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METHYLGLYOXAL AND GLYOXALASE1 (GLO1) IN PSYCHIATRIC DISORDERS

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Abstract

Many current pharmacological treatments for neuropsychiatric disorders, such as anxiety, alcohol use disorder (AUDs) and depression, are limited by a delayed onset of therapeutic effects, adverse side effects, abuse potential or lack of effects in many patients. Thus, identifying novel mechanisms and targets for treatment is very important. Glyoxalase 1 (GLO1) is a ubiquitous cytosolic enzyme primarily responsible for the clearance of methylglyoxal (MG) which is a competitive partial agonist at GABA-A receptors. Previous studies have implicated GLO1 in neuropsychiatric disorders, such as anxiety and depression. Here, we sought to build on these previous studies and assess the therapeutic potential of targeting GLO1 for the treatment of anxiety and depression. Additionally, as anxiety and depression are highly comorbid with AUD, a disorder in which GABA-A receptor function is strongly implicated, we also investigated the effects of GLO1 regulation in animal models of AUD.

Within the three chapters of this dissertation, genetic and pharmacological approaches were used to assess the impact of regulating GLO1 or MG concentrations in mouse models of anxiety, AUDs and depression. Chapter 2 details the investigation into the relevant neuroanatomical regions associated with GLO1 and MG regulation of anxiety-like behavior. As previous studies used ubiquitous overexpression, we sought to determine whether neuronal *Glo1* overexpression was sufficient to increase anxiety-like behavior. Furthermore, since previous administration of MG had been systemic, we sought to determine if direct microinjection of MG into the basolateral amygdala (BLA) was sufficient to reduce anxiety-like behavior. As expected, we found that anatomically specific manipulations of *Glo1* and MG were sufficient to induce changes in anxiety behavior. Chapter 3 explores the impact of *Glo1* expression on alcohol consumption and assesses the therapeutic potential of GLO1 inhibition for the treatment of

alcohol use disorders. We found that transgenic mice overexpressing *Glo1* on two different genetic backgrounds showed increased voluntary ethanol consumption compared to their wild-type littermates in a mouse model of binge drinking. Conversely, transgenic *Glo1* knockdown mice on a B6 background showed decreased voluntary ethanol consumption in DID. Furthermore, pharmacological GLO1 inhibition also reduced drinking in DID. Finally, Chapter 4 examines the role of GLO1 inhibition in depression-like behavior and evaluates the therapeutic potential of GLO1 inhibitors as novel fast-acting antidepressants. We found that GLO1 inhibition by either genetic knockdown or one of two structurally distinct GLO1 inhibitors reduced depression-like behavior in multiple mouse models of depression. We further found that GLO1 inhibitors may reduce depression-like behavior with subchronic (5 day) administration, suggesting GLO1 inhibitors may be fast-acting antidepressants. Together, the data presented within this dissertation strongly support a role for GLO1 inhibitors in the treatment of multiple highly comorbid psychiatric disorders.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Anxiety and depressive disorders affect about one in four adults at some point in their lifetime, while alcohol use disorders (**AUD**) have a twelve month prevalence of about one in ten (Grant et al. 2004; Kessler et al. 2012a; Kessler et al. 2012b). Although a variety of pharmaceuticals are available to treat these neuropsychiatric disorders, illness remains refractory in a significant portion of patients and many currently used drugs have adverse side effects and high abuse potential. Thus, identification of new biological targets and novel pharmaceuticals remains an important goal in treating these disorders (Machado-Vieira et al. 2008a; Rudolph and Knoflach 2011).

1.2 Anxiety, depression and AUDs

Anxiety is felt in response to aversive, dangerous or fearful situations, though anxiety disorders may develop when seemingly innocuous stimuli now induce excessive or inappropriate anxiety (Gross and Hen 2004; American Psychiatric Association 2013). Anxiety disorders are the most common psychiatric disorders and are divided within the Diagnostic and Statistical Manual of Mental Disorders (**DSM**) into subclasses that include generalized anxiety disorder, panic disorder and post-traumatic stress disorder (Kessler et al. 2005a; Kessler et al. 2005b; American Psychiatric Association 2013). The primary treatments for anxiety disorders include benzodiazepines, beta-blockers, barbituates and selective serotonin reuptake inhibitors (**SSRI**s). However, these treatments are limited by their abuse potential (benzodiazepines and barbituates), side effects (weight gain, sedation, etc.), and delayed onset (up to 4-6 weeks for SSRIs), pointing to a need for novel therapeutics (Kent et al. 2002; Rudolph and Knoflach 2011).

Depressive disorders, which include major depressive disorder, persistent depressive disorder and premenstrual dysphoric disorder, among others, are the second most common psychiatric disorders (Kessler et al. 2005a). Major depressive disorder (hereafter simply referred to as 'depression') is characterized by pervasive & persistent low mood, feelings of inappropriate guilt, worthlessness, hopelessness and/or an inability to feel pleasure in previously pleasurable activities (anhedonia)(American Psychiatric Association 2013). Depression can be extremely debilitating with adverse impacts on family, personal relationships and work lives and is also associated with a large economic burden (Greenberg et al. 2003; Willner et al. 2013). Similar to anxiety disorders, the most commonly prescribed class of antidepressants are SSRIs, though serotonin-norepinephrine reuptake inhibitors, tricyclics, monoamine oxidase inhibitors, and others are also commonly prescribed (Nestler and Carlezon 2006; Berton and Nestler 2006; Duman and Voleti 2012; Willner et al. 2013). Treatments for depression have primarily been limited by a lack of efficacy in ~50% of those treated and a delayed therapeutic onset of 2-4 weeks (Berton and Nestler 2006; Barbui et al. 2011; Willner et al. 2013).

Though previously thought to be impossible, antidepressants such as ketamine have recently demonstrated a rapid onset of therapeutic action in both humans and rodent models (Machado-Vieira et al. 2008b; Autry et al. 2011; Browne and Lucki 2013; Martinowich et al. 2013; Fischell et al. 2015). Though ketamine, in particular, has been limited by a high abuse potential, its ability to induce rapid antidepressant effects has generated considerable interest in the development of additional fast-acting antidepressants and the molecular pathways associated with these effects (Berton and Nestler 2006; Machado-Vieira et al. 2008b; Browne and Lucki 2013; Martinowich et al. 2013).

Alcohol use disorders (AUD) are characterized by "a problematic pattern of alcohol use

leading to clinically significant impairment or distress"(American Psychiatric Association 2013). AUDs are classified as mild, moderate or severe based on criteria presented in the DSM (American Psychiatric Association 2013). These criteria are associated with cravings for alcohol, persistent unsuccessful efforts to cut down or control alcohol use and alcohol use in either larger amounts or over a longer period of time than was intended and others (American Psychiatric Association 2013). While the most commonly prescribed treatments for AUD, naltrexone and acamprosate, have limited abuse potential and minimal side effects, their effect sizes are modest and show mixed efficacy in clinical trials when compared to placebo (Heilig et al. 2010; Maisel et al. 2012). Interestingly, studies investigating naltrexone have suggested that this is the result of the effects of naltrexone being limited to patients with a genetic variant of a gene for the m-opioid receptor (OPRM1) on which naltrexone acts (Heilig et al. 2010). The limited effects of acamprosate in the general population and naltrexone to a subpopulation, point to the importance of the identification and development of pharmacological treatments targeting alternative pathways that may effectively reach this largely non-responsive population.

Anxiety, depression and AUDs are highly comorbid and share high genetic liability, which may point to similar underlying mechanisms associated with risk of developing these disorders (Driessen et al. 2001; Grant et al. 2004; Bruce et al. 2005; Kessler et al. 2005b; Dawson et al. 2007; Kendler et al. 2007a; Smith and Book 2010; Boschloo et al. 2011; Rubio et al. 2011). While it is difficult to precisely determine rates of comorbidity, as many as 59% of patients with a lifetime diagnosis of depression will also be diagnosed with an anxiety disorder, 20.3% of those with anxiety and depression may present with alcohol dependence and in those with AUDs up to 38% will also be diagnosed with depression and 52% will be diagnosed with anxiety (Driessen et al. 2001; Boschloo et al. 2011; Demirkan et al. 2011). Further, comorbid

disorders are associated with worse treatment outcomes (Driessen et al. 2001; Bruce et al. 2005; Sullivan et al. 2005; Dawson et al. 2007), which point again to the benefit of identifying novel treatments with therapeutic potential for all three of these disorders.

1.3 *Glyoxalase1 (Glo1)* and Methylglyoxal (MG)

Recent studies have identified glyoxalase 1 (GLO1) as a new target for neurological and psychiatric conditions. Increased *Glo1* gene-expression is associated with anxiety- and depression-like behavior as well as seizure susceptibility in mice (Hovatta et al. 2005a; Williams et al. 2009; Benton et al. 2012a; Distler et al. 2012a; Distler et al. 2012b; Distler et al. 2013) and new evidence presented within this thesis also suggests a role for *Glo1* in regulating ethanol consumption (which will be discussed in more detail in later sections).

The role of GLO1 in psychiatric illness was somewhat unexpected as GLO1 is a ubiquitous cytosolic enzyme that catalyzes the reaction between glutathione and acyclic α-oxoaldehydes, particularly methylglyoxal (MG) (Thornalley 1990; Thornalley 1993; Thornalley 1996a; Thornalley 2003a). MG is formed as a byproduct during photosynthesis, protein and fatty acid catabolism and glycolysis; principally by the non-enzymatic degradation of acetone, aminoacetone and the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (**Figure 1.1a**; Thornalley 1996b). *In vitro* studies have demonstrated GLO1 is critical for clearing MG; indeed, overexpression of *Glo1* prevents MG accumulation, while GLO1 inhibition results in MG accumulation (**Figure 1.1b**; Thornalley 1990; Thornalley 1993; Thornalley 1996a; Thornalley 2003a).

Historically, most research on GLO1 has focused on the importance of detoxification of MG to prevent cellular damage due to the glycation of proteins and nucleic acids (Brownlee 2001; Thornalley 2003b). These studies have implicated high concentrations of MG and/or low

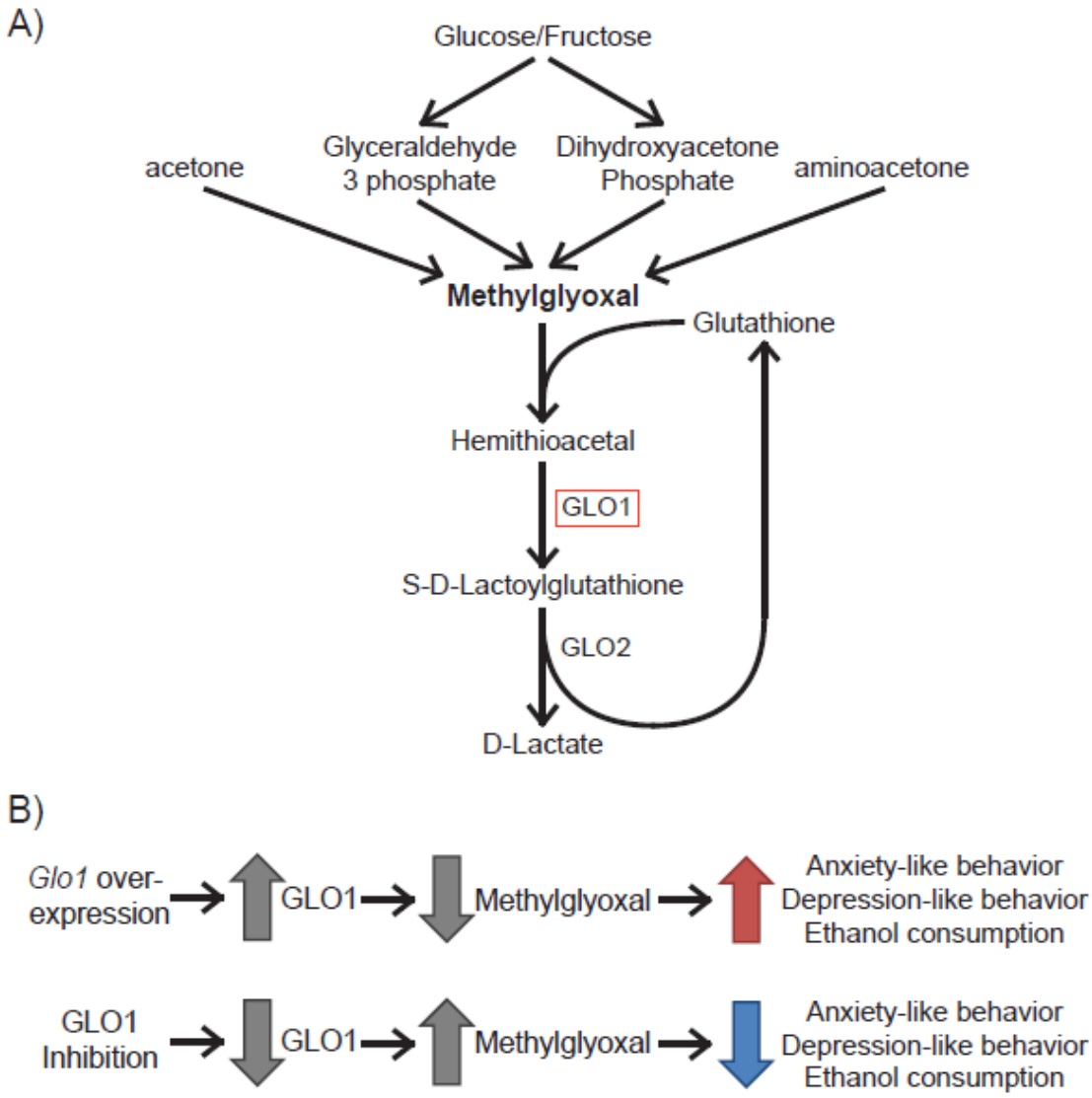

Figure 1.1 Formation and degradation of methylglyoxal and the role of the glyoxalase pathway in psychiatric disorders. (A) Methylglyoxal is principally formed by the non-enzymatic degradation of acetone, aminoacetone and the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Methyglyoxal and glutathione converge to form hemithioacetal, which is degraded by GLO1 and forms S-D-Lactoylglutathione. GLO2 then degrades S-D-Lactoylglutathione into D-lactate and glutathione. (B) Overexpression of *Glo1* results in increased enzymatic activity of GLO1 and an increased clearance of methylglyoxal. Increased clearance results in lower concentrations of methylglyoxal and subsequent increases in anxiety-like behavior, depression-like behavior and ethanol consumption. Correspondingly, GLO1 inhibition (genetic of pharmacological) reduces GLO1 activity, leading to increases in methylglyoxal and subsequent decreases in anxiety-like behavior, depression-like behavior and ethanol consumption.

GLO1 activity in the etiology of metabolic disorders, such as diabetes and in the development of cellular pathologies including aging (Thornalley 2003a; Thornalley and Rabbani 2011). At high levels, accumulation of MG adducts on proteins and nucleotides can lead to the formation of advanced glycation end-products that can cause these proteins to become dysfunctional and can trigger the production of reactive oxygen species which in turn can lead to apoptosis (Thornalley 1996a; Brownlee 2001; Thornalley 2003a; Ahmed and Thornalley 2007). Additionally, at high plasma levels MG is thought to evoke diabetes-associated neuropathic pain by stimulating TRPA1 or Nav1.8 receptors peripherally (Jack et al. 2012; Bierhaus et al. 2012; Andersson et al. 2013). Thus, strategies to reduce MG concentrations and/or enhance GLO1 activity may have therapeutic potential (Rabbani and Thornalley 2011; Jack et al. 2012). In contrast, many cancers exhibit enhanced GLO1 activity; it has been suggested that inhibition of GLO1 would therefore have anticancer properties (Brownlee 2001; Ahmed and Thornalley 2007; Morcos et al. 2008; Fleming et al. 2011).

In addition to the putative positive effects of GLO1 inhibitors in cancer treatment, recent studies from several labs indicated that modulation of MG concentrations and GLO1 activity can alter anxiety, depression, seizure, sleep, and pain phenotypes in mice (Hambsch et al. 2010; Distler et al. 2012a; Bierhaus et al. 2012; Distler et al. 2013; Jakubcakova et al. 2013). Therefore, increasing MG concentrations by inhibiting GLO1 may also represent a novel strategy for the treatment of neuropsychiatric disorders.

1.4 *Glo1* and methylglyoxal in neuropsychiatric disorders

A positive correlation between *Glo1* expression and anxiety-like behavior was first reported among a panel of inbred mouse strains, and has since been corroborated by numerous studies (Hovatta et al. 2005b; Reiner-Benaim et al. 2007; Williams et al. 2009; Loos et al. 2009;

Benton et al. 2012a). Subsequent studies confirmed a causal role for *Glo1* in anxiety-like behavior using viral vectors and transgenic mice to show that *Glo1* overexpression increased anxiety-like behavior, while knockdown decreased anxiety-like behavior (Hovatta et al. 2005b). Human genetic studies have yielded discrepant results regarding the association between *Glo1* and anxiety (Politi et al. 2006; Eser et al. 2011). However, interpretation of these data in humans is limited by small sample sizes and potential population stratification. Larger, well-controlled human genetic studies are required to elucidate the role of *Glo1* in human anxiety disorders.

In addition to anxiety, there is strong evidence that *Glo1* regulates other neuropsychiatric phenotypes in mice, including epilepsy, depression and neuropathic pain. For example, increased seizure susceptibility was associated with high *Glo1* expression among recombinant inbred mice and transgenic mice overexpressing *Glo1* (Distler et al. 2013). Another study found a clear, positive correlation between GLO1 protein levels and depression-like behavior (Benton et al. 2012a). While some studies in humans have suggested a role for *Glo1* in neuropsychiatric diseases other than anxiety, the evidence is usually less compelling and is limited by small sample size and a lack of replication. For example, one study reported a negative correlation between *Glo1* expression and depression; additional studies have reported negative correlation between *Glo1* expression and neuropathic pain, as well as associations between *Glo1* expression and autism, schizophrenia, and restless legs syndrome (Junaid et al. 2004; Sacco et al. 2007; Winkelmann et al. 2007; Stefansson et al. 2007; Rehnström et al. 2008; Fujimoto et al. 2008; Wu et al. 2008; Kemlink et al. 2009; Arai et al. 2010; Winkelmann et al. 2011; Toyosima et al. 2011; Jack and Wright 2012; Bierhaus et al. 2012; Skapare et al. 2013; Groener et al. 2013). At this time, rigorous analysis to determine the impact of *Glo1* expression levels, copy number variants or polymorphisms on the etiology or pathogenesis of human neuropsychiatric disorders is

lacking.

1.5 Mechanism of action - GABA receptors and MG

A previous study preformed in the lab reported that physiological levels of MG (low μM) are anxiolytic in mice by a simple mechanism: MG is a specific, partial, reversible agonist of GABA-A receptors in central neurons (Distler et al. 2012a). GABA-A receptors are pentameric, ligand-gated ion channels, and are comprised of two α-subunits (α1-6), two β-subunits (β1-4) and one γ1-4, δ, ε, θ, π or ρ1-3 subunit. The namesake ligand for GABA-A receptors is γ-aminobutyric acid (**GABA**). In the adult brain GABA serves as an inhibitory neurotransmitter. Binding of GABA to specific pockets at the interface of α and β-subunits opens a channel in the center of GABA-A receptors, this hyperpolarizes the membrane potential by passing Cl- ions. GABA-A receptors are present both at synapses and on the soma of neurons, and produce phasic and tonic currents, respectively (Kalueff and Nutt 2007; Vithlani et al. 2011; Brickley and Mody 2012). Application of MG to cerebellar granule or hippocampal neurons evokes Cl- currents that modulate the membrane potential and are blocked by the GABA-A specific antagonist SR-95531 (Distler et al. 2012a). MG evoked currents are ~⅓ of the magnitude of those evoked by GABA in the same cells and co-application with GABA is competitive, not additive, suggesting that both ligands act at the same binding site (Distler et al. 2012a). Importantly, the concentration of MG required to evoke currents in neurons is in the physiological range and the EC50 measured from the concentration-response relationship is ~10 μM, suggesting that small changes in concentration of MG will produce marked effects in the current magnitude. Based on these observations, MG can be described as an endogenously produced competitive partial agonist at GABA-A receptors at physiologically relevant concentrations (**Figure 1.1A**).

