to be the most effective out of the three, with P ≤ 0.01 when compared to the negative control mice.
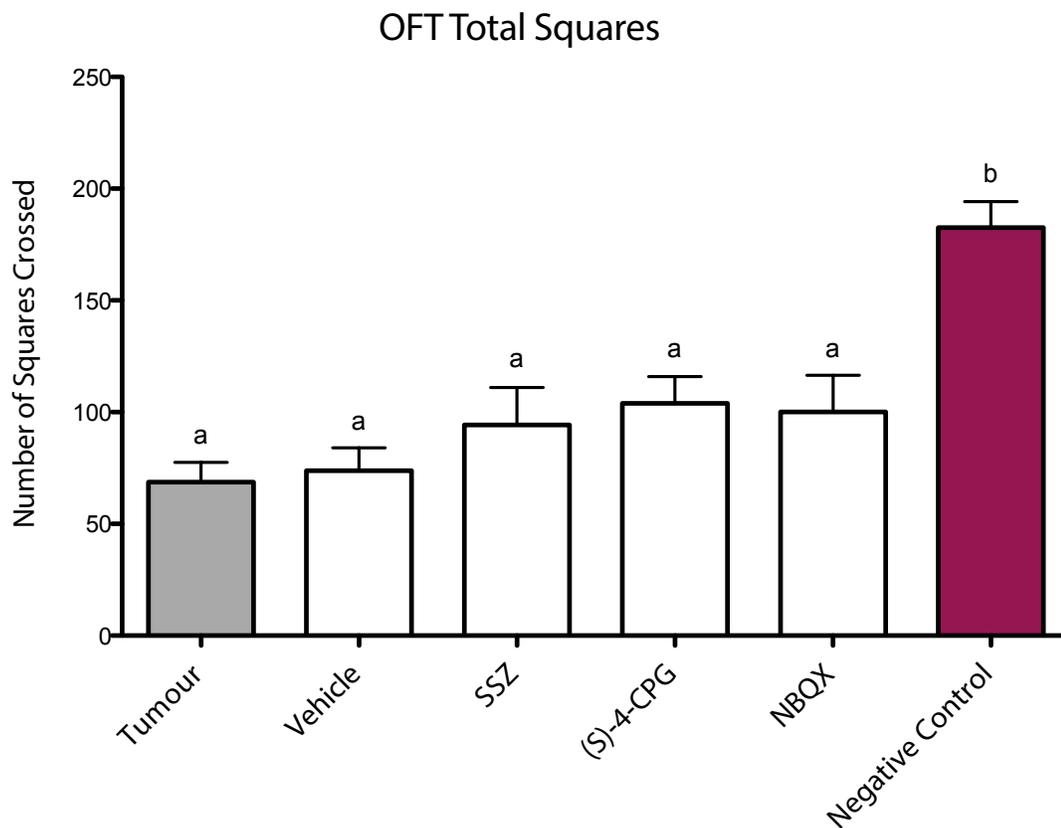
Figure 3.12: comparison of mean total squares crossed on the OFT. 6 experimental groups: tumour (n = 9), vehicle (n = 13), SSZ (n = 14), (*S*)-4-CPG (n = 12), NBQX (n = 13), negative control (n = 12). Negative control mice crossed the most squares, whilst untreated tumour-bearing mice crossed the least amount of squares. SSZ, (*S*)-4-CPG and

NBQX were able to increase the number of squares tumour-bearing mice crossed during the OFT, though not restoring it to the level of the negative control group.

The other outcome studied using the OFT was a preference for either the inner or outer area of the apparatus. As previously described, the former represents non-depressed behaviours while the latter reflects depressive-like symptoms. The tumour group was the only group to have a preference for the outside over the inside, while all other groups preferred the inner area. Both the SSZ and NBQX groups demonstrated significantly different preferences compared to the tumour group. For preference for the inner area, for instance, $P = 0.031$ and $P = 0.021$ for the SSZ and NBQX groups, respectively, compared to untreated tumour mice. It is of interest to note that the vehicle group behaved similarly to treatment groups and negative control mice, preferring the inner area over the outer area.
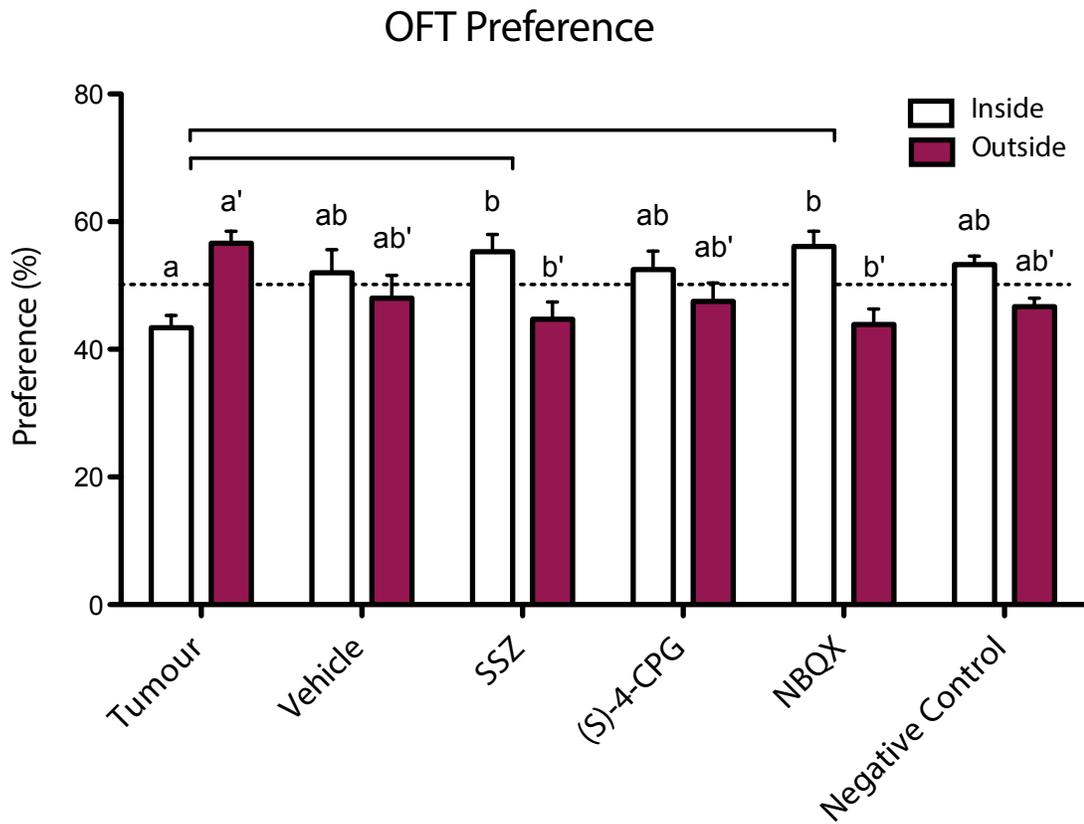
Figure 3.13: comparison of area preferences demonstrated by mice during the OFT. 6 experimental groups: tumour (n = 10), vehicle (n = 13), SSZ (n = 14), (*S*)-4-CPG (n = 12), NBQX (n = 13), negative control (n = 12). The tumour group preferred the outer area, while all other groups had a preference for the inner area. Statistically significant differences were indicated between the tumour group and the SSZ and NBQX groups for the inner area only in the figure, but the differences were also present for the same groups regarding outer area preference.

CHAPTER 4: Discussion

This study aimed to examine depression in the context of prostate cancer. It has been widely observed that the prevalence of depression is considerably higher in men who have been diagnosed with prostate cancer than in the healthy general population (Kiffel & Sher, 2015). Consequently, these patients experience a plethora of negative outcomes, including non-adherence to treatment regimens, suicidal ideation, and elevated mortality (Carlsson et al., 2013; Jayadevappa, Malkowicz, Chhatre, Johnson, & Gallo, 2012). Given the mixed reviews about the efficacy of traditional antidepressants in the cancer population, it is important to garner understanding of the biological underpinnings of prostate cancer-induced depression to develop new tailored therapeutic interventions.