Alterations in GABAergic signaling are implicated in numerous neurological and

psychiatric disorders, including depression, anxiety, AUD, panic, schizophrenia, Huntington's, Parkinson's, Alzheimer's, epilepsy, sleep, and chronic pain syndromes (Koob 2006; Kalueff and Nutt 2007; Kumar et al. 2009; Gajcy et al. 2010; Pizzarelli and Cherubini 2011; Luscher et al. 2011). Many commonly prescribed anxiolytic agents, such as the benzodiazepine, midazolam, target extrasynaptic GABA-A receptors with the aim of augmenting tonic inhibition (Yeung et al. 2003; Brickley and Mody 2012). Extracellular GABA-A receptors frequently contain α5/α6 and δ subunits; assemblies that are prominent in hippocampal and neocortical pyramidal neurons (α5βγ2) and CGN (α6βδ) (Nusser et al. 1999). The action of MG at extrasynaptic GABA-A receptors may be of particular relevance to pathophysiology because the concentration of GABA at extrasynaptic receptors is low (<μM), while MG has been measured at ~5 μM in mouse brain (Vithlani et al. 2011; Distler et al. 2012a).

Benzodiazepines are positive allosteric modulators of GABA-A receptors, augmenting inhibitory currents when GABA binds (Brickley and Mody 2012). Two such benzodiazepines (midazolam and diazepam) also augment GABAergic Cl- currents when MG binds to GABA-A receptors in hippocampal neurons. Similarly, the effects of MG are augmented by zolpidem, a non-benzodiazepine, imidazopyridine-based positive allosteric modulator of GABA-A receptors (**Figure 1.2**) (Distler et al. 2012). It is not yet known whether the activity or efficacy of benzodiazepines at specific GABA-A receptor subtypes differs between MG- and GABA-induced activation. However, the studies described above suggest that MG can activate GABA-A receptors that contain diazepam- and midazolam-sensitive α1-3 and α5 subunits as well as those with zolpidem-sensitive α1 and γ2 subunits. This array of subunits is common in brain areas associated with anxiety and depression, including hippocampal and cortical interneurons (α1β2γ2 receptors) and the limbic system (α2βXγ1 receptors) (Marowsky et al. 2004).

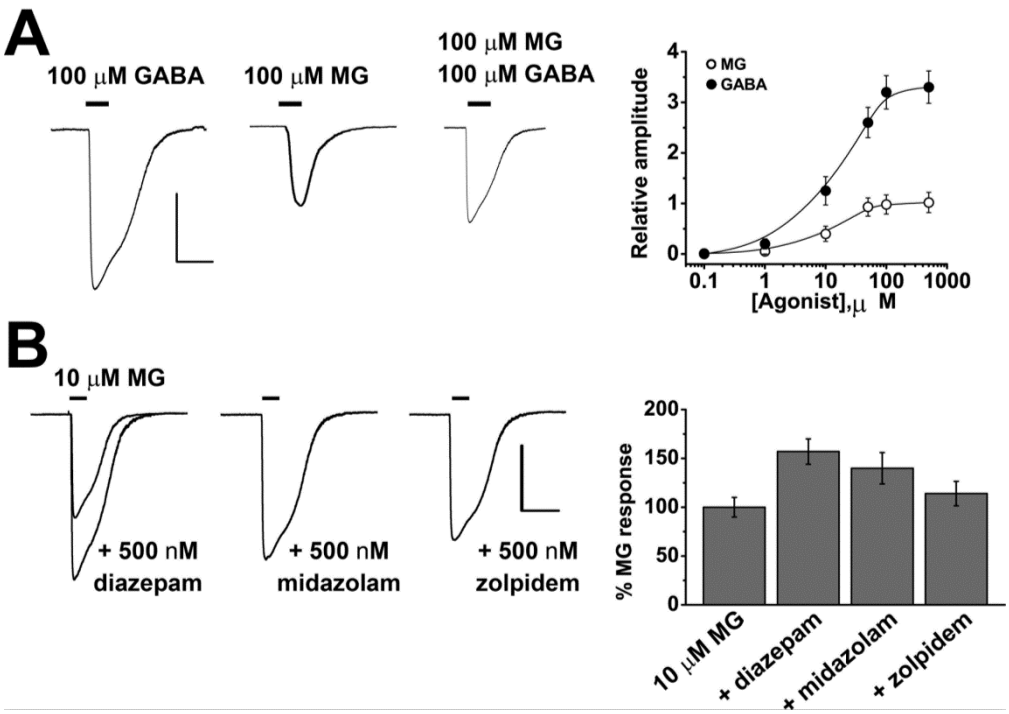

Figure 1.2 MG is an endogenous, partial agonist at neuronal GABA-A receptors. (A) The application of 100 μM MG to hippocampal neurons evokes Cl- currents through GABA-A receptors that are ~⅓ the magnitude of those evoked by 100 μM GABA in the same cells. The EC50 of the currents evoked by MG was 9.5 ± 1 μM and the physiological concentration of MG in rodent brain was measured at 5 μM. MG has a similar efficacy when applied to cerebellar granule neurons. **(B)** MG evoked currents in hippocampal neurons are augmented by co-application of classical anxiolytics that act as positive allosteric modulators of GABA-A receptors, such as the benzodiazapenes diazepam and midazolam and the imidazopyridine zolpidem. Scale bars represent 1 nA and 10 s. Adapted from Distler et al. 2012b.

GABA analogues have been considered as potential therapeutics, particularly for acute conditions, such as seizure or mania (Delorey et al. 1998; Liljelund et al. 2005; Gant et al. 2009). However, this strategy has been hampered by significant challenges; principally, that GABA is highly polar and flexible and activates GABA-B and GABA-C receptors in addition to GABA-A receptors. In contrast to GABA, MG does not activate neuronal GABA-B receptors; however,the effects of MG at GABA-C receptors have yet to be characterized. MG can easily cross the blood-brain barrier (Distler et al. 2012a); thus, MG precursors or MG bioisosteres might be clinically useful compounds.

In summary, activation of GABA-A receptors by MG is a promising approach for treatment of neuropsychiatric disorders and other diseases linked to GABA signaling. Possible approaches could include GLO1 inhibition or administration of MG precursors or bioisosteres.

1.6 Therapeutic potential of GLO1 inhibitors

Current drug-therapies for depression are limited by negative side effects, including sexual dysfunction, weight gain and insomnia, and require several weeks to produce their full therapeutic effect (Berton and Nestler 2006; Barbui et al. 2011). Similarly, anxiolytic and anti-epileptic drugs are limited by their sedating effects and abuse potential (Rudolph and Knoflach 2011). Those for alcohol use disorders are most importantly limited by their lack of effectiveness in a majority of patients (Dawson et al. 2007; Maisel et al. 2012; Liang and Olsen 2014). Identification of novel molecular targets may provide alternatives with fewer or different side effects. Additionally, the identification of targets with applications in multiple disorders is particularly beneficial as drug development is time consuming and expensive. Given its role in multiple neuropsychiatric disorders, agents that modulate MG levels might be of benefit as next generation treatments.

However, MG is highly bioreactive, modifying arginine and lysine residues in proteins and has been shown to be directly toxic to cells *in vitro*, inducing apoptosis when applied at concentrations > 100 μM. Thus, instead of direct administration of MG, an alternative and perhaps more promising strategy is to raise MG levels by inhibiting the GLO pathway. Application of a GLO1 inhibitor is expected to potentiate the activity of GABA-A receptors by reducing the degradation of MG to augment basal levels in the brain (**Figure 1.3**). This mechanism of action is fundamentally different to the action of commonly prescribed GABAergic drugs because it depends on the local accumulation of a competitive partial agonist rather that positive allosteric modulation of GABA-A receptors. Therefore, GLO1 inhibition is likely to cause anatomically and pharmacologically distinct responses to those observed following treatment with benzodiazepines and barbiturates.

Early studies already support a role for GLO1 inhibition in modulating behavioral phenotypes. For instance, GLO1 inhibition by S-bromobenzylglutathione cyclopentyl diester (BrBzGCp2) increased MG concentration in the brain and reduced anxiety-like behavior in mice (**Figure 1.4A-D**; Distler et al. 2012a) . Similarly, BrBzGCp2 attenuated epileptic seizures in mice (**Figure 1.4E**; Distler et al. 2013). However, these previous studies utilized either ubiquitious overexpress of *Glo1* or systemic administration to assess the effects of GLO1 inhibition on behavior.

Future studies are needed to more fully characterize and evaluate the therapeutic potential of GLO1/MG regulation on anxiety-like behavior. As we believe these anxiolytic effects are mediated through modulation of GABAergic neurophysiology by reducing the rate of MG clearance in the CNS, it is therefore important to determine whether the effects of *Glo1* and MG on anxiety-like behavior are peripherally or centrally mediated. Additionally, it is important to

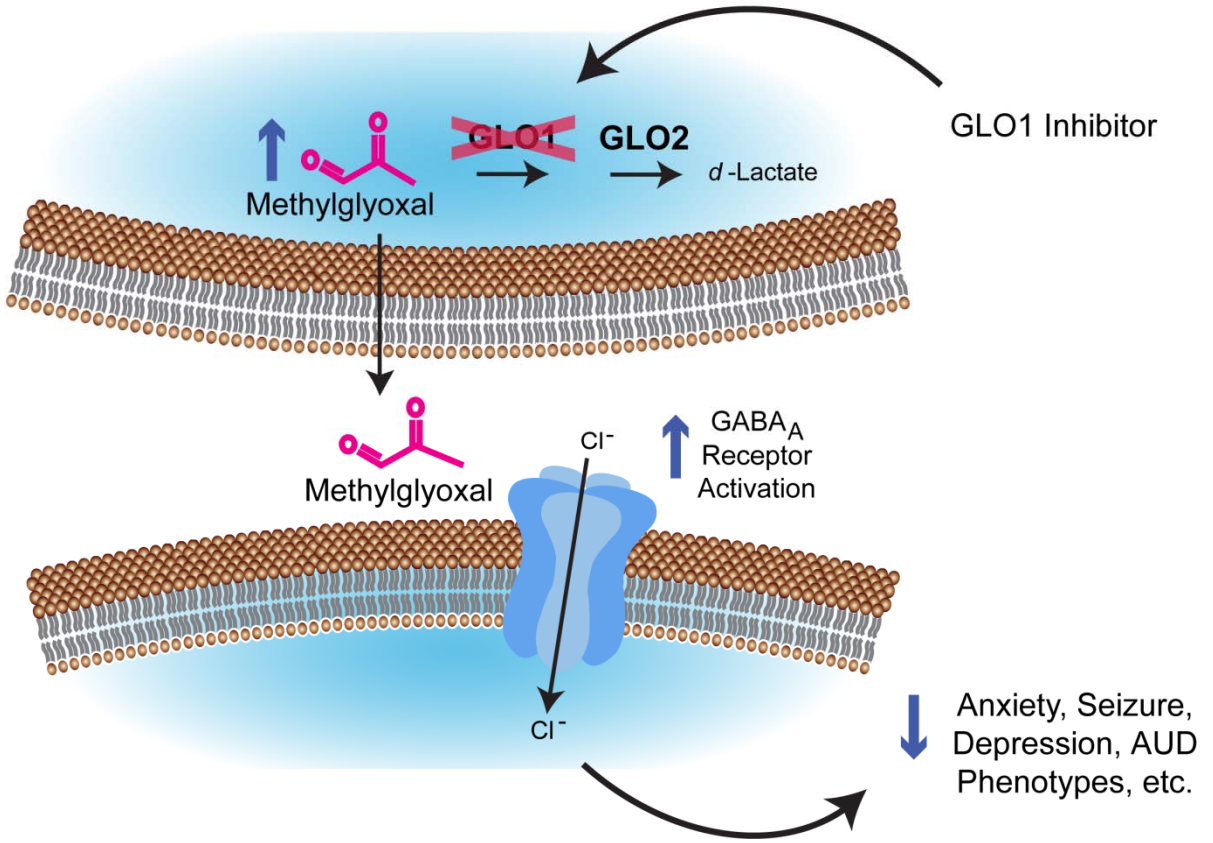

Figure 1.3: A model for GLO1 Inhibition in the treatment of neuropsychiatric disorders and epilepsy. Treatment with GLO1 inhibitors will increase concentrations of methylglyoxal due to decreased clearance by GLO1. Increased methylglyoxal will result in increased activation of GABA-A receptors and subsequently, a decrease in neuropsychiatric disorder phenotypes (ie. reduced anxiety, depression and seizure). Adapted from McMurray et al. 2014.

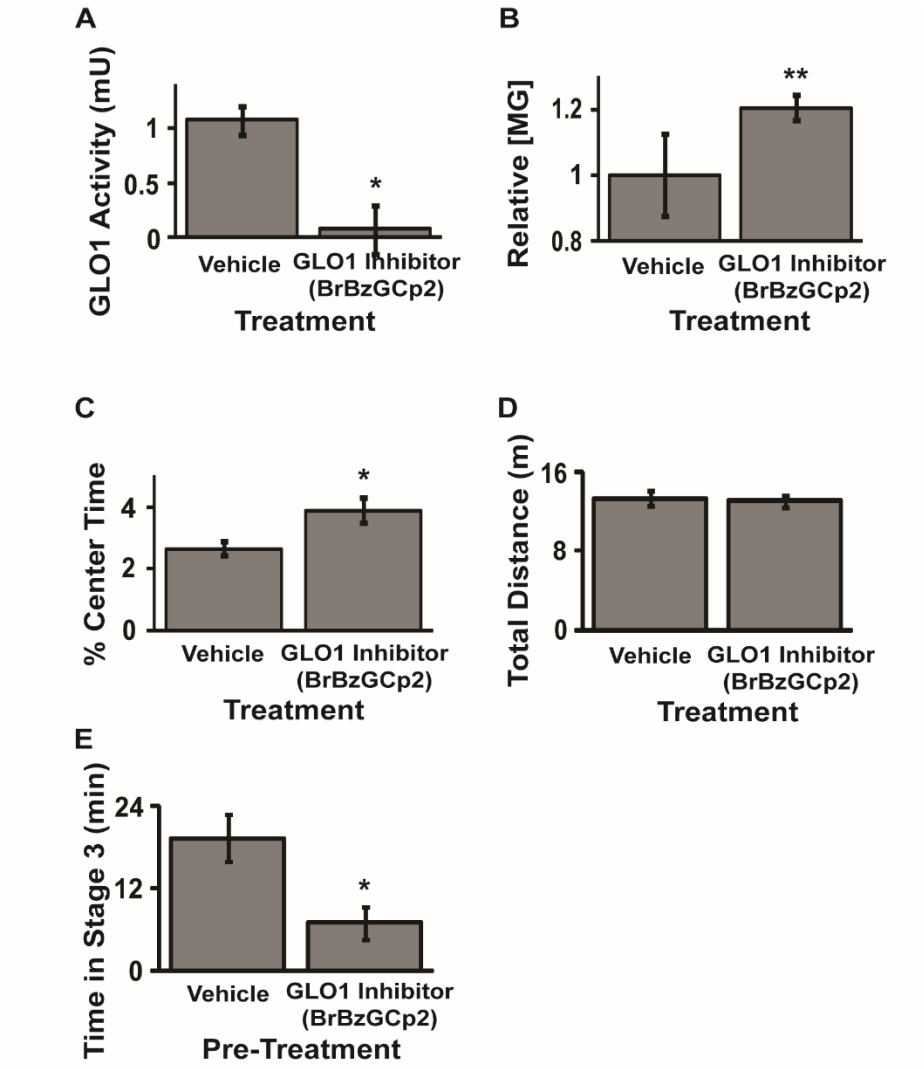

Figure 1.4 Systemic administration of a GLO1 inhibitor regulates MG concentration, reduces anxiety-like behavior and attenuates seizure in mice. Pharmacological inhibition of GLO1 by BrBzGCp2 **(A)** reduces GLO1 enzymatic activity; **(B)** increases concentrations of MG in whole brain of mice 2 hrs after i.p. treatment; **(C)** reduces anxiety-like behavior in the open field test (C57BL6/J mice) without affecting total distance traveled **(D)**; and **(E)** attenuates pilocarpine-induced seizures (50 mg/kg prior to pilocarpine (250mg/kg)). From McMurray et al. 2014, adapted from Distler et al. 2012a; Distler et al. 2013.

compare MG activation to that of currently used GABAergic agents, such as midazolam, to determine whether MG acts by similar mechanisms and through similar neurocircuitry.

While no association has been found between *Glo1* and alcohol use disorders, there is an extensive literature associating GABA-A receptor activation with alcohol use disorders (Grobin et al. 1998; Chester and Cunningham 2002; Koob 2006; Kumar et al. 2009; Yang et al. 2011). It is therefore possible that *Glo1* expression could regulate ethanol consumption through changes in MG concentrations and subsequent activity at GABA-A receptors. Further, given the high comorbidity between anxiety and alcohol use disorders, pharmacotherapies used to treat both disorders would particularly desirable.

Benton and colleagues reported a positive correlation between *Glo1* expression and depression-like behavior in mice (Cryan et al. 2005; Benton et al. 2012a). This observation appears surprising in light of the link between Glo1, MG and GABA, since other GABA-A receptor agonists (e.g. barbiturates and benzodiazepines) do not generally alter depression-like behaviors (Cryan et al. 2005). While GLO1 inhibitors have not been evaluated for their efficacy in depression-like behaviors, these data suggest that GLO1 inhibition may have antidepressant activity, likely by increasing MG levels. Anxiolytic drugs that modulate GABAergic signaling, such as benzodiazepines, have not been found to be effective for treatment of depression in humans (Rudolph and Knoflach 2011; Möhler 2012a). However, recent evidence shows that co-administration of the serotonin-selective reuptake inhibitor fluoxetine with eszopiclone (a partial agonist at GABA-A receptors that contain α_1, α_2 or α_3 subunits) has a greater antidepressant effect than fluoxetine alone (Fava et al. 2011). Additionally, a new study found that a negative modulator of α5-containing GABA-A receptors showed antidepressant-like efficacy in rats (Fischell et al. 2015). Together, these studies suggest a potential role for GABAergic drugs in the

treatment of depression that has been proposed elsewhere (Gajcy et al. 2010; Rudolph and Knoflach 2011; Möhler 2012a). In conjunction with the correlation between *Glo1* and depression-like behavior, these data reflect a role for GABA-A receptors in the treatment of depression and highlight the potential utility of GLO1 inhibition versus classical anxiolytics for regulating GABAergic signaling. Thus, it will be important to assess the effects of GLO1 inhibitors in models of depression-like behavior.

1.7 Overview

Within the three chapters of this thesis, genetic and pharmacological approaches are utilized to assess the impact of regulating GLO1 or MG concentrations in mouse models of anxiety, depression and alcohol use disorders. Chapter 2 investigates the relevant neuroanatomical regions associated with GLO1 and MG regulation of anxiety-like behavior. Chapter 3 explores the impact of *Glo1* expression on alcohol consumption and assesses the therapeutic potential of GLO1 inhibition for the treatment of alcohol use disorders. Finally, Chapter 4 examines the role of GLO1 inhibition in depression-like behavior and evaluates the therapeutic potential of GLO1 inhibitors as novel fast-acting antidepressants. Overall, the work presented herein attempts to investigate the role of GLO1 and MG three highly comorbid disorders: anxiety, alcohol use disorders and depression and assesses the therapeutic potential of GLO1 inhibition as a novel pharmacotherapy.

CHAPTER 2

NEURONAL OVEREXPRESSION OF *Glo1* OR AMYGDALAR MICROINJECTION OF METHYLGLYOXAL IS SUFFICIENT TO REGULATE ANXIETY-LIKE BEHAVIOR IN MICE

This chapter was formatted for 'Behavioural Brain Research' where it is currently under review.

2.1 Abstract

GLO1 (Glyoxalase1) is a ubiquitous cellular enzyme that detoxifies methylglyoxal (MG), which is a byproduct of glycolysis. Previously, we showed that ubiquitous overexpression of *Glo1* reduced concentrations of MG and increased anxiety-like behavior, whereas systemic injection of MG reduced anxiety-like behavior. We further showed that MG is a competitive partial agonist at GABA-A receptors. Based on those data we hypothesized that modulation of GABAergic signaling by MG underlies *Glo1* and MG's effects on anxiety-like behavior.

As previous studies used ubiquitous overexpression, we sought to determine whether neuronal *Glo1* overexpression was sufficient to increase anxiety-like behavior. We generated knock-in mice with a floxed-stop codon upstream from human *Glo1* (FLOXGlo1KI) and bred them with mice expressing CRE recombinase under the direction of the S*ynapsin 1* promoter (Syn-CRE) to limit overexpression of *Glo1* specifically to neurons.

Furthermore, since previous administration of MG had been systemic, we sought to determine if direct microinjection of MG into the basolateral amygdala (BLA) was sufficient to reduce anxiety-like behavior. Thus, we performed bilateral microinjections of saline, MG (12μM or 24μM), or the positive control midazolam (4mM) directly into the BLA.

FLOXGlo1KIxSyn-CRE mice showed significantly increased anxiety-like behavior compared to their FLOXGLO1xWT littermates. In addition, bilateral microinjection of MG and midazolam significantly decreased anxiety-like behavior compared to saline treated mice. These

studies suggest that anatomically specific manipulations of *Glo1* and MG are sufficient to induce changes in anxiety-like behavior.