The first objective of this project was to develop a validated animal model for depression. In essence, the aim was to see if the inoculation of RM1 murine prostate cancer cells into male mice would induce depressive-like symptoms. Three experimental groups allowed us to study these effects: a negative control group that was sham-injected with dead RM1 cells; a positive control group that was sham-injected, as well as administered CORT to induce a depressed state as per the chronic mild stress model of depression; and a tumour group, that would be compared to both the negative and positive control groups. If the tumour group behaved more similarly to the positive control group, it would indicate that these animals were experiencing depressive-like symptoms. Thus, a battery of behavioural tests were used: the SPT, measuring anhedonia; the TST and FST, both measuring despair; and the OFT, measuring depressive-like behaviour.

Results showed that the tumour group behaved more similarly to the positive control group than the negative control group. On the TST, the tumour group had the

highest mean immobility time, which alludes to a despair-like state. On the OFT, the tumour group crossed the fewest squares in the apparatus. Both tumour and CORT-treated mice displayed a stronger preference for the protected outer area of the open field than the exposed inner area. Altogether, these results made us confident that the subcutaneous injection of RM1 murine prostate cancer cells into C57BL/6 mice produced an appropriate model of depression that could be used to test the effect of various drugs.

Some limitations did exist with regards to this first phase of the project. A prior optimization experiment using the same animals and cell line suggested that 1% and 2% sucrose solutions did not detect any differences in sucrose preference between groups, or were not capable of establishing a sufficient baseline preference. Therefore, a 3% sucrose solution was used in this animal model experiment. However, 3% sucrose solution was found to be too high. Although there may have been differences between groups with regards to anhedonia, these were not detected as the preference for such a concentrated solution were too robust to be affected by any present depressive-like behaviours. It is possible that the reason why the animals in the optimization experiment didn't respond to the 1% and 2% sucrose solutions wasn't due to the concentrations of the solutions, but to other factors to do with the animals themselves. It was in that cohort of animals that a large portion of animals had to be sacrificed early due to the presence of abscesses. It was later determined that these animals had *Staphylococcus aureus* infections, likely attributed to the lack of a cage washer where they were housed at Charles River. Future animals were ordered from a location that had a cage washer and abscesses no longer presented as an issue.

Another limitation with this experiment was with the FST. Several animals had to be removed from testing after they began to drown. It is possible that the size of their tumours was weighing them down and impairing their ability to stay afloat; however, some animals with bigger tumours did not have this issue while others, with smaller tumours, did. It is hard to tell which animals will/will not be able to successfully complete this test; therefore, in the future, it may be best to run this test slightly earlier than endpoint in order to reduce the confounding effects of tumour burden on performance.

The second objective of this project was the one that addressed the main hypothesis: whether or not blocking glutamate release and/or signaling could alleviate depressive-like symptoms in our animal model. Three different drugs were selected: SSZ, (S)-4-CPG, and NBQX. SSZ is a known inhibitor of the cystine/glutamate antiporter system x_c^-, which releases glutamate into the extracellular environment and is highly expressed in cancer cells. (S)-4-CPG was first identified as an mGluR1 and mGlur5 antagonist, but was later discovered to double as a potent inhibitor of system x_c^-. Finally, NBQX is an AMPA receptor antagonist, which plays an important role in glutamatergic neurotransmission. Doses for all three drugs were chosen based on existing *in vivo* literature and reflected results from our own *in vitro* experiments. We found that higher concentrations of SSZ and NBQX were necessary to decrease extracellular glutamate concentrations measured from culture media; however, higher concentrations of (S)-4-CPG were found to be cytotoxic, increasing glutamate in media. Further, NBQX was effective in higher concentrations relative to SSZ. Therefore, concentrations of 8 mg/kg, 5 mg/kg, and 10 mg/kg for SSZ, (S)-4-CPG, and NBQX, respectively, were selected.

The same behavioural tests from Objective 1 were used, with the exception of the FST. Unfortunately, technological failures with the testing software rendered us unable to complete the test. For all remaining behavioural tests – the SPT, TST, and OFT – all three drugs improved depressive-like symptoms to an extent, though not always to statistical significance. In the SPT, the tumour group experienced a large decline in sucrose preference between the baseline and endpoint SPTs. This would suggest that the tumours were able to induce an anhedonic-like state in these animals. With the three treatment groups, preference still decreased from baseline to endpoint, but not to the extent that it did in the tumour group. (*S*)-4-CPG and NBQX mitigated the preference decline more effectively than did SSZ, and NBQX was the most effective. This would suggest that the three drugs were able to limit the extent to which the animals were affected by anhedonia.

With regards to immobility times on the TST, the tumour group had the highest mean immobility time, representative of despair-like behaviours and a lack of active efforts to escape from testing conditions. The SSZ and (*S*)-4-CPG groups had lower immobility times than the tumour group, though not fully restoring it to the performance level of the negative control group. Meanwhile, the NBQX group was able to improve immobility on the TST such that their mean immobility time was slightly lower than that of the negative control group (139.6 seconds versus 140.2 seconds, respectively). Taken together, the results suggest that the three treatments were able to improve despair-like symptoms as measured on the TST, spending more time trying to escape.

On the OFT, the tumour group had the lowest mean for total squares crossed, while the negative control group had the highest. The SSZ group had a higher mean than

the tumour group, the NBQX-treated mice had a higher average than the SSZ group, and the mice administered (*S*)-4-CPG had the highest mean squares crossed out of all treatment groups. However, all of the treatment groups were still statistically different from the negative control group, having been unable to sufficiently restore activity. With regards to area preference, all groups except for the tumour group preferred in the inner area. It is thought that a preference for the inner area is characteristic of anxiolytic and exploratory behaviour, which are tied to a less depressed state; meanwhile, a preference for the outer area, which is more sheltered with the sides of the apparatus acting as protection, is indicative of depressive-like symptoms. The SSZ and NBQX groups had a significantly higher preference for the inner area relative to the tumour group, while the (*S*)-4-CPG group had a higher, but not statistically significant, preference relative to the untreated tumour mice. Altogether, the OFT results suggest that the treatment groups were less depressed than the tumour group, engaging in more exploratory and locomotive activity.

The results from the three behavioural tests invite further investigations into the role of glutamate in inducing PCaID, as inhibiting the release and/or signaling of glutamate was able to improve depressive-like symptoms relative to untreated counterparts in some instances but not others. Since these drugs differ in how they impede glutamatergic signaling, we are afforded insight into the possible mechanisms that may be at play in PCaID. As discussed earlier, it is not yet elucidated whether the effects of glutamate are direct, via transport of glutamate from the periphery into the brain, or indirect, via the action of glutamate in the periphery that is transmitted centrally to the

brain. Since (*S*)-4-CPG and NBQX are both glutamate receptor antagonists, and generally seemed to be more efficacious relative to SSZ in behavioural testing, it would seem that the latter, indirect mechanism plays a large role in PCaID. However, we must also consider that we are unable to tease apart the effect of (*S*)-4-CPG on system x_c^- and group 1 mGluRs, as well as the fact that SSZ still had a positive effect on depressive-like symptoms. Therefore, the role of glutamate released from peripheral tumours cannot be ignored.

It is of interest to note that NBQX largely seemed to be the most beneficial of the three treatment options. Recall that previous explorations of NBQX in the context of depression studied its ability to block the antidepressant properties of ketamine and other NMDA antagonists (Park, Niciu, & Zarate, 2015; Pereira, Romano, Wegener, & Joca, 2015). Or, when NBQX was studied independent of other therapeutics, only single doses were used a short time prior to behavioural testing. Here, the effects of chronically administered NBQX were studied and proved to be more efficacious than acute administrations discussed in the literature. Therefore, further investigations into the properties of NBQX as an antidepressant and how it interacts with other drugs is warranted. Its efficacy as an antidepressant may also further support the significance of the NMDA receptor in glutamate-based depression; the initial activation of AMPA receptors is largely responsible for removing the Mg^{2+} block in NMDA receptors and, therefore, the antagonism of AMPA receptors may significantly impact NMDA receptor neurotransmission and limit excitotoxicity.