2.2 Assessment of anxiety-like behavior following neuronal overexpression of *Glo1* or microinjection of MG into BLA

Mounting evidence supports a role for *Gyloxalase 1* (*Glo1*) and its substrate methylglyoxal (MG) in the regulation of anxiety-like behavior (Hovatta et al. 2005b; Williams et al. 2009; Hambsch et al. 2010; Distler et al. 2012b). GLO1 is a ubiquitous cytosolic enzyme primarily responsible for catalyzing the reaction between glutathione and acyclic a-oxoaldehydes; particularly, MG (Thornalley 1996b). MG is a byproduct of glycolysis that is mainly formed from the nonenzymatic degradation of the glycolytic intermediates dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Thornalley 1996b). GLO1 has a critical role in the clearance of MG with overexpression of *Glo1* preventing MG accumulation and GLO1 inhibition resulting in MG accumulation (Thornalley 1996b; Distler et al. 2012b).

We previously demonstrated that a duplication of a region containing 4 genes that included *Glo1* was associated with increased *Glo1* mRNA and increased anxiety-like behavior (Williams et al. 2009). Additionally, we found that transgenic mice ubiquitously overexpressing *Glo1* alone showed a copy-number-dependent increase in anxiety-like behavior (Distler et al. 2012b). Conversely, acute administration of MG or a GLO1 inhibitor, S-bromobenzylglutathione cyclopentyl diester (pBBG), decreased anxiety-like behavior in wild-type animals (Distler et al. 2012b). Electrophysiological recordings indicted that MG was a competitive partial agonist at GABA-A receptors and that MG activates these receptors at physiologically relevant concentrations (Distler et al. 2012b). Based on these data we hypothesized that the action of MG at GABA-A receptors likely contributes to its anxiolytic effects.

Many studies have implicated the basolateral amygdala (BLA) in both normal and pathological anxiety (Davis 1992; Earnheart et al. 2007; Tye and Deisseroth 2012; Janak and Tye 2015). Neuroimaging studies have reported differences in amygdala-prefrontal circuitry in patients with anxiety disorders (Möhler 2012b). Additionally, direct injection of midazolam, a positive allosteric modulator at GABA-A receptors (benzodiazepine), into the BLA reduces anxiety-like behavior in mice (Heldt and Ressler 2006). However, because *Glo1* expression and MG production occur in all tissues and all brain regions, the role of the BLA in mediating the effects of MG on anxiety-like behavior have not been explored.

The studies performed here aimed to determine whether the effects of *Glo1*/MG on anxiety-like behavior are peripherally or centrally mediated and if central, to determine whether the BLA was sufficient for the anxiolytic effects of MG. All studies used male mice that were group housed on a standard light cycle (12L/12D) and given unlimited access to standard food and water. Data were analyzed using Student's *t*-test or ANOVA as appropriate. Holm-Sidak multiple comparisons procedures were used to determine which treatments yielded significantly different responses. p-values < 0.05 were considered significant.

In the first experiment, tissue-specific overexpression of *Glo1* was achieved on a C57BL/6J (B6) background by knock-in of human *Glo1* with an upstream floxed STOP to the ROSA26 locus (Fig.1A; FLOXGlo1KI; Albert Einstein College of Medicine). Insertion of the FLOXGlo1KI construct was confirmed by genotyping DNA from mice using the following primers: Fwd: ACTGAAGATGATGCGACCCAG; Rev: CACCTGTTCAATTCCCCTGC. Mice homozygous for FLOXGlo1KI were bred at The University of Chicago to hemizygous mice expressing CRE recombinase under the direction of the *synapsin 1* promoter (Syn-CRE; B6.Cg-Tg(Syn1-cre)671Jxm/J, obtained from The Jackson Laboratory; generated on B6;CBAF1

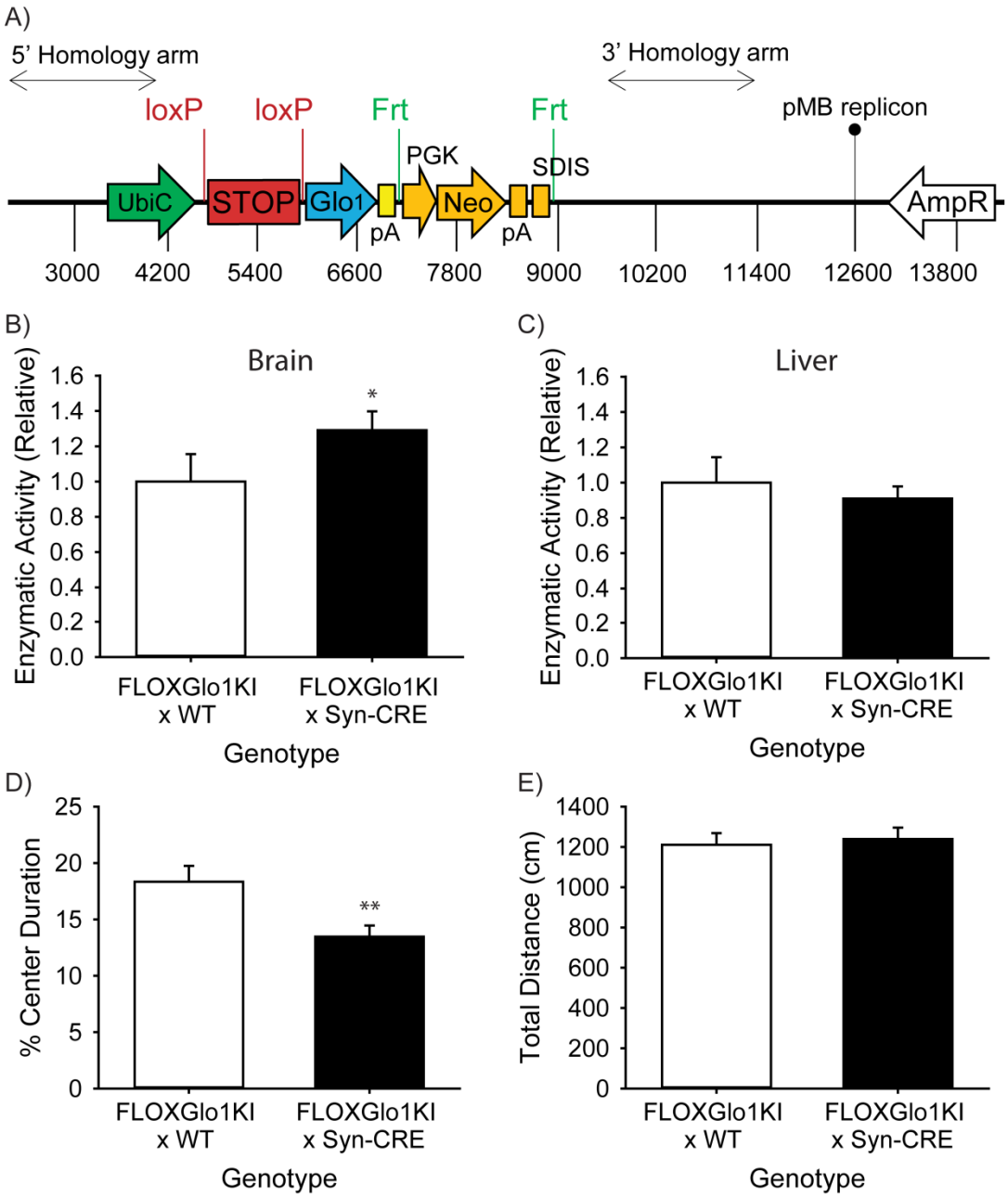

Figure 2.1. Neuron specific overexpression of *Glo1* increases anxiety-like behavior in the open field test (OFT). A) Construct map illustrating the generation of FLOXGlo1KI mice wherein Glo1 with a preceding floxed STOP was knocked-in to ROSA26 locus. B) Increased GLO1 enzymatic activity was seen in the brain, (C) but not liver of FLOXGLO1KI x SynCRE mice relative to their FLOXGLO1KI x WT littermates. D) Mice overexpressing Glo1 in neurons (FLOXGlo1KI x Syn-CRE) show reduced center duration in the OFT, indicating increased anxiety-like behavior. E) Mice did not differ in total distance traveled. Data are mean ± SEM. *p <0.05, **p<0.01

background, founders bred to C57BL/6NHsd) which is only expressed in neurons. Thus, in behavioral studies we used littermates (8-13 weeks old) that were always positive for FLOXGlo1KI, but were either CRE positive (overexpress *Glo1;* n=26) or CRE negative (not overexpress *Glo1;* n=19). These mice allowed us to assess the impact of over-expressing *Glo1* only in neurons on anxiety-like behavior in the open-field test (OFT).

No deficits were seen in the general health for either FLOXGlo1KI mice or FLOXGlo1KIxSyn-CRE mice. For example, there was no effect of genotype on weight in these mice (p=0.889 by Two-tailed t-Test). To confirm overexpression of *Glo1* in the brain, we assayed GLO1 enzymatic activity by measuring the rate of formation of S-D-lactoylglutathione as previously described (Distler et al. 2012b). Briefly, brain (n=8 per group) or liver (n=4 per group) homogenate (50 μg protein) was added to a hemithioacetal substrate (incubate 2 mM MG and 2 mM Glutathione at 37°C for 10 minutes), and the absorbance at 240 nm was measured every 30 seconds for 4 minutes. FLOXGlo1KIxSyn-CRE mice showed significantly increased GLO1 enzymatic activity in the brain compared to their FLOXGlo1KI x WT littermates (Fig.1B; $F(1,15)=5.823$; $p<0.05$). There was also an effect of cohort for brain enzymatic activity ($F(1,15)=21.845$; $p<0.001$), likely due to the extended freeze time (~1year) of samples from cohort 1 in comparison to those of cohort 2 (~4 hrs frozen; 4 mice per cohort). However, there was no significant cohort x genotype interaction ($F(1,15)=0.506$; p=0.491). There was also no difference in GLO1 enzymatic activity in the liver between FLOXGlo1KIxSyn-CRE and their FLOXGlo1KI x WT littermates (Fig.1C; Two-tailed t-test, p=0.605). Thus, FLOXGlo1KIxSyn-CRE mice showed about a ~30% increase in GLO1 enzymatic activity that was limited to the brain (central; Fig.1B), but not liver (periphery; Fig.1C).

To assess anxiety-like behavior in the OFT, mice were placed into chambers (AccuScan,

Colombus, OH, USA) surrounded by infrared detection beams on the X, Y and Z-axes which tracked the animals' activity. Locomotor activity and center duration were assessed using automated Versamax software. Chambers measured 43 x 43 x 33 cm (width x depth x height) and had dim overhead fluorescent lighting (14 lux). Center size was 26 x 26cm. We found that FLOXGlo1KIxSyn-CRE spent significantly less time than their FLOXGlo1KI x WT littermates in the center during the first 5 minutes (Fig.1D, Two-tailed *t*-test, $p<0.01$). Importantly, there was no difference in total distance traveled (Fig.1E; Two-tailed *t*-test, $p=0.729$) suggesting that differences in anxiety-like behavior are not due to changes in overall activity.

Prior studies with ubiquitously overexpressed *Glo1* indicated that higher levels of GLO1 activity were needed to induce behavioral changes (Distler et al. 2012). The somewhat modest increase in GLO1 enzymatic activity observed in this study (~30%) may reflect our use of whole brain homogenate. This homogenate includes other cell types (e.g. glia) that do not overexpress GLO1 and thus dilute the increased enzymatic activity induced in neurons. Regardless of the reason for the modest increase in enzymatic activity observed in this study, these levels were sufficient to alter anxiety-like behavior in the OFT.

In a second experiment intended to further assess neuroanatomical specificity, we implanted bilateral cannula directed to the BLA. The BLA has long been shown to be involved in the regulation of anxiety-like behavior (Kent et al. 2002; Vyas and Chattarji 2004; Heldt and Ressler 2006; Tye and Deisseroth 2012; Janak and Tye 2015). As MG is a GABA-A receptor agonist, we hypothesized that direct injection of MG into the BLA would reduce anxiety-like behavior in the OFT. Thus, we performed bilateral microinjections of vehicle, MG (12μM or 24 μM) or midazolam, a benzodiazepine, as a positive control (Heldt and Ressler 2006) directly into the BLA (Fig.2A) and then measured anxiety-like behavior in the OFT.

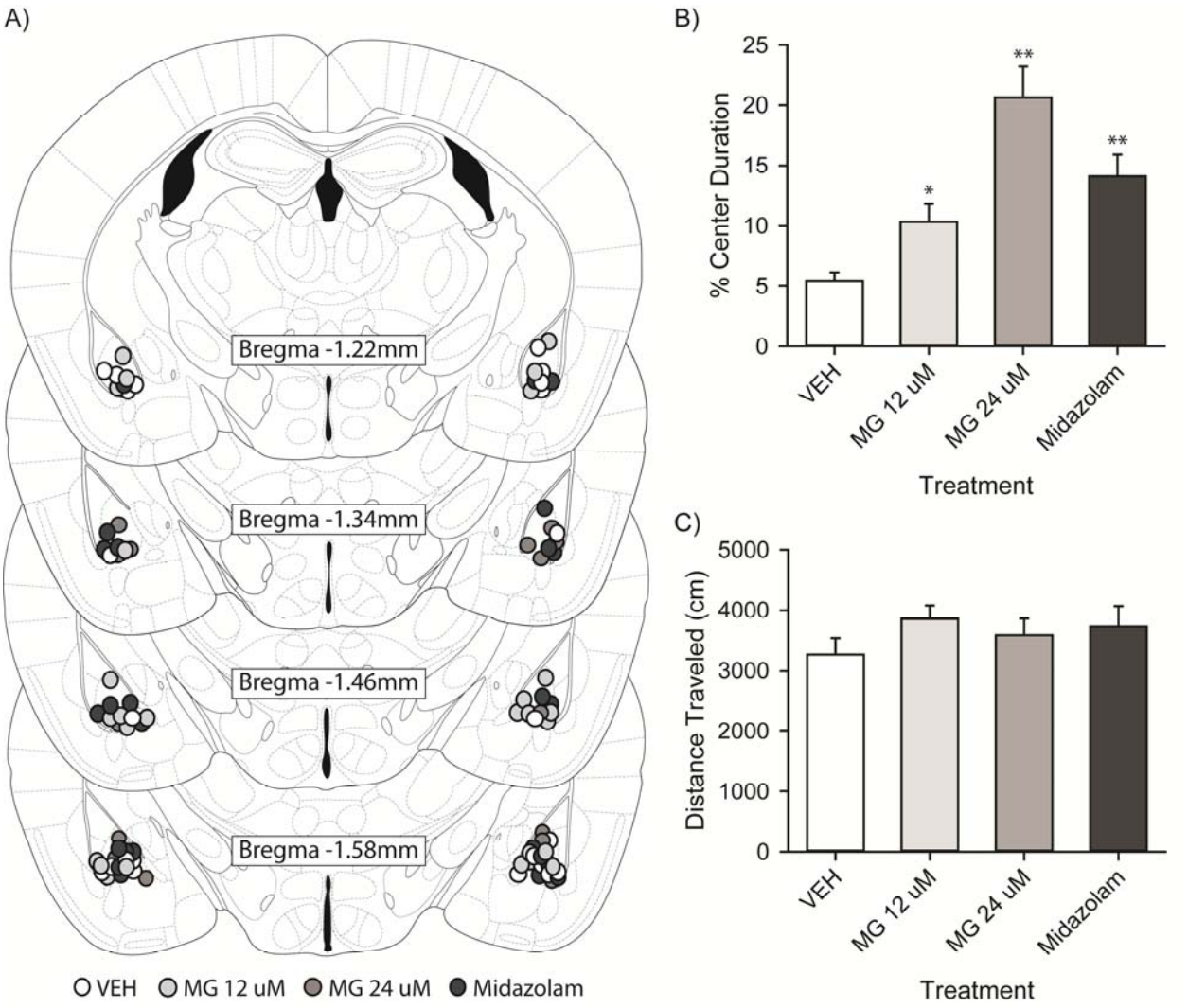

Figure 2.2. Bilateral microinjection of methylglyoxal into the basolateral-amygdala (BLA) reduces anxiety-like behavior in the OFT.A) Schematic representation of bilateral microinjections sites. Images were adapted from Paxinos and Franklin (2004), the color of the circles is defined below the figure and corresponds to column color in panels B and C indicating the nature of the injection. B) Bilateral microinjection of MG (12 μM or 24 μM) or Midazolam (4mM) directly into the BLA increased center duration in the OFT over 30min compared to vehicle (VEH) treatment, indicating reduced anxiety-like behavior. C) There was no effect of these treatments on total distance traveled. Data are mean ± SEM. *p <0.05, **p<0.01 by Holm-Sidak Post-hoc comparisons to vehicle.

In the microinjection study, B6 mice were purchased from the Jackson Laboratory (7-9 weeks old; JAX). Mice were anesthetized with ketamine/xylazine (88/1.3 mg/kg I.P.; Sigma-Aldrich) and bilaterally implanted with guide cannula targeting the BLA (AP -1.4, ML ± 3.2, DV 5.1). Animals were allowed to recover for 5-6 days. On test day, injection cannulas were inserted into guide cannula with a 1 mm projection past guide cannula. Vehicle (0.9% saline; n=15), 4 mM midazolam (positive control, n=15; UC429 Sigma-Aldrich), 12 µM MG (n=15; M0252 Sigma-Aldrich), or 24 µM MG (n=9) was bilaterally microinjected at a constant flow rate of 0.25µl/min for 2 minutes (0.5 µl total) with an additional 2 minutes allowed for diffusion. Following microinjection, mice were placed directly into the OFT. Treatments significantly increased center duration in the OFT over 30 minutes (Fig.2B; $F(3, 45)=13.765$; $p<0.001$). Individual post-hoc tests revealed increases in center duration compared to vehicle for all treatments (MG 12µM, $p<0.05$; MG 24µM, $p<0.01$ and midazolam, $p<0.01$ by Holm-Sidak post-hoc comparisons). Importantly, there was no effect on total distance traveled (Fig.2C; $F(3,45)=0.887$; $p=0.456$). Differences in center duration and distance traveled between the VEH treated mice within this study and the FLOXGlo1KIxWT mice in the previous study are likely due to the increased stress associated with microinjection directly before testing.

Overall, these data suggest *Glo1*'s effects on anxiety-like behavior are centrally mediated as overexpression of *Glo1* in neurons was sufficient to increase anxiety-like behavior. They also suggest that MG is able to modulate anxiety-like behavior in the OFT through direct application into the BLA as there was a dose dependent increase in center duration after direct injection of MG into the BLA that was comparable to that of midazolam. Importantly, the doses of MG used (12 µM and 24 µM) are within a physiologically relevant range based on previous reports of MG concentration in the brain (Hambsch et al. 2010; Distler et al. 2012b).

These data are consistent with previous studies suggesting that expression within the brain is sufficient for regulating *Glo1* and MG mediated anxiety-like behavior in mice. Hovatta et al. (2005) found that within anterior cingulate cortex, lentiviral mediated overexpression of *Glo1* increased anxiety-like behavior and lentiviral mediated knockdown of *Glo1* reduced anxiety-like behavior in 129S6/SvEvTac mice. While lentiviral knockdown of *Glo1* also reduced anxiety-like behavior in B6 mice, the study failed to see increased anxiety-like behavior with lentiviral mediated overexpression in the anterior cingulate cortex in this strain. In the study presented here, FLOXGlo1KIxSynCRE mice (B6 background) that overexpress *Glo1* in neurons showed increased anxiety-like behavior which may suggest that lentiviral mediated overexpression within the anterior cingulate cortex was insufficient to alter anxiety-like behavior in B6 mice, but that broader overexpression (e.g. all neurons) can induce increased anxiety-like behavior in this strain.

Hambsch et al. (2010) previously found that i.c.v. administration of MG for 6 days reduced anxiety-like behavior in the elevated plus maze. The studies presented here build on those of Hambsch et al. (2010) by administering MG to a more specific neuroanatomical region that is associated with anxiety-like behavior, the BLA (Kent et al. 2002; Vyas and Chattarji 2004; Heldt and Ressler 2006; Tye and Deisseroth 2012; Janak and Tye 2015). Taken together with prior studies our data suggest that *Glo1* regulates anxiety-like behavior through neurocircuitry typically associated with anxiety-like behavior.

Our data also support the therapeutic potential of modulating MG levels for the treatment of anxiety disorders. MG accumulation is fundamentally different from that of treatment with currently used anxiolytic drugs, such as benzodiazepines because MG is an endogenously produced competitive partial agonist, rather than a positive modulator, such as midazolam (Kent

et al. 2002; Distler et al. 2012b). Additionally, MG production increases with increased metabolic load (Brownlee 2001; Ahmed and Thornalley 2007) which may lead to more region specific increases in MG with treatment. Thus, treatments that increase MG concentrations may have qualitatively different effects as compared to existing approaches.