Unexpectedly, though the vehicle control group behaved on par with the tumour group in certain instances, like the SPT and for total squares crossed in the OFT, at other times they acted similarly to the SSZ group, as in the TST and for area preference in the OFT. This observation invites further investigation into the effects of the vehicle, as it's possible that these outcomes could be attributed to its basic nature. Given the fact that glutamate has acidic side chains, it is possible that there may be an interaction or neutralization reaction between the basic vehicle and glutamate. It may also be interesting to see whether dissolving SSZ and (S)-4-CPG in other solvents affects their efficacy, either positively or negatively.

There were a few challenges encountered while conducting these experiments. As already noted, difficulties with the FST software made it impossible to administer the test to animals and, therefore, data was not collected for that particular behavioural test. Future investigations may benefit from having this additional test to corroborate the results of other behavioural data. Furthermore, a negative control group was missing from SPT analysis, as well as a number of tumour control mice, since these animals were administered 3% sucrose solution as per Objective 1 and did not have usable data to analyze against treatment groups. Consequently, future experiments would benefit from the inclusion of a negative control group for the SPT in order to gain an understanding of how their sucrose preference changes over time, if at all.

CHAPTER 5: Conclusion

Men who are diagnosed with prostate cancer are more prone to develop depression than the general population. A biological mechanism that could contribute to this finding is the fact that cancer cells release excessive amounts of glutamate into the extracellular environment. It is thought that this is facilitated, at least in part, by the antiporter system x_c^-, which secretes glutamate in exchange for importing cystine, a precursor to the antioxidant GSH that protects cells against oxidative stress. When glutamate exists in excessive concentrations extracellularly, it can lead to excitotoxicity whereby neurons are hyperactivated and neurodegeneration occurs as a result.

Given these unique properties that exist in cancer cells and the cancer environment, new therapeutics beyond SSRIs and traditional antidepressants are warranted for the depressed cancer population. This project aimed to assess the efficacy of glutamatergic inhibitors in the treatment of depressive symptoms. The results of our experiments support the idea that glutamate contributes to the onset of PCaID. Both the peripheral release of glutamate from cancer cells and the central action of glutamate on its receptors was inhibited. Behavioural data suggests that the central modulation of glutamate may be more effective in treating PCaID compared to targeting cancer-derived glutamate. Taken together, this project supports the idea that targeting glutamate could be an effective strategy to treat depression in the cancer population, either as a standalone intervention or as an adjuvant to current treatment options.

Future Directions

As mentioned in the Methods section, blood samples were collected from each animal at the time of sacrifice via intracardial exsanguination. Samples were then spun down to separate the various components of whole blood, following which serum plasma was collected and stored for each animal. At present, the Amplex Red glutamate assay is not optimized for use with serum samples in which antioxidants are present, or serum where endogenous proteases and oxidases are active. It has previously been shown that various biomarkers of oxidative stress are present in colorectal cancer patients, including antioxidants (Wu et al., 2017). Therefore, while the Amplex Red assay performs optimally with extracellular solutions (as in our *in vitro* drug treatments, where media was collected from cell samples and measured for glutamate release), it is limited in its ability to quantify *in vivo* serum concentrations.

Our lab is currently in the process of optimizing high-performance liquid chromatography (HPLC) to measure glutamate in *in vivo* serum samples, which has been shown in the past to be an effective technique for that purpose (Janik, Kalbarczyk, Gutowicz, Barańczyk-Kuźma, & Kwieciński, 2010). When the HPLC parameters have been fully optimized for glutamate, it may prove to be an effective means for glutamate quantification in animal samples. When we are able to accurately measure glutamate concentrations in serum, we will be more equipped to corroborate our behavioural data with information about how much, if at all, our drugs were able to affect glutamate *in vivo*.