2.3 Acknowledgements

We wish to thank Margaret Distler and Naomi Gorfinkle for help with the microinjection studies. This work was supported by NIH grant MH079103. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago and performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

CHAPTER 3

GENETIC AND PHARMACOLOGICAL MANIPULATIONS OF *GLYOXALASE 1* REGULATES VOLUNTARY ETHANOL CONSUMPTION IN MICE

This chapter was formatted for 'Addiction Biology' where it is currently in press.

3.1 Abstract:

Previous studies have identified an association between the gene glyoxalase 1 (*Glo1*) and anxiety-like behavior in mice and have shown that the substrate of GLO1, methylglyoxal, is a competitive partial agonist at $GABA_A$ receptors. Given the well-established role of $GABA_A$ receptors in the behavioral effects of ethanol (EtOH), we investigated the role of *Glo1* in voluntary EtOH consumption in mice using the drinking in the dark (DID) paradigm. Transgenic mice overexpressing *Glo1* on both FVB/NJ (FVB) or C57BL/6J (B6) backgrounds showed increased voluntary EtOH consumption compared to their wild-type littermates in DID. Furthermore, transgenic *Glo1* knockdown mice on a B6 background showed decreased voluntary EtOH consumption in DID. These genetic manipulations of *Glo1* had no effect on sucrose, saccharin or water consumption. Finally, we found that a small molecule GLO1 inhibitor (S-bromobenzylglutathione cyclopentyl diester (pBBG; 6.25, 12.5 mg/kg) reduced EtOH consumption compared to vehicle treated B6 mice without altering saccharin or water consumption. Sucrose consumption was only reduced by the higher (12.5 mg/kg) dose of pBBG. We did not observe differences in the loss of righting reflex (LORR) or EtOH-induced foot slips on the balance beam in response to acute EtOH administration (LORR: 4g/kg, Balance Beam: 1.25g/kg) in B6 or FVB mice overexpressing *Glo1*, nor in B6 mice treated with pBBG. These data are the first to implicate *Glo1* in EtOH-related behaviors and suggest that GLO1 inhibitors may have therapeutic potential for the treatment of alcohol use disorders.

3.2 Introduction

Alcohol use disorders (AUD) are characterized by "a problematic pattern of alcohol use leading to clinically significant impairment or distress" (DSM V). There are a dearth of pharmacological treatments for AUDs and those that exist are only modestly effective and may even be ineffective in certain individuals (Dawson et al. 2007; Maisel et al. 2012). Further, AUDs share high comorbidity with several psychiatric disorders including generalized anxiety disorder (GAD) (Grant et al. 2004; Boschloo et al. 2011; Smith and Randall 2012) and these comorbid disorders are associated with worse treatment outcomes (Driessen et al. 2001; Bruce et al. 2005; Smith and Book 2010). Thus, identifying novel treatments for AUD, especially ones that might also address psychiatric co-morbidities is of critical importance.

While it is impossible to fully recapitulate AUDs in model organisms, key aspects of AUD can be modeled and may be used to evaluate the potential effectiveness of novel therapeutic targets. Binge drinking is defined as drinking enough to obtain a blood alcohol concentration (BAC) of 0.08g/dL or above (National Institute on Alcohol Abuse and Alcoholism 2004). Binge drinking is a risk factor for the development of AUDs (Viner and Taylor 2007) and accounts for a large portion of harm that is associated with AUDs (Bouchery et al. 2011). The drinking in the dark (DID) paradigm was developed to model binge drinking in rodents (Rhodes et al. 2005). DID takes advantage of the tendency of mice to voluntarily consume large amounts of ethanol (EtOH) when it is presented for a limited period of time during the dark phase of the light cycle. Under these conditions several inbred mouse strains will freely consume enough EtOH to achieve BAC greater than 0.08 g/dL, and will thus demonstrate overt signs of behavioral intoxication (Rhodes et al., 2005). Importantly, current treatments for AUDs such as naltrexone and acamprosate reduce EtOH consumption in this model without altering water or sucrose consumption, illustrating the strong predictive validity of DID (Kamdar et al. 2007;

Gupta et al. 2008).

Several previous studies have identified an association between expression of the gene, glyoxalase 1 (*Glo1*) and anxiety-like behavior in mice (Hovatta et al. 2005b; Williams et al. 2009; Distler et al. 2012a). *Glo1*'s protein product, GLO1, is a ubiquitous cytosolic enzyme that mediates the detoxification of methylglyoxal (MG), which is a non-enzymatic by-product of glycolysis (Thornalley 1996b). We previously showed that transgenic overexpression of *Glo1* increased anxiety-like behavior and that direct administration of MG decreased anxiety-like behavior in mice. Further, a pharmacological inhibitor of GLO1, S-bromobenzylglutathione cyclopentyl diester (pBBG), increased MG concentrations in brain and reduced anxiety-like behavior. We subsequently determined that MG is a competitive partial agonist at $GABA_A$ receptors, likely explaining the effect of GLO1 on anxiety-like behavior (Distler et al. 2012a).

Many of the behavioral effects associated with EtOH use are mediated through the actions of EtOH at $GABA_A$ receptors and modulation of $GABA_A$ receptor activation alters both the behavioral effects of EtOH and voluntary EtOH consumption (Grobin et al. 1998; Moore et al. 2007; Kumar et al. 2009; Liang and Olsen 2014). Because GLO1 regulates the concentration of MG, which is a competitive partial agonist at GABA-A receptors, we hypothesized that increased *Glo1* expression and corresponding decreases in MG would increase EtOH consumption, while reduced *Glo1* expression or reduced enzymatic activity of GLO1, which would increase MG concentrations, would decrease EtOH consumption.

3.3 Materials and Methods

Mice: Transgenic (TG) mice overexpressing *Glo1* on either a FVB/NJ (FVB) or C57BL/6J (B6) background were generated by insertion of a BAC transgene, as previously described (Distler et al. 2012a). FVB TGs used in this paper had approximately 35 copies of the transgene while B6

TGs had approximately 8 copies; previously published estimates of brain mRNA suggest that these transgenes induced 17-fold (FVB) and 5-fold (B6) increases in brain *Glo1* mRNA relative to wild-type (WT) littermates (Distler et al. 2012a). *Glo1* knock-down (KD) mice were generated on a C57BL/6 background in the lab of Dr. Michael Brownlee (Albert Einstein College of Medicine, Bronx, NY) and show an approximately 45-65% reduction in GLO1 enzymatic activity as previously described (El-Osta et al. 2008). KD mice have been maintained in our lab by continuing to backcross to B6 for more than 5 generations. In all studies of mutant mice, TG, KD and their corresponding WT littermates were tested at ages 10-16 weeks; both males and females were used. For studies using the GLO1 inhibitor (pBBG), male B6 mice were purchased from The Jackson Laboratory (JAX) and tested when they were 8-12 weeks old. All mice were group housed on a reverse light cycle (12/12 hour light/dark, lights on at 22:30) for at least 2 weeks prior to testing. All mice were singly housed beginning exactly 5 days before the start of DID testing. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago and performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Drinking in the dark (DID): Our studies were performed using the two day DID model of binge drinking as described in Rhodes et al. (2007). Briefly, three hours into the dark cycle, mice were given access to 20% EtOH (vol/vol) for 2 hours on day 1 and for 4 hours on day 2. The same animals were tested to determine their consumption of other solutions using the same 2 day DID paradigm. We examined 10% sucrose (wt/vol), 0.2% Saccharin (wt/vol) and water. Mice had at least 1 day of rest between studies. For example, in one week, sucrose testing took place on a Monday and Tuesday, the mice were undisturbed on Wednesday, then water testing took place Thursday and Friday. For studies using the GLO1 inhibitor pBBG, mice followed a similar

testing schedule, but received injections of the inhibitor 2 hours prior to testing on day 2. This 2 hour time-point was used to allow time for accumulation of MG in the brain through a reduction in MG clearance; we have previously shown increases of MG in the brain and anxiolytic behaviors effects 2 hours after administration of pBBG (Distler et al. 2012a; Distler et al. 2013). TG and KD mice underwent testing in the following order: sucrose, water, saccharin and EtOH. Mice receiving the GLO1 inhibitor pBBG underwent testing in a different order: sucrose, saccharin, water and EtOH. There was no particular reason for this difference. Blood samples were taken for BEC analysis immediately following the 4 hour exposure to EtOH on day 2 of DID.

Loss of Righting Reflex (LORR): Separate cohorts of treatment naïve mice (FVB TG, B6 TG, and their WT littermates or WT male B6 from JAX for pBBG studies) were used for the LORR studies. pBBG injections occurred 2 hours before mice received EtOH injections. LORR testing began with a 4 g/kg IP injection of EtOH; the injection consisted of a 20% EtOH solution that was prepared by diluting a 95% EtOH with 0.9% saline. LORR was defined as the time at which a mouse could no longer right itself twice within 30 seconds. Mice taking longer than 3.5 minutes to lose their righting reflex were deemed to have received a misplaced injection and were excluded from analysis. Of 112 mice tested, 8 were excluded for either failing to lose their righting reflex (5 of 8) or for taking longer than 3.5 minutes to lose their righting reflex (3 of 8). Of the 8 that were excluded, 4 were FVB (1 TG, 3 WT) and 4 were B6 that were purchased from JAX for the pharmacological studies. No mice were excluded from the B6 Glo1 overexpressing line based on these criteria. Duration of LORR was defined as the time at which a mouse regained the ability to right itself 3 times in 60 seconds minus the time it achieved LORR.

Balance Beam: Separate cohorts of treatment naïve mice (FVB TG, B6 TG and their

corresponding WT littermates or WT male B6 from JAX for pBBG studies) were used for the balance beam study. The day before testing, mice were trained to traverse a balance beam (97cm length, 16 mm wide, suspended 56 cm above the floor) by placing mice at one end of the balance beam and encouraging them, if necessary, to walk to the other side of the balance beam by a light nudge at the base of the tail using the eraser end of a pencil. Previous studies (Linsenbardt, Moore, Griffin, Gigante, & Boehm nd, 2011; Rhodes et al., 2007) have shown that this training is sufficient to have mice traverse the beam during testing without encouragement. FVB and B6 TG mice were tested on the balance beam over 2 days. On day 1 they received no injections and baseline foot slips were assessed. On day 2, all mice received 1.25g/kg EtOH 10 minutes before being placed on the balance beam. In a separate study, we used a 3x2 experimental design to assess interactions between drug (VEH, MG or pBBG) and EtOH (saline or EtOH) on ataxia (foot slips). WT B6 JAX mice received injections of either VEH or 6.25mg/kg pBBG 2 hours before testing and then received another injection of either saline, 50mg/kg MG, 1.25g/kgEtOH or 50mg/kg MG + 1.25g/kg EtOH 10 minutes before testing. Mice were then placed on one end of the balance beam and allowed to traverse to the other end while hind foot slips were recorded by an observer blind to treatment conditions.

EtOH Metabolism: Novel sets of EtOH naïve mice (FVB TG, B6 TG, B6 KD and their corresponding WT littermates) were used to assess EtOH metabolism. A 2 g/kg dose of EtOH was administered IP using a 20% EtOH solution that was prepared by diluting a 95% stock solution with 0.9% saline. Blood (20 µl) was taken from the tail at 15, 30, 60 and 120 minutes post injection and blood EtOH concentrations (BECs) were determined as described below.

BEC: Blood samples were processed by the laboratory of Professor John C. Crabbe at Oregon Health & Sciences University using procedures described previously (Barkley-Levenson and

Crabbe 2012). For the DID study, 20 µl blood samples were taken immediately after the 4 hour EtOH exposure on day 2. For the EtOH metabolism study 20 µl blood samples were taken at the indicated time points. For the LORR study, 20 µl blood samples were taken upon the regain of the righting reflex. All samples were placed into microcentrifuge tubes containing 50 µl zinc sulfate on ice. Following collection of all blood samples, 50 µl of 0.3N barium hydroxide and 300 ul distilled water were added and samples were centrifuged at 12,000 RPM for 5 minutes. The supernatant was then removed, placed in a sealed, air-tight container, frozen and subsequently analyzed by gas chromatography. Samples were compared to a standard EtOH concentration curve.

Drugs: S-bromobenzylglutathione cyclopentyl diester (pBBG) was synthesized in the laboratory of Professor Alexander Arnold at the University of Wisconsin Milwaukee as follows: In a dry glass vial, L-Glutathione (307 mg, 1 mmol) was dissolved in water (2 mL) at room temperature for 5 minutes. 2 mL of 6N NaOH was added slowly followed by the dropwise addition of a solution of 4-bromobenzyl bromide (1.1 mmol) in methanol (2 mL). The reaction mixture was stirred at room temperature for 3 hours. The product was precipitated by adding 6N HCL (2 mL). The precipitate was washed with water and dried. The crude product was dissolved in cyclopentanol (10 ml). To the solution, few drops of concentrated sulfuric acid were added and stirred for 48 hours. The completion of the reaction was monitored by LC/MS until the disappearance of the starting material. The product was precipitated by adding hexanes. The precipitation step was repeated three times giving 122 mg (20% yield). ^{1}H NMR (DMSO-d6) δ 8.52 (t, 1H, J = 5.9 Hz, NH), 8.27 (d, 1H, J = 5.9 Hz, NH), 7.49 (d, 2H, J = 8.6 Hz), 7.29 (d, 2H, J = 8.6 Hz), 7.09 (s (broad), 2H, NH2) 5.18 (m, 1H), 5.05 (m, 1H), 4.56 (m, 1H), 3.95, (m, 1H), 3.79 (d, J = 5.6 Hz, 2H), 3.73 (s, 2H), 2.75 (m, 1H), 2.53 (m, 1H), 2.42 (m, 2H), 2.09 (m, 2H),

1.6-1.9 (m, 16H); ESI MS (+ve) 613.16 m/z; found 614.36 (M+H). pBBG was dissolved in vehicle (8% DMSO/18% Tween80/74% PBS) and administered IP. Methylglyoxal (Sigma-Aldrich, M0252) was dissolved in 0.9% saline or 20% EtOH in 0.9% saline and administered IP. Statistical Analysis: Data were analyzed using *t*-Test or ANOVA. Holm-Sidak multiple comparisons procedures were used to determine which doses yielded significantly different responses. *p*-values less than 0.05 were considered significant.

3.4 Results

TG mice on both FVB and B6 backgrounds showed significantly increased EtOH consumption over the 4 hours of drinking on day 2 compared to their WT littermates in DID (**Figure 3.1a-b**). Mice on a FVB background showed significant effects of genotype and sex (**Figure 3.1a**; $F(1,30) = 4.643$ $p<0.05$, $F(1,30) = 6.913$; $p<0.05$) with TGs drinking more EtOH than WTs and females drinking more than males. There was no significant interaction between genotype and sex ($F(1,33)=0.575$ $p>0.05$). Mice on a B6 background also showed a significant effect of genotype (**Figure 3.1b**; $F(1,38) = 4.251$; $p<0.05$), but showed no significant effect of sex ($F(1,41)=2.26$; $p>0.05$) or interaction between genotype and sex ($F(1,41)=0.334$; $p>0.05$). Conversely, KD mice showed significantly reduced EtOH consumption (**Figure 3.1c**). There was a significant effect of both genotype and sex (**Figure 3.1c**; $F(1,45) = 4.633$, $p<0.05$; $F(1,45) = 8.951$, $p<0.01$), with KDs drinking less EtOH than WTs and females drinking more than males. There was no significant interaction between genotype and sex ($F(1,48)=0.554$; $p>0.05$). BECs were positively correlated with EtOH consumption in all strains (FVB: $R^2 = 0.3$, $p<0.01$; B6: $R^2 = 0.2$, $p<0.05$; KD: $R^2 = 0.2$, $p<0.01$). Importantly, genotype had no effect on water, sucrose or saccharin consumption (**Figure S3.1a-l**) in FVB (water $F(1,15)=1.543$; $p>0.05$; sucrose $F(1,16)=0.502$; $p>0.05$; saccharin $F(1,16)=0.0123$; $p>0.05$) or B6 (water $F(1,41)=0.334$; $p>0.05$;

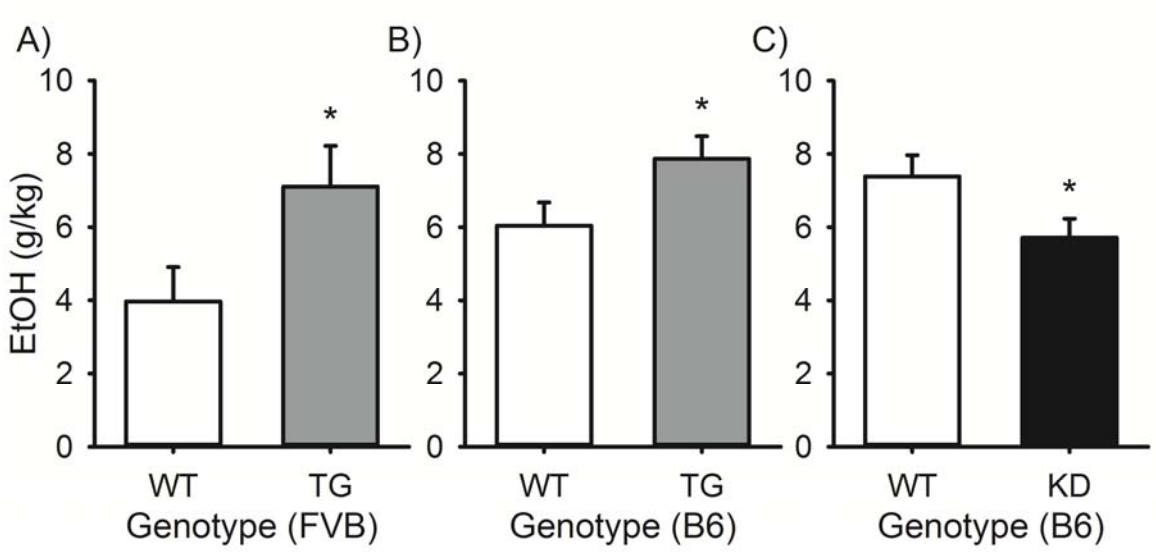

Figure 3.1 *Glo1* expression regulates EtOH consumption. Mice overexpressing Glo1 (TG) on a (A) FVB background (n=20 WT, 16 TG) and (B) B6 background showed increased EtOH consumption over a 4 hr period in DID (n=21 per genotype). (C) Glo1 knockdown (KD; B6 background) mice show reduced EtOH consumption over a 4 hr period in DID (n=21 WT, 26 KD). *$p<0.05$ by Two-Way ANOVA.

sucrose F(1,41)=0.334; p>0.05; saccharin F(1,41)=0.334; p>0.05) TG mice. Genotype also had no effect on water or sucrose consumption in KD mice, though there was a non-significant trend towards reduced saccharin consumption (**Figure S3.1a-i**; water F(1,14)=1.027 p>0.05; sucrose F(1,14)=0.699 p>0.05; saccharin F(1,16)=4.097, p=0.064). There was no difference in EtOH metabolism in FVB or B6 TG mice or in KD mice (data not shown; FVB Genotype F(1, 58)=1.082 p>0.05; B6 Genotype F(1,53)=0.123 p>0.05; KD Genotype F(1,59)=0.162 p>0.05). Because *Glo1* knockdown mice showed reduced EtOH consumption, we next investigated the therapeutic potential of GLO1 inhibition by using a pharmacological inhibitor of GLO1, pBBG (Thornalley et al. 1996; Distler et al. 2012a). Male B6 WT mice received an IP injection of pBBG (0, 6.25, or 12.5 mg/kg) 2 hours before testing on day 2 of the DID paradigm. There was a significant effect of treatment on EtOH consumption (**Figure 3.2a**; F(2,43) = 4.712; p<0.05). Post hoc tests revealed that both doses significantly reduced EtOH consumption compared to vehicle treatment (p<0.05). BECs were positively correlated with EtOH consumption (R^2 = 0.3, p<0.001). There was no effect of pBBG on water consumption (**Figure 3. 2b**; F(2,43)=0.866 p>0.05) or saccharin consumption (**Figure 3.2c**; F(2,43)=0.969 p>0.05), but sucrose consumption was reduced following the 12.5 mg/kg dose of pBBG (**Figure 3.2d**; Sucrose F(2, 41)= 8.354; p<0.001; post hoc for 0 vs 12.5 mg/kg p<0.002). However, the 6.25 mg/kg dose of pBBG did not change sucrose consumption.

In a separate set of studies we found that there was a significant effect of treatment on EtOH consumption when using higher doses of pBBG (**Figure S3.2a**; 25mg/kg and 50mg/kg; F(2,42) = 9.113; p<0.001). However, these doses also changed consumption of water and sucrose, which confounds the interpretation of DID. Specifically, there was a significant main effect of treatment on water consumption (F(2,41) = 3.522; p<0.05); post hoc tests were

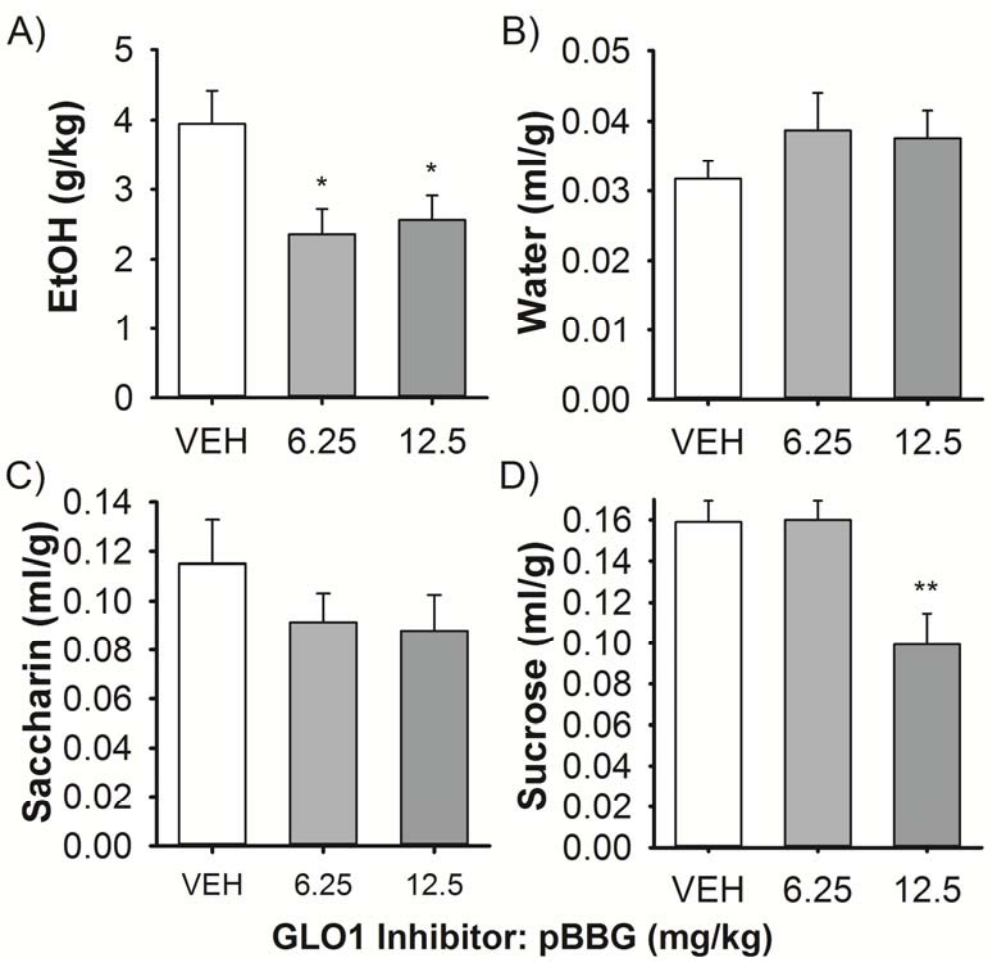

Figure 3.2 The GLO1 inhibitor pBBG reduces EtOH consumption. Acute IP injection (2hrs before testing) with the indicated doses of pBBG reduces (A) EtOH consumption at multiple doses, but has no effect on (B) water or (C) 0.2% saccharin consumption. (D) Sucrose consumption was reduced only at the 12.5 mg/kg dose. n=14-15 per group for each test *$p<0.05$, ** $p<0.01$ by Holm-Sidak (comparisons to VEH).

suggestive for both doses (**Figure S3.2b**; 0 vs 25mg/kg: p=0.079; 0 vs 50mg/kg: p=0.053). There was a non-significant trend of treatment on saccharin consumption (**Figure S3.2c**; F(2,43)=3.05; p=0.058) and a significant effect of treatment on sucrose consumption (**Figure S3.2d**; F(2,42) = 6.201; p<0.01). For sucrose, post hoc tests were suggestive for 25mg/kg pBBG (p=0.064) and were significant for the 50mg/kg pBBG (p<0.05).

Finally, we performed the LORR and balance beam tests to determine whether manipulations of GLO1 altered sensitivity to the sedative or ataxic effects of EtOH. In LORR, we observed no significant differences in duration of LORR between TG *Glo1* overexpressing and WT mice on either FVB (**Figure 3.3a**; Genotype: F(1,19)=1.458 p>0.05; Sex: F(1,19)=2.775 p>0.05; Interaction F(1,19)=0.0797 p>0.05) or B6 backgrounds (**Figure 3.3b**; Genotype F(1,25)=0.15 p>0.05; Sex F(1,25)=0.203 p>0.05; Interaction F(1,25)=2.224 p>0.05). Similarly, we did not observe a significant effect of treatment (VEH, 6.25mg/kg or 12.5mg/kg pBBG) on duration of LORR in male B6 WT mice (**Figure 3.3c**; F(2,53)=0.207 p>0.05). No differences in BECs were seen upon the regain of righting reflex between genotypes in either the FVB or the B6 strains, nor after pBBG treatment in B6 WT mice (data not shown; FVB Genotype F(1, 21)=0.0009 p>0.05; B6 Genotype F(1,25)=0.0761 p>0.05; pBBG F(2, 54)=1.697 p>0.05).

On the balance beam, mice overexpressing *Glo1* (TG) showed no differences in foot slips at baseline or following EtOH injections on either an FVB background (**Figure 3.3d**; Baseline: Genotype F(1,16)=0.284 p>0.05; Sex F(1,16)=0.0178 p>0.05; Interaction F(1,16)=0.0178 p>0.05; EtOH: Genotype F(1,16)=1.458 p>0.05; Sex F(1,16)=2.775 p>0.05; Interaction F(1,16)=0.0797 p>0.05), or B6 background (**Figure 3.3e**, Baseline: Genotype F(1,16)=0.006 p>0.05; Sex F(1,16)=0.746 p>0.05; Interaction F(1,16)=0.746 p>0.05; EtOH: Genotype F(1,16)=0.171 p>0.05; Sex F(1,16)=0.501 p>0.05; Interaction F(1,16)=0.0139 p>0.05). In WT

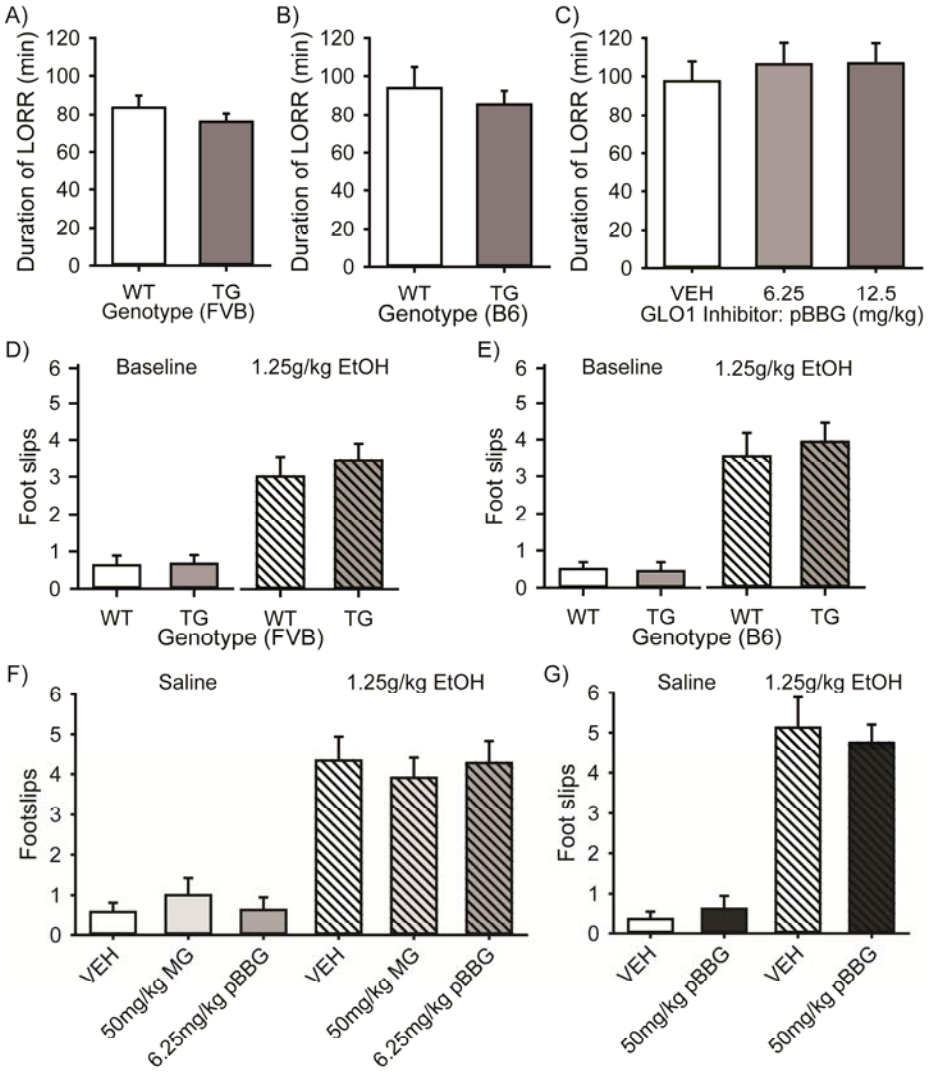

Figure 3.3 EtOH induced LORR and balance beam foot slips are not altered in *Glo1* TG overexpressing mice or in mice treated with MG or pBBG. Mice overexpressing Glo1 (TG) on an (A) FVB background (n=9 WT, 13 TG), or (B) B6 background (n=15 WT, 11 TG) show no differences in duration of LORR following a 4g/kg EtOH injection. (C) WT male B6 mice treated with 0, 6.25 or 12.5 mg/kg pBBG 2 hours before EtOH injections showed no differences in duration of LORR (n=19-20 per group). On the balance beam, mice overexpressing Glo1 (TG) showed no differences in foot slips at baseline or following 1.25g/kg EtOH injections on either an (D) FVB background (n=8 WT, 9 TG), or (E) B6 background (n=8 WT, 9 TG). (F) In WT B6 mice (n=11-12 per group) EtOH treatment significantly increased foot slips ($p<0.001$ Two-way ANOVA), but there was no interaction between drug (VEH, 50mg/kg MG or 6.25mg/kg pBBG) and EtOH treatment (G) Using a higher dose of pBBG (50mg/kg) in WT B6 mice (n=8 per group), EtOH treatment significantly increased foot slips ($p<0.001$ by Two-way ANOVA), but again, there was no interaction between drug and EtOH treatment.

male B6 mice, EtOH treatment significantly increased foot slips (**Figure 3.3f**; $F(1,68)=84.479$, $p<0.001$), but there was no effect of drug treatment (VEH, 50mg/kg MG or 6.25mg/kg pBBG; $F(2, 68)<0.0001$ $p>0.05$) nor was there an interaction ($F(2,68)=0.501$ $p>0.05$). In a separate study in WT male B6 mice using a higher dose of pBBG (50mg/kg), EtOH treatment significantly increased foot slips (**Figure 3.3g**; $F(1,31)=84.621$, $p<0.001$), but again, there was no effect of pBBG treatment ($F(1,31)=0.0168$ $p>0.05$) nor was there an interaction ($F(1,31)=0.42$ $p>0.05$).

3.5 Discussion

Our data demonstrate a novel role for GLO1 in the regulation of EtOH consumption. We observed increased EtOH consumption in FVB and B6 TG mice overexpressing *Glo1*. Conversely, we observed decreased EtOH consumption following both genetic knockdown of *Glo1* (KD) and pharmacological inhibition of GLO1 by pBBG. To the best of our knowledge, these are the first studies to demonstrate that manipulations of *Glo1* expression and enzymatic activity can alter voluntary EtOH consumption. These data suggest that pharmacological inhibition of GLO1 could be used to reduce voluntary EtOH consumption.

Importantly, neither overexpressing nor knocking down *Glo1* affected general consummatory behavior as there was no effect on 10% sucrose or water consumption. Additionally, genotype did not alter EtOH metabolism. While all doses of the GLO1 inhibitor (pBBG) reduced EtOH consumption, the higher doses altered consumption of sucrose (12.5, 25 and 50 mg/kg) and water (25, 50 mg/kg) consumption. No doses of pBBG altered saccharin consumption nor did genotype have an effect on saccharin consumption in TG mice. While there was a non-significant trend towards an effect of KD on 0.2% saccharin consumption, the consistent effects of *Glo1* manipulations on EtOH consumption and lack of general effect of

Glo1 manipulation on other consummatory behaviors suggests that *Glo1* manipulations are not leading to decreased EtOH drinking through changes in their tastant sensitivity. Additionally, the DID studies may be limited by effects from either repeated testing or order of testing. However, this again seems unlikely given the complimentary and inverse effects of *Glo1* overexpression versus *Glo1* knockdown or GLO1 inhibition.

Correlations between EtOH drinking and BEC were somewhat modest, though they were not dissimilar to those seen by others (e.g. Wilcox et al., 2013) and may be a reflection of the extended access (4 hrs) wherein mice will show different patterns of drinking. For example, Wilcox et al (2013) showed that in DID mice may "front-load" or drink EtOH at the highest rate during the first 15 minutes of EtOH access which could lead to high overall drinking, but lower than expected BECs at the end of the session.

In studies using *Glo1* overexpressing mice or *Glo1* knockdown, both males and females were used. We saw no interactions between sex and genotype in any of our measures. A limitation of the GLO1 inhibitor studies is that we did not use females. While the lack of female subjects in the inhibitor study makes it unclear whether the GLO1 inhibitor would reduce EtOH consumption in females, the effects seen in the transgenic animals suggest EtOH consumption in females would respond to GLO1 inhibition.

We have previously shown that MG, which is metabolized by GLO1, is a competitive partial agonist at $GABA_A$ receptors (Distler and Palmer 2012; Distler et al. 2012a; McMurray et al. 2014). We suspect that the changes in MG concentrations, which are caused by manipulations of *Glo1* expression or enzymatic inhibition (Distler et al. 2012a), modulate EtOH consumption via the action of MG at $GABA_A$ receptors. There is a well-established role of the $GABA_A$ receptor system in regulating EtOH consumption (Kumar et al. 2009). Indeed, GABAergic drugs

such as muscimol and THIP reduce EtOH consumption as measured using DID, though they also reduce other consummatory behavior such as sucrose and water consumption (Moore et al. 2007). Additionally, a recent mouse study found that $GABA_A$ receptor-mediated signaling was depressed in the striatum following repeated EtOH consumption through 6 weeks of DID (Wilcox et al. 2013). Our data are consistent with those supporting a role for $GABA_A$ receptors in the regulation of EtOH consumption in DID and show that these effects can be obtained via manipulation of *Glo1*.

It is possible that MG and GLO1 are involved in the normal regulation of alcohol consumption through the activity of MG at $GABA_A$ receptors. MG is an endogenously produced byproduct of glycolysis (Thornalley 1996b). However, MG is also found in almost all foods and in many alcoholic beverages (Nemet et al. 2006; Angeloni et al. 2014; Ojeda et al. 2014). Whether concentrations of MG reach pharmacologically meaningful levels is unknown, but it raises the possibility that direct ingestion of MG may be an important component of the pharmacological properties of fermented beverages. MG may provide negative feedback on alcohol consumption whereby alcohol increases MG levels both through endogenous production and exogenous ingestion. High levels of MG may occupy $GABA_A$ receptors and lead to a reduction in EtOH consumption. This reduction may be the result of antagonistic-like properties of MG by reducing the maximal amplitude of GABAergic currents because of its actions as a partial agonist. This may be similar to decreased EtOH consumption seen after systemic administration $GABA_A$ antagonists (Koob et al. 1998; Chester and Cunningham 2002; Koob 2006). Alternatively, increased activation from baseline could increase sensitivity to the hypnotic or ataxic effects of alcohol use and lead to early termination of drinking similar to the reduction in consumption others have seen using $GABA_A$ receptor agonists such as muscimol (Moore et al.

2007). However, this theory is not supported by our observation that there were no differences in LORR or footslips in either TG mice or mice treated with the GLO1 inhibitor.

The ability of GLO1 inhibitors to reduce EtOH consumption in DID suggest GLO1 inhibitors may be a viable for the treatment of alcohol use disorders. We previously showed that GLO1 inhibitors reduce anxiety-like behavior in mice and have suggested that GLO1 inhibitors could be used for the treatment of anxiety disorders (Distler and Palmer 2012; Distler et al. 2012a; McMurray et al. 2014), which are highly comorbid with AUDs (Smith and Randall 2012). Current pharmacological treatments for anxiety disorders include selective serotonin reuptake inhibitors (e.g. fluoxetine), serotonin-norepinephrine reuptake inhibitors and benzodiazepines (e.g. diazepam) that are positive allosteric modulators that do not directly activate $GABA_A$ receptor (e.g. diazepam, a benzodiazepine) (Smith and Randall 2012). Drugs most commonly used for the treatment of AUDs act either as a mu-opioid antagonist or a NMDA receptor modulator (e.g. naltrexone, acamprosate respectively) (Yahn, Watterson, and Olive 2013). Based on our pre-clinical models, GLO1 inhibition reduces anxiety-like behavior and EtOH consumption by a mechanism that may be distinct from those currently in use.

In summary, the studies presented here suggest that manipulation of *Glo1* can influence EtOH consumption, thus offering a novel target for the treatment of AUDs. Our previous studies have established a therapeutic potential for GLO1 inhibition in the treatment of anxiety disorders, which in conjunction with the data presented here, suggest GLO1 inhibition may be of particular interest for treatment of comorbid AUD and anxiety disorder.

3.6 Acknowledgements

The authors would like to thank John Crabbe, Stephanie Spence, Pamela Metten, Igor Ponomarev, Charissa Newkirk and Stephanie Dulawa for technical advice and assistance related

to DID procedures and BEC analysis. This work was supported by MH079103 (AAP), NS076517 (JMC), MH096463 (JMC) and DA031090 (LAA).

3.7 Supplementary Information

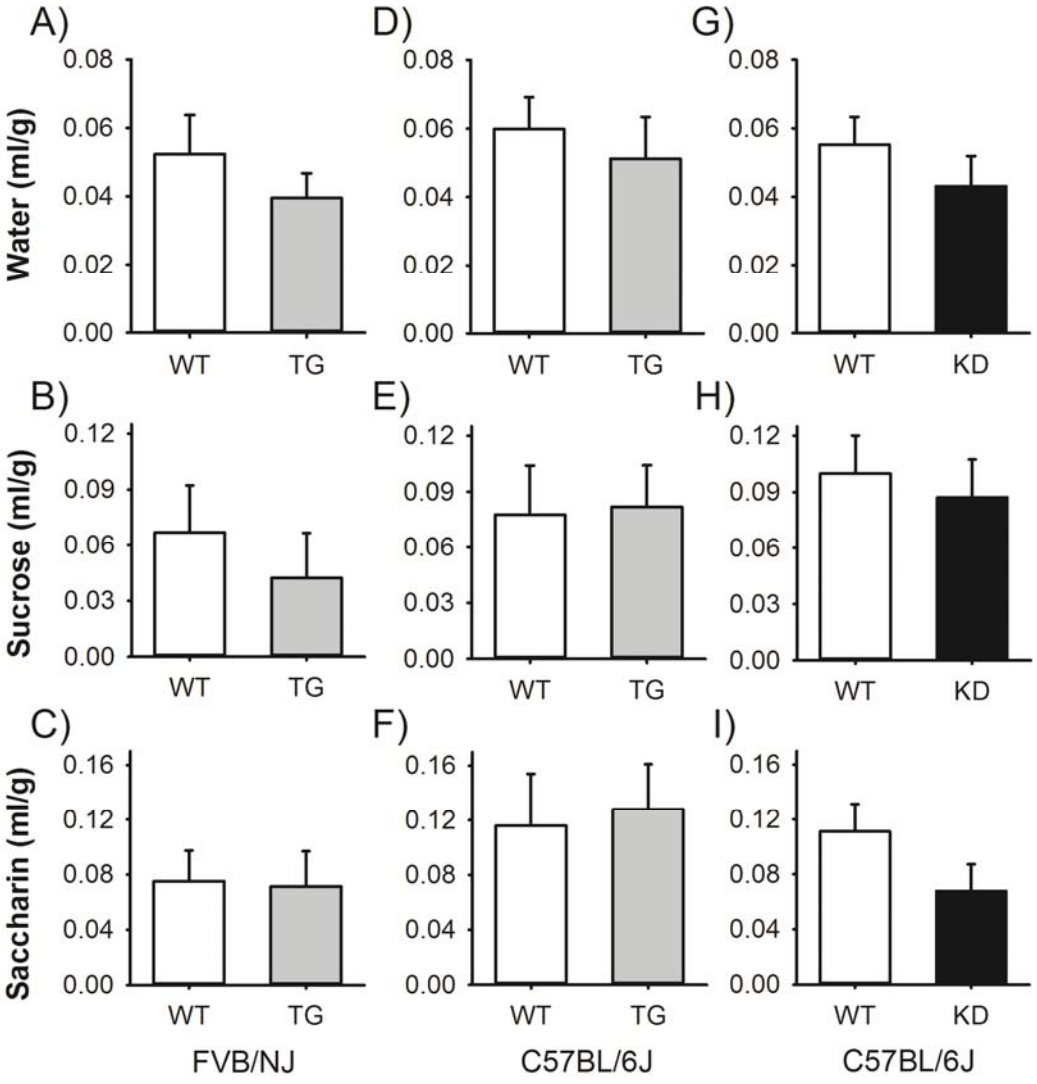

Figure S3.1 *Glo1* expression does not alter general consummatory behaviors in DID. *Glo1* expression had no effect on water, 10% sucrose, or 0.2% saccharin consumption over a 4hr period in DID for mice overexpressing *Glo1* (TG) on a FVB background (A-C; n=9 per genotype), B6 background (D-F; n=8 per genotype) , or in Glo1KD (KD) mice on a B6 background (G-I; n=8 WT, 9 KD).