Yet, even after we develop appropriate means to measure serum glutamate, we will have to be cautious about interpreting the data. We know that under normal conditions, there is a unidirectional movement of glutamate from the brain to the blood via EAATs on the BBB. However, it has also been shown that under pathological states, breakdown of the BBB allows for bidirectional flow of glutamate. Given the drastic concentration gradient between the extracellular fluid (ECF) in the brain, where glutamate exists in concentrations between 0.5-2 μmol/L, and the blood, where concentrations range from 50-100 μmol/L, plasma glutamate is likely to move from the periphery to the ECF. Therefore, making inferences about the effects of our drugs based on serum glutamate concentrations will be complicated by the potential bidirectional movement of glutamate across the BBB. Factors like whether or not some peripheral glutamate has moved into the brain, and therefore is not quantified in our samples, will need to be considered.

Furthermore, tumour tissues were collected and stored for each animal. It would be of interest to measure xCT – the functional subunit of system x_c^- – in these samples to see if its expression changes with the administration of our drugs. For example, does the antagonism of system x_c^- by SSZ and (*S*)-4-CPG lead to the upregulation of xCT in prostate cancer cells? This could be a mechanism through which cancer cells try to protect themselves against oxidative stress and accommodate for the limited cystine import from blocked system x_c^- antiporters.

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APPENDIX 1: ALZET Micro-osmotic Pumps

Model 1002 ALZET micro-osmotic pumps (DURECT Corporation, Cupertino, CA, USA) were chosen as a means of drug delivery in order to avoid stressful daily i.p. injections. All pumps are constructed with three distinct layers: 1) the innermost layer, an impermeable drug reservoir, 2) the middle layer, an osmotic layer, and 3) the outermost layer, a semipermeable membrane **(Figure A1)**. After implantation into an animal, fluids from the intraperitoneal cavity begin to pass through the outer semipermeable membrane. These fluids are then absorbed by the osmotic layer. The osmotic layer then expands as a result and pushes against the drug reservoir which, though impermeable, is made of flexible material. The forces that push the wall of the drug reservoir inwards pushes the drug out of the release channel at a controlled rate that is determined by the specifications of the pump. In the case of the model 1002 pumps, this rate was 0.25 ± 0.05 μL per hour.
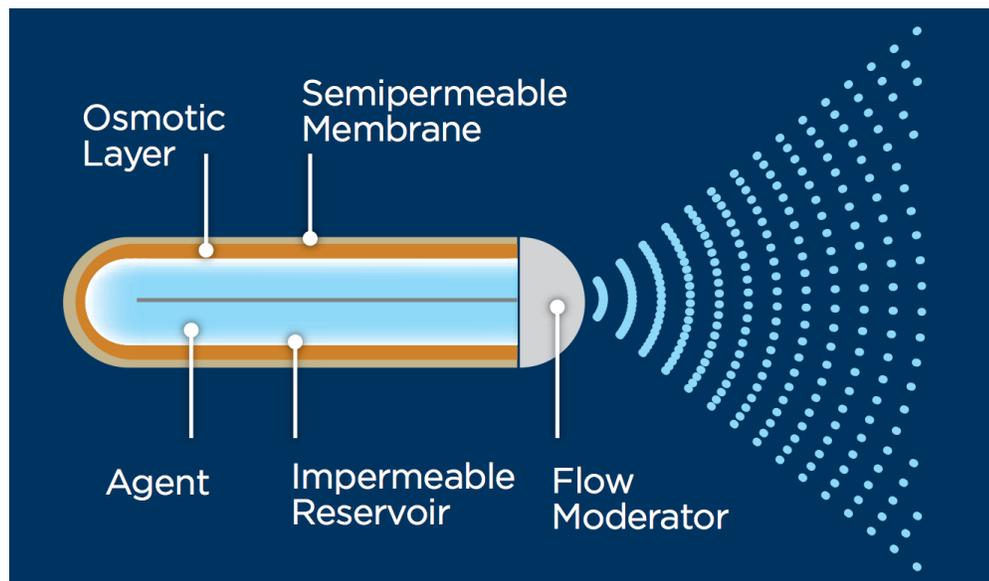
Figure A1: the layers of an ALZET micro-osmotic pump. Image from ALZET.