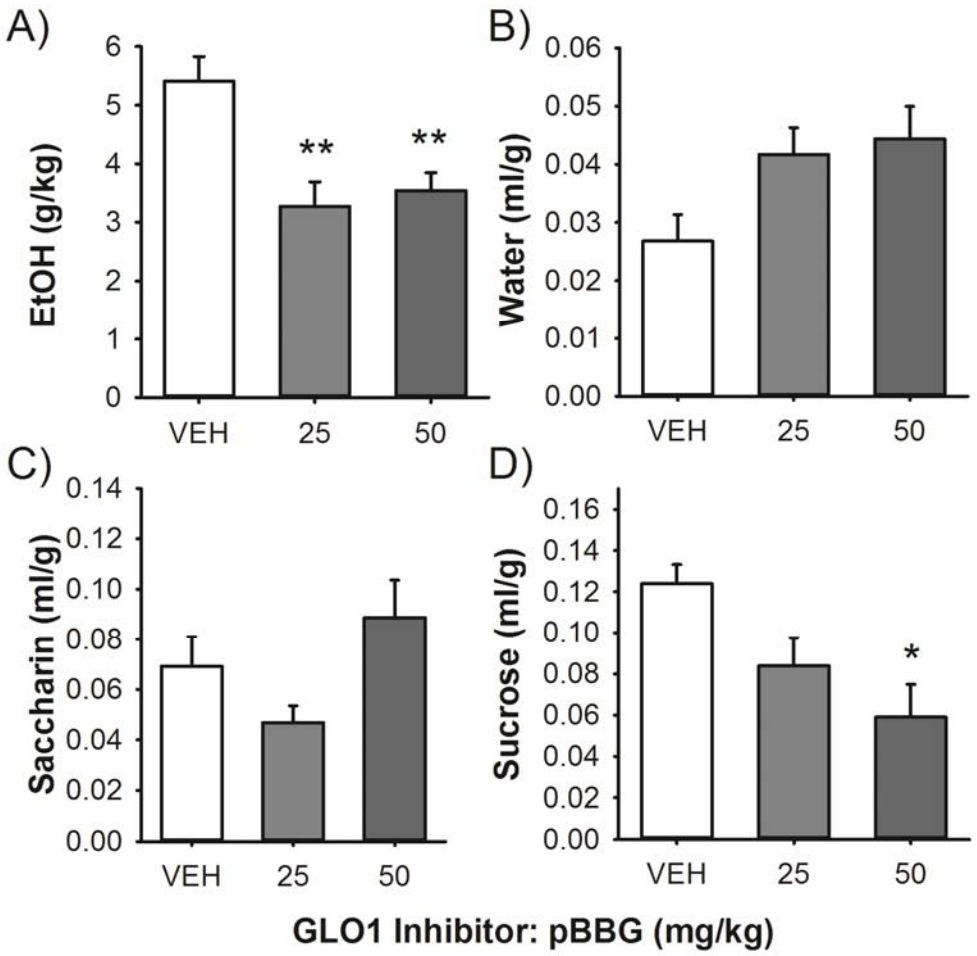

Figure S3.2 Effects of larger doses of GLO1 inhibitor pBBG on consummatory behaviors. **(**A) Relative to vehicle (VEH) treated mice, ethanol consumption was reduced by pBBG. (B) There was an effect the 25 and 50 mg/kg doses of pBBG on water consumption, although the posthoc tests were not quite significant (VEH vs 25 p=0.079 and VEH vs 50 p=0.053). (C) There was no effect of pBBG on saccharin consumption. D) There was an overall effect of pBBG on sucrose consumption; posthoc tests were significant reduction for 50 mg/kg ($p<0.05$) and suggestive for the 25 mg/kg dose (p=0.064). n=14-15 per treatment for all tests. *$p<0.05$, **$p<0.01$

CHAPTER 4

IDENTIFICATION OF A NOVEL, FAST-ACTING ANTIDEPRESSANT

4.1 Abstract

Currently available pharmacotherapies for depression are not effective in all patients, have relatively slow onset, and are limited by side effects. GLO1 is a ubiquitous cellular enzyme responsible for the detoxification of the glycolytic byproduct methylglyoxal (MG), which is a competitive partial agonist at GABA-A receptors. We examined the effects of genetic and pharmacological inhibition of GLO1 in two antidepressant assay models: the tail suspension test (TST) and the forced swim test (FST). We also examined the effects of GLO1 inhibition in three models of antidepressant onset: the chronic FST (cFST), chronic mild stress (CMS) and olfactory bulbectomy (OBX). Genetic knockdown of Glo1 or pharmacological inhibition by two structurally distinct GLO1 inhibitors (S-bromobenzylglutathione cyclopentyl diester (pBBG) or methyl gerfelin (MeGFN)) reduced immobility in the TST and acute FST. Both GLO1 inhibitors, but not fluoxetine, reduced immobility in the cFST after 5 days; all three compounds reduced immobility after 14 days of treatment. Furthermore, 5 days of treatment with either GLO1 inhibitor blocked the depression-like effects induced by CMS on the FST and coat state. Finally, pBBG also attenuated the locomotor hyperactivity induced by OBX after 5 days of treatment. We also found that 5 days of treatment with a GLO1 inhibitor, but not the SSRI fluoxetine, induced classical molecular markers of the antidepressant response, such as increased BDNF in the hippocampus and mPFC and the pCREB to CREB ratio in the hippocampus. Our findings indicate that inhibition of GLO1 may provide a novel and fast-acting pharmacotherapy for depression.

4.2 Introduction

Depression affects about one in six adults at some point in their lifetime (Kessler et al. 2005b; Kessler et al. 2012b). Current treatments for depression are limited by negative side effects, slow onset of therapeutic effects, and limited efficacy (Berton and Nestler 2006; Martinowich et al. 2013). Thus, identification of novel targets for antidepressant drug development is urgently needed.

Here we examined potential antidepressant effects of Glyoxalase 1 (GLO1) inhibitors, and their temporal onset of action. GLO1 is a ubiquitous cytosolic enzyme that catalyzes the reduction of methylglyoxal (**MG**) , which is a non-enzymatic side product of glycolysis (Thornalley 1996a). Therefore, MG concentrations are inversely proportional to GLO1 enzymatic activity. Previous studies have shown that increased expression of *Glo1* increases anxiety-like behavior in mice (Hovatta et al. 2005a; Williams et al. 2009; Distler et al. 2012b). Additionally, administration of MG or a GLO1 inhibitor, S-bromobenzylglutathione cyclopentyl diester (**pBBG**), decrease anxiety-like behavior in mice (Distler et al. 2012b). Electrophysiological recordings from primary neuronal cultures demonstrated that MG is a competitive partial agonist at GABA-A receptors (Distler et al. 2012b). Therefore, we hypothesize that GLO1 inhibitors and direct administration of MG reduce anxiety-like behavior via increased GABA-A receptor activation.

Anxiety and depression are highly comorbid and genetic studies have also identified a significant shared genetic liability between the two (Kendler et al. 2007b; Demirkan et al. 2011). There is also significant overlap in the neurobiology and neural circuitry underlying anxiety and depression, including the hippocampal-prefrontal circuitry, which strongly suggests a common underlying mechanism (Nestler et al. 2002; Kendler et al. 2007a; Martinowich et al. 2007; Krishnan and Nestler 2008; Möhler 2012a; Willner et al. 2013). Moreover, classical

antidepressants such as selective serotonin reuptake inhibitors (SSRIs), tricyclics, and MAOIs are also effective for treating anxiety disorders (Kent et al. 2002; Gross and Hen 2004; Dulawa and Hen 2005; Bandelow et al. 2015). However, GABAergc anxiolytic drugs, such as benzodiazepines, are not generally effective for the treatment of depression (Barbui et al. 2011) and typically do not show antidepressant effects in preclinical animal models (Cryan et al. 2002; Cryan et al. 2005), although recent studies have identified exceptions (Fava et al., 2011, Fischell et al. 2015). Nevertheless, depression is associated with reductions in GABA in cerebrospinal fluid and reductions in the number of GABA-A receptors in cortical regions, and chronic antidepressant treatment correlates with an increase in GABA (Sanacora et al. 2002; Klumpers et al. 2010), supporting a potential role for GABAergic signaling in depression (Kalueff and Nutt 2007; Möhler 2012a).

A recent study reported increased depression-like behavior in mice overexpressing *Glo1* in the tail suspension test (TST) (Benton et al. 2012b), a highly reliable screen for antidepressant drug activity (Cryan et al. 2005). Therefore, we investigated the effect of genetic and pharmacological GLO1 inhibition in acute preclinical screens for antidepressant efficacy using *Glo1* knockdown mice and two structurally distinct GLO1 inhibitors. We then assessed the time-course of antidepressant action of the two GLO1 inhibitors using the cFST, CMS, and OBX models of antidepressant onset. Finally, we assessed whether 5 days of treatment with GLO1 inhibitors induce upregulation of classical molecular markers of the antidepressant response, including BDNF induction and CREB phosphorylation in hippocampus and medial prefrontal cortex (mPFC).

4.3 Materials and Methods

Mice: *Glo1* knock-down (KD) mice were generated on a C57BL/6J (B6) background in the lab

of Dr. Michael Brownlee (Albert Einstein College of Medicine, Bronx, NY) and have been reported to show an approximately 45-65% reduction in GLO1 enzymatic activity(El-Osta et al., 2008). Male and female KD mice and their WT littermates were tested at ages 8-14 weeks.

For studies using the GLO1 inhibitors (pBBG and Me-GFN), male and female B6, BALB/cJ (BALB) or FVB/NJ (FVB) mice were purchased from The Jackson Laboratory (JAX) and tested at ages 8-15 weeks. All mice were group housed on a standard 12/12 hour light/dark cycle unless otherwise noted (e.g. during CMS) and underwent behavioral testing in the second half of their light cycle (12-5pm). Separate cohorts were used in each behavioral study unless otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago and performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs: S-bromobenzylglutathione cyclopentyl diester (pBBG) and Methyl gerfelin (MeGFN) were synthesized as previously described (Thornalley et al. 1996; Kawatani et al. 2008; Kanoh et al. 2013). In acute tests of depression-like behavior, mice received 50mg/kg pBBG, 12.5mg/kg,or vehicle (pBBG: 8% DMSO, 18% Tween80; MeGFN: 4%DMSO/9%Tween80 in H_2O) by I.P. injection 2 hours before testing. In chronic studies, minipumps were filled with pBBG, methyl gerfelin or vehicle (50% DMSO, 50% PEG400) and inserted into a small subcutaneous incision made on the back (Opal et al. 2013). Fluoxetine (FLX) was delivered via the drinking water in opaque water bottles at a concentration of 160mg/L to achieve a dose 18 mg/kg/day (Dulawa et al. 2004)..

Behavioral Studies**:**

TST: Male and female B6, FVB, Glo1KD and their WT littermates were suspended upside-down by the tail for 6 min and immobility was quantified as a measure of depression-like behavior.

FVB mice were scored using the Noldus EthoVision (Leesburg VA) software; scoring from this system was strongly correlated with scores from human observers in the subset that were scored by both. B6 and Glo1KD mice were scored using am observer who was blind to their treatment/genotype. Mice that responded to the TST by climbing their tails were excluded from analysis (3 FVB, 9 B6, 2 KD).

Acute FST: FST procedures were performed as previously described (Jiao et al. 2012). Briefly, male and female B6, FVB, BALB, Glo1KD and their WT littermates were placed into round buckets 22cm across and 20cm deep that were filled with water (23-25˚C; 16cm deep) for 10 min. On day 2, mice were placed in the same buckets for 6 min. The final 4 min on the second day were scored for immobility.

Chronic Forced Swim Test (cFST): cFST was performed as previously (Opal et al. 2013). Male and female BALB/cJ mice were implanted with minipumps delivering pBBG (5, 10, 15, 30 or 60 mg/kg/day), MeGFN (5, 10, 15 mg/kg/day) or vehicle for either 5 or 14 days then underwent FST.

Open-field Test (OFT): Center duration and locomotor activity was assessed in mice that underwent cFST using automated Versamax software as previously described (Distler et al. 2012a; Opal et al. 2013). Chambers measured 43 x 43 x 33 cm (width x depth x height) and had dim overhead fluorescent lighting (14 lux). Center was defined as the middle 26 x 26cm.

Olfactory Bulbectomy (OBX): OBX was performed as previously (Opal et al. 2013). Female B6 or male BALB mice were anesthetized using isoflurane and mounted in a stereotaxic frame. A midline sagittal incision was made to expose bregma. Small holes were drilled in the skull to expose the olfactory bulbs (AP +7 mm, ML ±2.5 mm). Olfactory bulbs were removed using a vacuum pump and holes were filled with haemostatic sponge. For sham operated animals an

identical protocol was followed such that holes were drilled to expose the olfactory bulbs, but the vacuum pump was not used. Incisions were closed using wound clips and mice were allowed to recover for 14 days after which minipumps containing vehicle or pBBG were implanted. Five days after minipump insertion, mice were placed into the OFT for 30 minutes to assess locomotion.

Chronic Mild Stress (CMS): Female BALB mice were exposed to a series of stressors that varied daily and repeated weekly, as described previously (Opal et al. 2013). Following 6 weeks of stress, mice were surgically implanted with minipumps delivering vehicle (50% DMSO, 50% PEG400), 10mg/kg/day pBBG or 10mg/kg/day MeGFN. Mice continued to receive stressors following surgery. After 5 day of treatment coat state was evaluated, immediately followed by the sucrose preference test and finally the splash test; the next day mice underwent the FST. Non-stressed control animals were housed in a separate room under standard housing conditions.

Coat state: Coat state evaluation was similar to Dournes et al. (2013) and Nollet et al. (2013). Following CMS, photos were taken and coat state was evaluated on the head, neck, dorsal coat, and tail by an experimenter blind to treatment condition. For each area, a score of 0 was given for a coat state in good condition (clean/fluffy), 0.5 for a mildly disheveled coat (oily appearance), or a 1 for a severely disheveled coat (very oily and ruffled in appearance). Total score was the sum of the scores for each region.

Sucrose preference: Mice were placed into individual cages and given access to water and 2% sucrose for 2 hours. Preference was defined as the amount of sucrose consumed divided by the total amount of water and sucrose.

Splash Test: Splash test was modeled after Nollet et al. (2013). Mice were sprayed twice on their back with a 10% sucrose solution and placed back into the cage in which they had just

undergone the sucrose preference test. Videos were recorded and grooming behavior was scored by blind observers for the next 5 minutes. A bout was defined by self-grooming characterized by any number of leg strokes along the body, a minimum of 2 arm strokes over the face/head, or any amount of time spent licking/biting the fur. Latency to groom was the time of the first grooming bout minus the time of placement back into the cage following spraying.

Western Blots: Westerns were performed as previously described (Distler et al. 2012b; Opal et al. 2013). Briefly, 1.5mm tissue punches were taken from medial prefrontal cortex (mPFC) or hippocampus and snap frozen. Tissue was homogenized in ice cold RIPA buffer and quantified using the BCA Protein Assay Kit (Pierce; Rockford IL). Twenty μg of protein was separated by SDS-PAGE. Membranes were probed with primary antibodies against phosphorylated cyclic-AMP response binding protein (pCREB), CREB, brain-derived neurotrophic factor (BDNF), and α-tubulin then labeled with peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Blots were developed using Pierce ECL Plus (Thermo Fisher Scientific), digitized and band intensity was measured using ImageJ (NIH; http://rsbweb.nih.gov/ij/).

Statistical Analysis: Data were analyzed using ANOVA. Tukey posthoc tests were used to determine which doses yielded significantly different responses. *p*-values <0.05 were considered significant.

4.4 Results

TST: In the TST, male and female *Glo1* knockdown mice (**GLO1KD**) showed significantly less immobility than their WT littermates (**Figure 4.1a**; $F(1,44)=7.447$, $p<0.01$). There was no significant effect of sex on immobility nor was there a significant interaction between sex and genotype. IP injection of pBBG significantly reduced immobility in male and female B6 and FVB mice (**Figure 4.1b-c**; B6 $F(1,20)=12.022$, $p<0.01$; FVB $F(1,39)=4.642$, $p<0.05$). There was

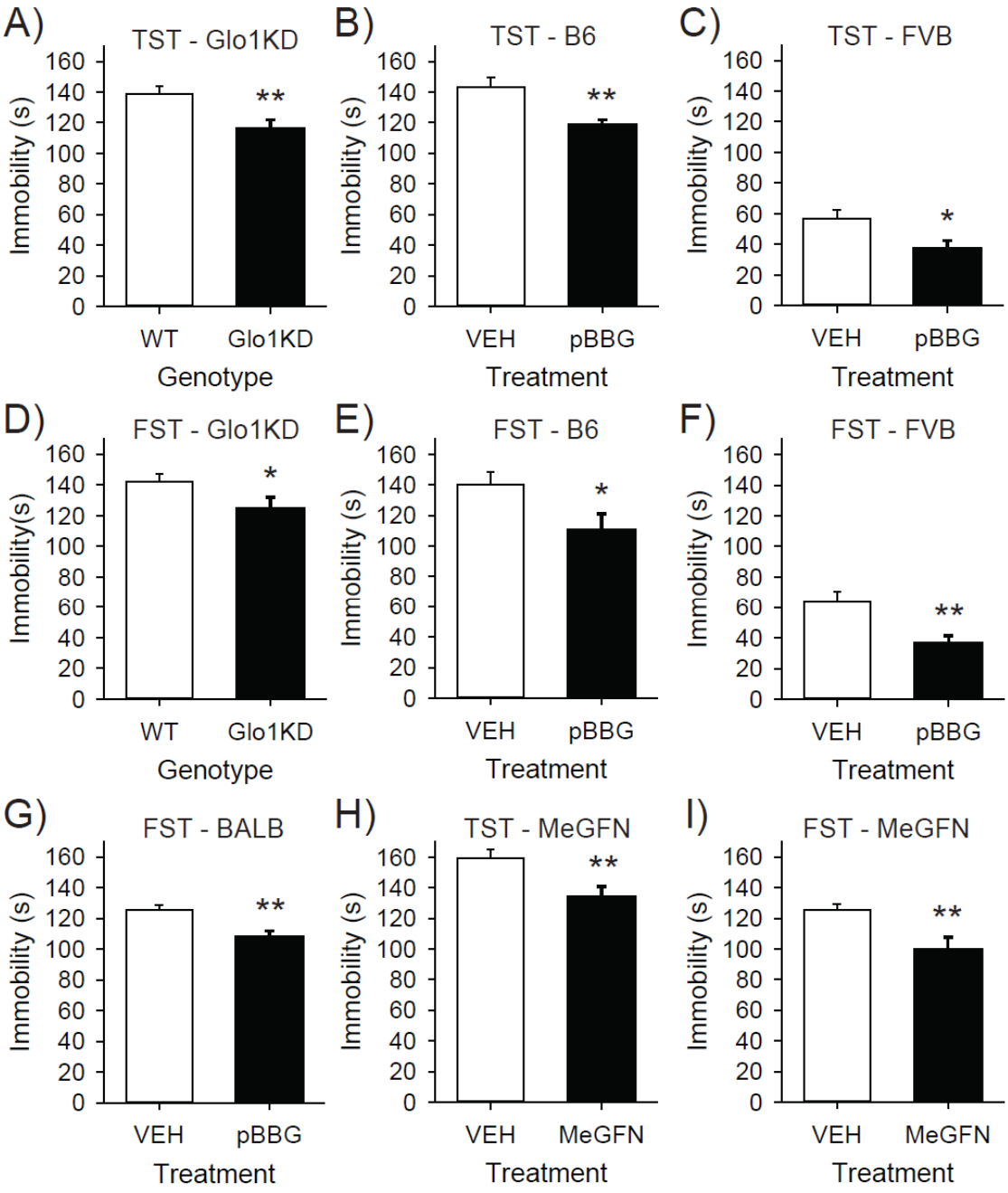

Figure 4.1 Reductions in GLO1 reduce depression-like behavior acutely in the TST and FST. In the TST, immobility is reduced in (a) GLO1 knockdown (KD; n=20) mice compared to their wild-type (WT; n=25) littermates or after I.P. pBBG (50 mg/kg) in b) B6 (n=9 VEH, 12 pBBG), (c) FVB (n=20). In the FST, immobility was reduced in (d) KD (n=29WT, 18KD) mice. pBBG also reduced immobility in (e) B6 (n=14 VEH, 16 pBBG), (f) FVB (n=22) and (g) BALB/cJ mice (n=30 VEH, 29 pBBG). A pharmacologically distinct GLO1 inhibitor, MeGFN, (12.5 mg/kg) was also able to reduce immobility in male B6 mice in both the (h) TST (n=14, 12) and (I) FST (n=18 VEH, 19 MeGFN). **p<0.05, **p<0.01.*

no effect of sex on immobility nor was there an interaction between treatment and sex in either B6 or FVB mice.

Acute FST: GLO1KD mice also showed significantly less immobility than their WT in the FST (**Figure 4.1d**; $F(1,46)=5.256$, $p<0.05$). Females showed significantly greater immobility ($F(1,39)=2.926$, $p<0.05$), but there was no interaction between sex and genotype ($p>0.05$). pBBG significantly reduced immobility in 3 different mouse strains, B6, FVB and BALB (**Figure 4.1e-g**; B6: $F(1,29)=3.681$, $p<0.05$; FVB: $F(1, 43)=10.105$, $p<0.01$; BALB: $(1,58)=10.989$, $p<0.01$). There was no significant effect of sex on immobility nor was there a significant interaction between treatment and sex in B6 or BALB mice ($p>0.05$). There was a significant effect of sex in FVB mice ($F(1,43)=7.895$, $p<0.01$), but the interaction between sex and treatment was not significant. A second GLO1 inhibitor, MeGFN, (12.5mg/kg; IP 2 hours before testing) also reduced immobility in both the TST **(Figure 4.1h**; $F(1,25)=8.233$, $p<0.01$) and FST (**Figure 4.1i**; $F(1,36)=7.803$, $p<0.01$) in male B6 mice.

cFST (14 days)

When treated by continuous infusion (e.g. minipump) rather than acute bolus injection, BALB mice show reduced immobility in the FST in response to chronic (14 days), but not subchronic (5 days) treatment with SSRIs (Dulawa et al. 2004). After 14 days of treatment with pBBG (0, 5, 10, 15, 30 or 60 mg/kg/day), there was a significant effect of treatment on the cFST (**Figure 4.2a**; $F(5,113)=6.738$, $p<0.001$). Posthoc testing indicated that the 5, 10 and 15 mg/kg/day doses all significantly reduced immobility compared to vehicle, the 30 mg/kg/day significantly increased immobility and 60 mg/kg/day had no significant effect. We did not observe any effect on the apparent health or weight (data not shown; $F(3, 58)=0.0115$, $p>0.05$; $F(3, 59)=1.002$, $p>0.05$) of mice treated for 14 days with pBBG. In a separate cohort of mice treated with

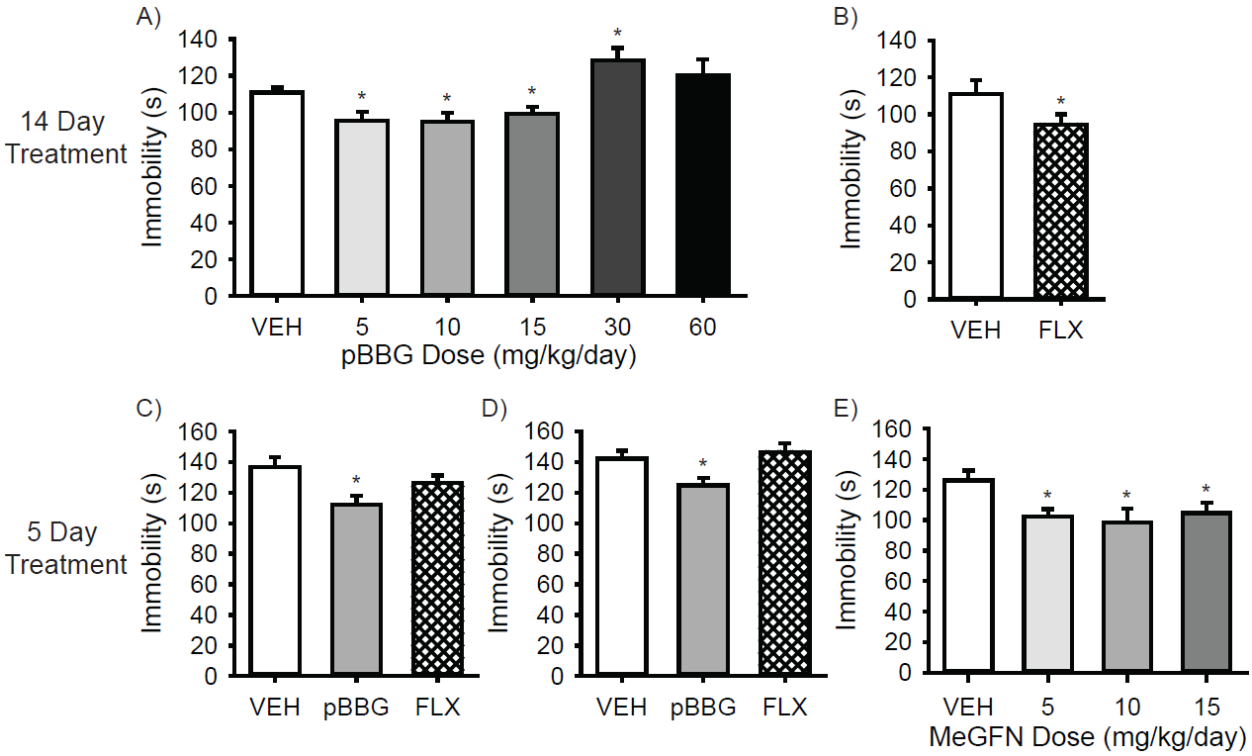

Figure 4.2 The GLO1 inhibitor pBBG reduces immobility in the cFST. (a) Chronic (14 day) treatment with pBBG reduces immobility at multiple doses in the cFST, though higher doses may become aversive. (b) Fluoxetine is also able to reduce immobility following chronic treatment. Following subchronic (5 day) treatment, pBBG, but not fluoxetine, reduced immobility by 5 days in BALB (c) males and (d) females. (e) MeGFN also reduced immobility by 5 days in female BALB mice. *n=10-15 per group except in panel 'a' VEH (n=42) and 15 mg/kg/day (n=23); *p<0.05.*

fluoxetine in the drinking water we confirmed that 14 days of treatment with fluoxetine reduced immobility (**Figure 4.2b**; $t=1.851$, $p<0.038$ by one-tailed t-test).

These same animals were also tested in the OFT prior to cFST to determine whether they showed anxiolytic effects after 12 days of treatment. There was a significant effect of treatment with pBBG on center duration (**Figure S4.1a**; $F(5, 110)=4.447$ $p<0.01$). Post hoc tests revealed that 10mg/kg/day significantly increased center duration compared to VEH treatment ($p<0.001$); no other treatments showed significant effects. None of the doses of pBBG altered locomotor activity in the OFT (**Figure S4.1b**; $F(5,116)=0.796$ $p>0.05$). Fluoxetine-treated mice were not tested in the OFT.

Next, we investigated subchronic (5 day) treatment with pBBG and MeGFN in cFST to determine whether GLO1 inhibition might have a faster onset of antidepressant effects; these studies used separate cohorts of male and female BALB mice. pBBG (10 mg/kg/day) significantly reduced immobility in both male (**Figure 4.2c**; $F(2,38)=4.526$, $p<0.05$) and female (**Figure 4.2d**; $F(2,41)=4.775$, $p<0.05$) mice. Posthoc tests confirmed that pBBG but not fluoxetine reduced immobility compared to the vehicle treatment. In these mice, there was a non-significant trend of treatment on center duration in the OFT (**Figure S4.2a**; $F(2,42)=3.107$, $p=0.056$). However, there was an overall significant effect of treatment on locomotor behavior in the OFT on day 4 of treatment (**Figure S4.2c**; $F(2,41)=4.764$, $p<0.05$). Post hocs revealed that fluoxetine significantly increased locomotor behavior compared to VEH ($p<0.05$), but there was no significant increase in activity following treatment with pBBG.

In a separate cohort of mice, we examined the effect of 5 days of MeGFN treatment (5, 10 and 15 mg/kg/day) on female mice in the cFST. All three doses of MeGFN significantly reduced immobility in the cFST (**Figure 4.2e**; $F(3,55) =3.395$, $p<0.05$; post hocs all $p<0.05$).

There were no significant effects of 4 days of MeGFN treatment on center duration or locomotor activity in the OFT (**Figure S4.2b,d**; $F(3,56)=1.568$, $p>0.05$; $F(3,57)=0.24$, $p>0.05$).

CMS: CMS is a commonly used model of depression-like behavior that responds to chronic but not subchronic treatment with classical antidepressants (Cryan and Holmes 2005). Following CMS, we performed the sucrose preference test which is thought to model a common symptom of depression in humans, anhedonia. There was no significant effect of treatment with pBBG (10mg/kg/day) or MeGFN (10mg/kg/day) on sucrose preference (**Figure 4.3a**; $F(3,47)=0.546$ $p>0.05$) or total consumption (data not shown; $F(3,59)=1.858$ $p>0.05$). Because no difference was seen between the stressed and unstressed groups the results of the pBBG treatment on this test are uninterpretable.

However, there were significant effect of treatment with pBBG or MeGFN on the FST following CMS **(Figure 4.3b**; $F(3,52)=2.94$ $p<0.05$). Post-hoc tests revealed that stress increased immobility relative to unstressed mice ($p<0.05$), but there was no difference between unstressed mice and mice treated with either pBBG or MeGFN ($p>0.05$). There was also a significant effect of treatment with both GLO1 inhibitors on coat state (**Figure 4.3c-d**; $F(3, 59)=5.713$ $p<0.01$). Stress led to a significantly deteriorated coat (indicated by an increased score) compared to unstressed mice ($p<0.001$) which was rescued by treatment with either pBBG ($p<0.05$) or MeGFN ($p<0.05$). We also performed the splash test and found a significant effect of treatment on the number of grooming bouts (**Figure S4.3b**; $F(3,59)=3.194$ $p<0.05$). Post-hoc tests revealed that stressed mice had fewer bouts relative to unstressed mice ($p<0.05$), while there were no differences between unstressed mice and mice treated with either pBBG or MeGFN ($p>0.05$). However, there was no significant effect of stress or treatment with either GLO1 inhibitor on the total duration of grooming (**Figure S4.3a**; $F(3,56)=0.396$ $p>0.05$) or the latency to begin

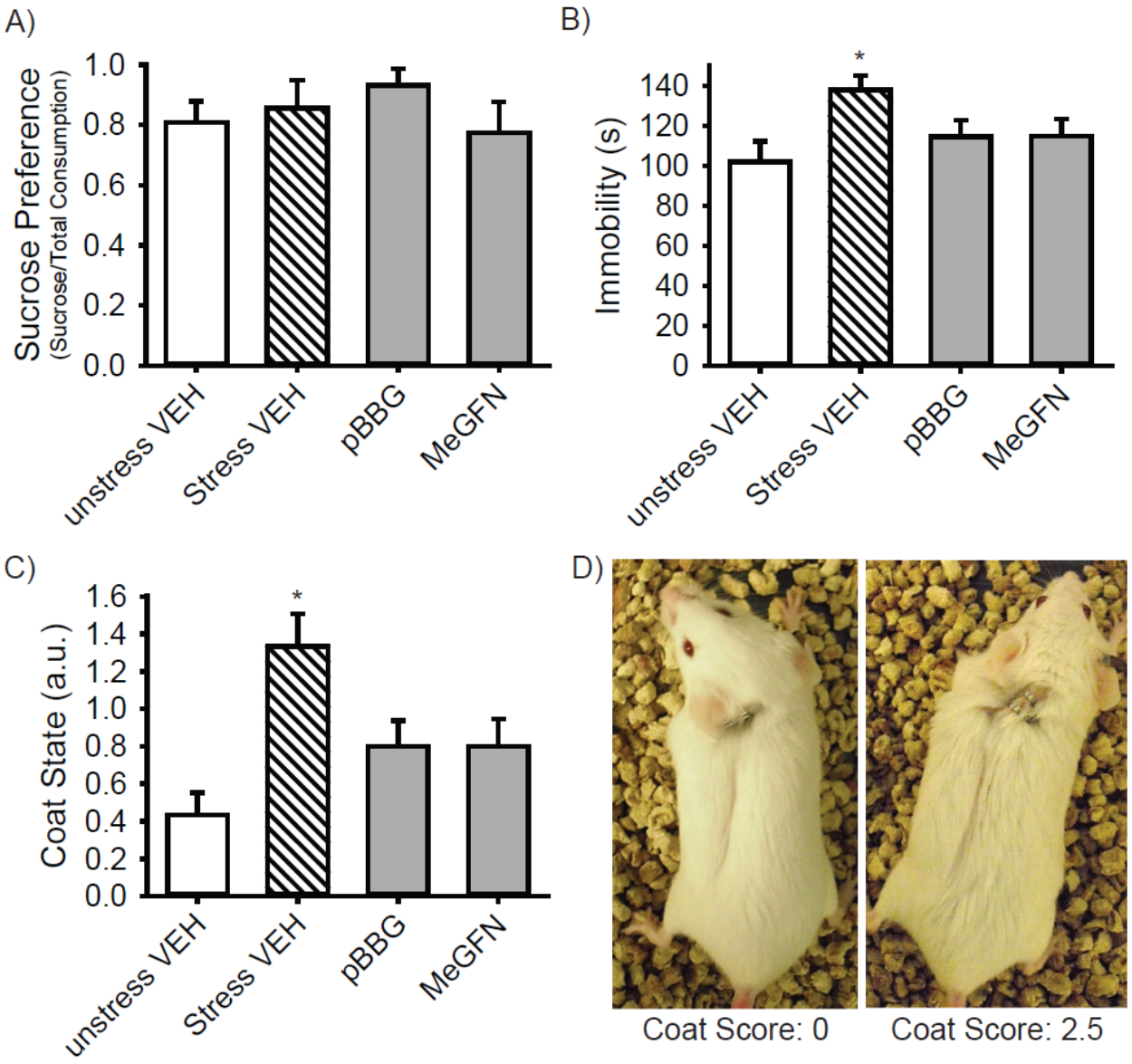

Figure 4.3 Chronic Mild Stress: Following 6 weeks of CMS, (a) there were no differences in sucrose preference between stressed and unstressed mice. However, CMS did induce increases in (b) immobility in the FST and (c) poor coat state that were rescued by 5 days of treatment with GLO1 inhibitors, pBBG and MeGFN; *n=13-15 per group *p<0.05*

grooming (**Figure S4.3c**; F(3,55)=1.914 p=0.139).

OBX: We found that pBBG reversed OBX induced hyperactivity in both male B6 and female BALB mice (**Figure 4.4a-b**). There was a significant interaction between OBX and treatment in male B6 mice (**Figure 4.4a**; F(1,47)=4.927p<0.05). Posthoc tests revealed a trend towards pBBG reducing locomotor hyperactivity in the OBX group (p=0.07), while there was no effect of pBBG in SHAM operated animals. There was also a significant interaction between OBX and pBBG treatment in female BALB mice (**Figure 4.4b**; interaction F(1,45)=4.506 p<0.05). Post hoc tests revealed that pBBG reduced immobility in the OBX group (p<0.01) but not in the SHAM SHAM group (p>0.05).

Western Blots: Finally, we examined whether pBBG treatment could upregulate BDNF and the ratio of pCREB to CREB (pCREB/CREB) in the hippocampus and mPFC, which are associated with antidepressant onset (Duman and Voleti 2012; Browne and Lucki 2013; Opal et al. 2013). There was a significant effect of treatment on BDNF expression in the hippocampus (**Figure 4.5a**; F(2,27)=3.87, p<0.05) and the mPFC (**Figure 4.5b**; F(2, 29)=7.577, p<0.01). Post hoc tests revealed that pBBG significantly upregulated BDNF in both mPFC and hippocampus in comparison to VEH (mPFC: p<0.01; hippocampus: p<0.05). There was also a significant effect of treatment on the ratio of pCREB/CREB in the hippocampus (**Figure 4.5c**; F(2,29)=3.781; p<0.05). Post hoc tests revealed that pBBG significantly upregulated pCREB/CREB in comparison to VEH (p<0.05) and showed a non-significant trend towards upregulation compared to FLX (p=0.079). There was no significant effect of treatment on pCREB/CREB within the mPFC (**Figure 4.5d** p=0.176).

4.5 Discussion

These results suggest that inhibition of GLO1 has anti-depressant like effects in multiple

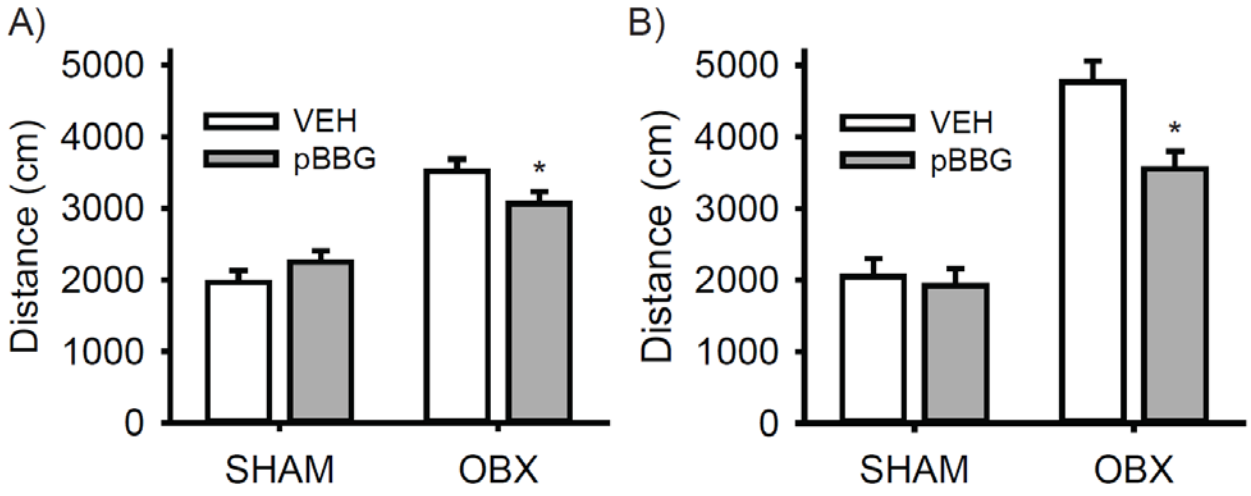

Figure 4.4 OBX: OBX induces hyperactivity that is reduced by 5 day pBBG treatment in (a) male B6 mice and (b) female BALB mice. *n=11-14 *p<0.05*

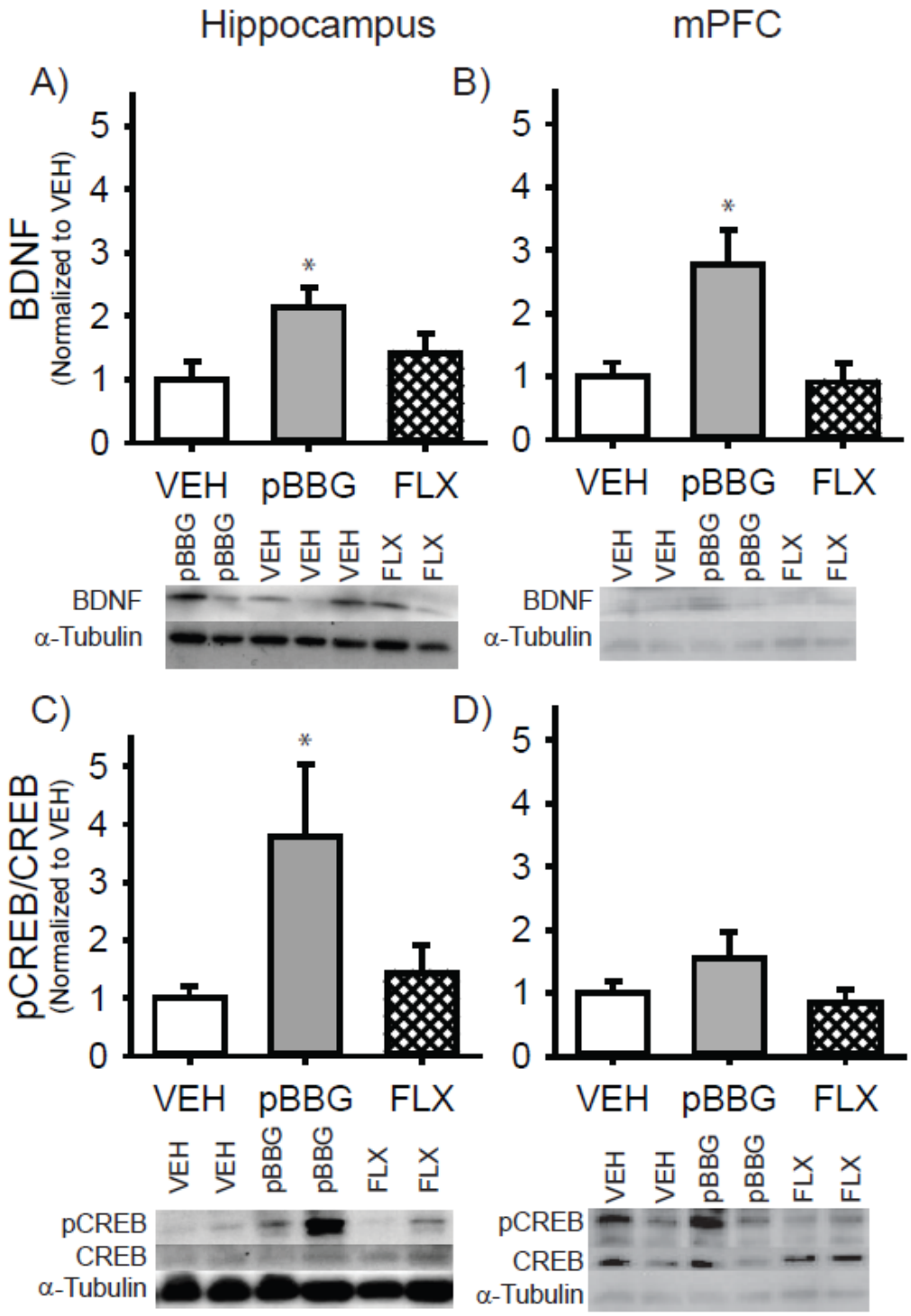

Figure 4.5. Western Blots. 5 day treatment with GLO1 inhibitor, pBBG, but not fluoxetine increases proteins associated with antidepressant onset. BDNF was upregulated in (A) hippocampus and (B) mPFC. pCREB/CREB was upregulated in (C) hippocampus, but not (D) mPFC. *n=10 per group; *p<0.05*

acute and chronic preclinical paradigms. Importantly we used both genetic and pharmacological tools to inhibit GLO1. Moreover, we used two chemically distinct GLO1 inhibitors. Based on these convergent results we conclude that the effects are not due to non-specific effects of these treatments. Both pharmacological and genetic GLO1 inhibition reduced immobility in both the TST and FST in male and female mice from multiple different inbred strains. These high-throughput acute screens have high predictive validity and are commonly used to screen for compounds with antidepressant activity (Cryan et al. 2005; Petit-Demouliere et al. 2005). These findings are consistent with a previous study that found a positive association between GLO1 expression and immobility in the TST (Benton et al. 2012b). We also examined three behavioral paradigms that are designed to assess chronic anti-depressant like effects. The cFST allowed us to investigate the speed of onset of GLO1 inhibition. Whereas the prototypic SSRIs fluoxetine required 14 days to reduce immobility in the cFST, both pBBG and MeGFN were effective after 5 days of treatment, suggesting that GLO1 inhibitors might be fast acting antidepressants. We also used CMS to examine the effects of chronic GLO1 inhibition. Five days of treatment with pBBG and MeGFN reduced immobility and improved coat state following 6 weeks of CMS, though neither altered sucrose drinking, which is commonly used to measure anhedonia following CMS (Willner 1997; Cryan et al. 2002). Finally, 5 days of treatment with pBBG reduced the locomotor hyperactivity induced by OBX. These results suggest that GLO1 inhibition has anti-depressant-like effects.

GLO1 inhibition was effective in the cFST, CMS and OBX models after only 5 days of treatment. Tricyclic and SSRIs antidepressants require 14 days of treatment before they become effective in these tests (Figure 4. 2b-d) (Cryan et al. 2002; Cryan and Holmes 2005; Dulawa and Hen 2005; Opal et al. 2013). Additionally, we observed an upregulation of BDNF and

pCREB/CREB after just 5 days of pBBG treatment, whereas similar upregulation requires 14 days of treatment with fluoxetine (Duman and Voleti 2012; Browne and Lucki 2013; Opal et al. 2013). These observations were consistent with previous study that found incubation with rat hippocampal cultures with MG upregulated BDNF expression (Di Loreto et al. 2008). Increased BDNF in mPFC and hippocampus is associated with an antidepressant-like response in behavioral models of depression; deletion or blockade of BDNF prevents antidepressant efficacy (Shirayama et al. 2002; Duman and Voleti 2012; Browne and Lucki 2013). Taken together, these data suggest that in addition to having antidepressant like activity, GLO1 inhibition has a faster onset than fluoxetine.

We hypothesize that GLO1 inhibition reduces depression-like behavior through increases in MG, which is an endogenously produced competitive partial agonist at GABA-A receptors; but no data presented in this paper directly tests this hypothesis. If correct, it is in contrast to a significant literature suggesting that other GABA-A acting compounds (i.e. barbiturates and benzodiazepines) are not effective for the treatment of depression in humans (Barbui et al. 2011) and do not typically show antidepressant-like effects in animal models of depression (Willner 1997; Cryan et al. 2002; Cryan et al. 2005; Petit-Demouliere et al. 2005). We saw no evidence of locomotor depressant effects of in GLO1KD mice or in mice treated with pBBG or MeGFN. It is important to note that antidepressant effects are characterized by increased activity in TST, FST and cFST, but by decreased locomotor hyperactivity in OBX; thus, nonspecific locomotor stimulant or depressant effects could not easily explain our observations. Our hypothesis that the action of MG at GABA-A receptors is responsible for the observed antidepressant effects is consistent with a recent study in humans that showed that co-administration of fluoxetine with eszopiclone (preferential GABA-A partial agonist at $\alpha 1$, $\alpha 2$ and $\alpha 3$ subtypes) has greater

antidepressant effects than fluoxetine alone (Fava et al., 2011). Additionally, a more recent study found that α5-selective negative modulators of GABA-A receptors show fast-acting antidepressant-like effects in rats (Fischell et al. 2015). Our data may provide further evidence that modulation of GABA-A signaling may be a promising approach for the development of novel antidepressants or as a means of augmenting the effects of classical antidepressants.

Anxiety and depression are highly comorbid, show shared genetic liability, and are treated with some of the same drugs (Kent et al. 2002; Kendler et al. 2007b; Demirkan et al. 2011). We have previously shown that genetic and pharmacological GLO1 inhibition, as well as MG administration are anxiolytic (Distler et al 2012). These same manipulation have also been shown to have anti-seizure effects (Distler et al. 2013). The current results show that GLO1 inhibition might provide a unique strategy for treating both depression with comorbid anxiety and epilepsy, which would constitute a unique class of therapeutic compounds. Inhibition of GLO1 is predicted to increase MG concentrations and thus GABA-A activation, in proportion to the amount of local glycolysis. Because MG is a competitive partial agonists at GABA-A receptors, GLO1 inhibitors may have qualitatively different effects as compared to all other GABA-A acting compounds; these differences may increase or decrease the therapeutic potential of GLO1 inhibition (Distler and Palmer 2012; McMurray et al. 2014).

4.6 Acknowledgements

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4.7 Supplemental Material

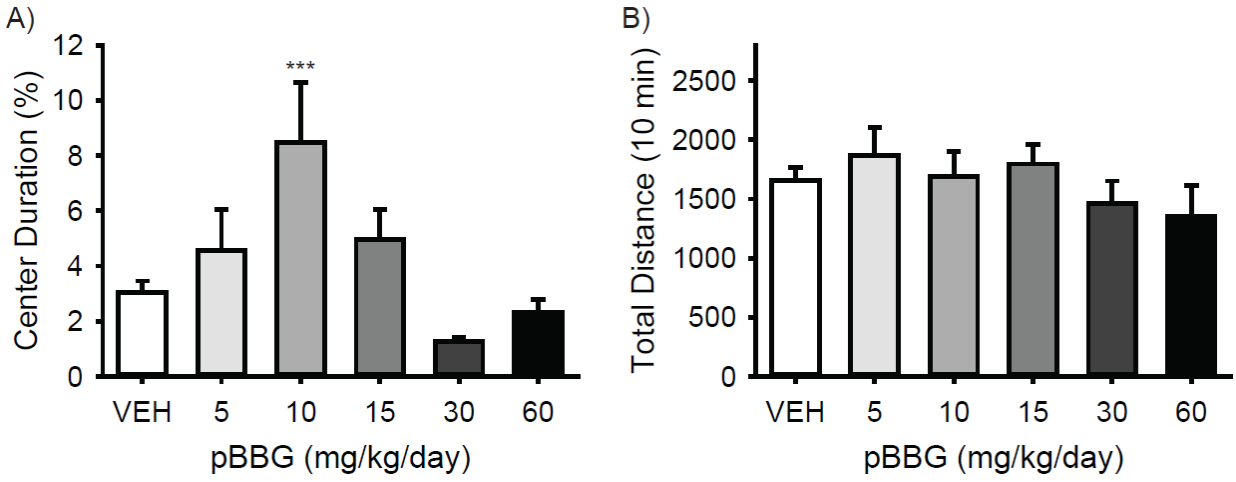

Figure S4.1 OFT (12 day). 12 Day treatment with GLO1 inhibitors (a) increases center duration and (b) has no effect on total distance traveled. n per group: veh=42, 5=14, 10=15, 15=25, 30=12, 60=10; *$p<0.05$.

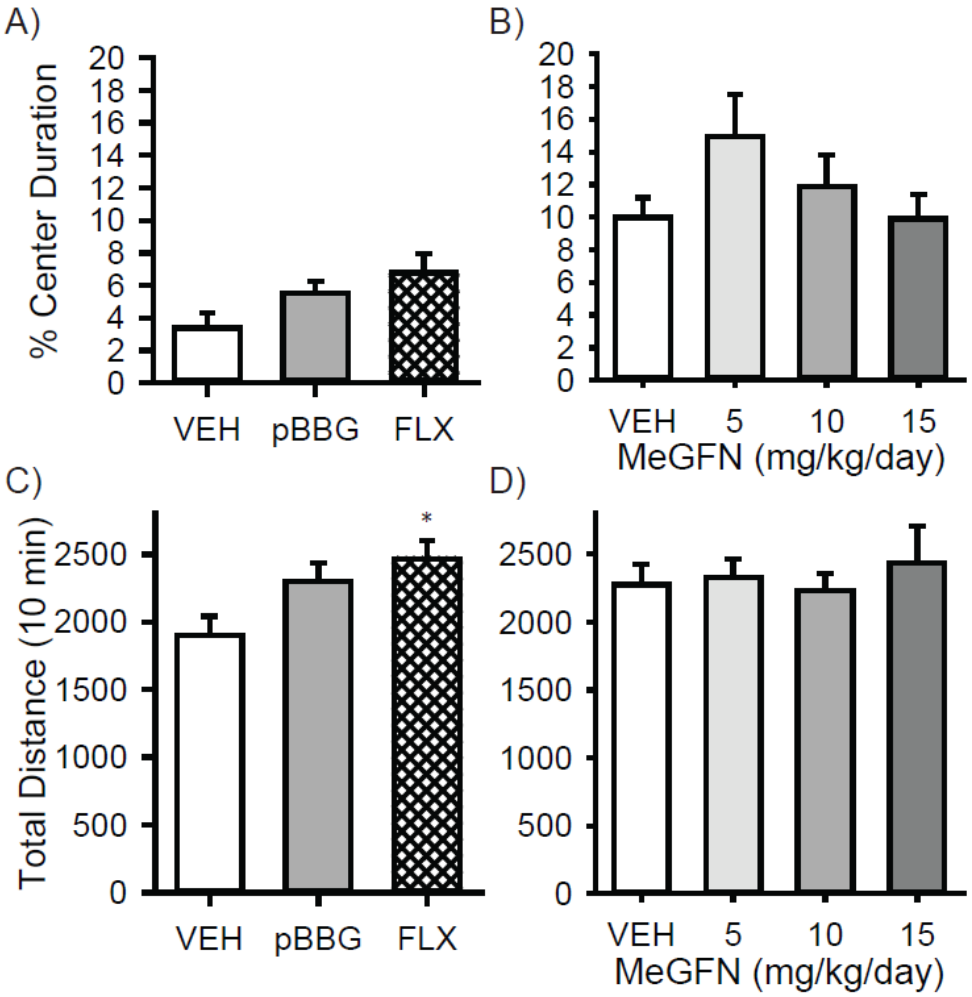

Figure S4.2 OFT (4 day) There were no significant effects of 4 Day treatment with (a) pBBG or fluoxetine (FLX) or (b) MeGFN on % center duration in the OFT (10 minutes). There was a significant effect of (c) fluoxetine, but not pBBG or (d) MeGFN on total distance traveled in the OFT n=12-15 per group, *$p<0.05$

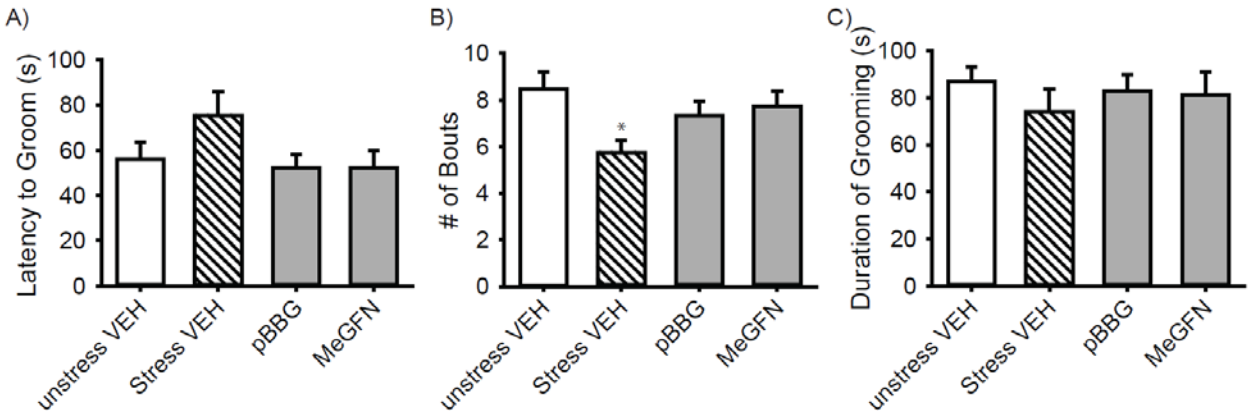

Figure S4.3 Splash Test: Following CMS and subchronic (5 days) treatment with GLO1 inhibitor *(*a) stress did not alter latency to groom. (b) While stress significantly decreased the number of grooming bouts, (c) there was no effect on the total duration of grooming. *n=13-15 per group; *p<0.05 compared to unstress VEH*

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Summary

This thesis has focused on the role of GLO1 in mouse models of anxiety, depression and alcohol use disorders. These psychiatric disorders are highly comorbid, have considerable shared genetic liability and the development of each disorder subsequently confers higher risk for development of the others (Driessen et al. 2001; Grant et al. 2004; Kessler et al. 2005b; Kendler et al. 2007b; Smith and Book 2010; Boschloo et al. 2011). Thus, pharmacotherapies that could be used to treat any or all of these disorders would be particularly appealing and could lead to advancements in our understanding of the underlying pathology.

The goal of chapter 2 was to build on previous studies in the lab and elsewhere that found associations between anxiety-like behavior and increased *Glo1* expression in mice (Hovatta et al. 2005b; Williams et al. 2009; Benton et al. 2012a; Distler et al. 2012b). Further, we wanted to begin characterizing GLO1's substrate, MG, which is a competitive partial agonist at GABA-A receptors by comparing MG to other anxiolytic pharmaceuticals that target GABA-A receptors within the BLA (Kent et al. 2002; Heldt and Ressler 2006; Möhler 2012a; Distler et al. 2012b). In this study we used mice that overexpress *Glo1* only in neurons to determine that limiting overexpression to the brain is sufficient to increase anxiety-like behavior. Additionally, we found that microinjection of MG into the BLA was sufficient to reduce anxiety-like behavior and that it did so to a similar extent as a positive control midazolam (benzodiazepine). These studies support the idea that *Glo1* overexpression increases anxiety-like behaviors through actions within the brain and that MG administration can reduce anxiety-like behavior through typical neuroanatomy associated with anxiety (BLA).

In chapter 3, we determined that GLO1 can regulate EtOH consumption in mice using a model of binge drinking, DID. Overexpression of *Glo1* increased EtOH consumption in DID, while GLO1 inhibition by knockdown of *Glo1* or the pharmacological inhibitor, pBBG, reduced EtOH consumption in DID. In an effort to determine the mechanism by which GLO1 inhibition reduces EtOH consumption we also investigate additional phenotypes known to be altered by EtOH administration. Though GABA-A receptor agonists or positive modulators like muscimol or diazepam increase sedation in LORR and ataxia on the balance beam (Liljequist and Engel 1982; Kumar et al. 2012; Milić et al. 2012), we saw no differences in the response to EtOH in either test when mice either overexpressed *Glo1* or were treated with GLO1 inhibitors. These data suggested that GLO1 inhibition was not reducing EtOH consumption through increases in the less rewarding or potential aversive consequences of EtOH consumption like sedation or ataxia.

In chapter 4, we evaluated the therapeutic potential of GLO1 inhibitors for the treatment of depression. First, we saw that GLO1 inhibition reduced depression-like behavior in assays of antidepressant efficacy that respond to acute treatments with current antidepressants, the TST and FST. This was seen using both genetic knockdown and pharmacological inhibition by two different GLO1 inhibitors. Second, we saw that GLO1 inhibitors reduced depression-like behavior in models of antidepressant onset, chronicFST, CMS and OBX, that are sensitive to chronic (14 day) treatment with current antidepressants by 5 days. Finally, the GLO1 inhibitor, pBBG, was able to increase proteins associated with antidepressant onset, BDNF and pCREB/CREB in hippocampus and BDNF in mPFC. Together, these data strongly suggest that GLO1 inhibitors are a novel class of fast-acting antidepressants.

While the primary purpose of this thesis was to investigate the role of GLO1 in each

disorder individually, the ability of GLO1 to alter behaviors in multiple mouse models of each of these disorders supports the idea that all three disorders are highly associated with each other. **Figure 5.1** illustrates the general interrelated nature of anxiety, depression and alcohol use disorders and highlights both the shared role of GLO1 in each of these disorders and the potential therapeutic value of targeting GLO1 for treatment.

5.5 Limitations and Future Directions:

One limitation of the studies in chapter 2 was the lack of specificity achieved with the FLOXGlo1KI mice that overexpress *Glo1* in the presence of CRE. Anxiety-like behaviors are generally associated neuroanatomically with the amygdala (Davis 1992; Heldt and Ressler 2006), yet currently there are no mice that express CRE using promoters that are specific to the amygdala. In order to address this lack of specificity we chose to microinject MG directly into BLA which reduces anxiety-like behavior. However, a corresponding study overexpressing *Glo1* only in amygdala would complement our previous work. In order to better target the amygdala and other neuroanatomical regions associated with anxiety-like behavior such as the bed nucleus of the stria terminalis or mPFC, future studies could use viral vectors to drive CRE expression specifically in these regions (Tye and Deisseroth 2012; Kim et al. 2013). It may also be interesting to determine if overexpressing *Glo1* at different time points during development has different effects on anxiety-like behavior using an inducible CRE mouse.

In chapter 3, lingering questions about the mechanism by which GLO1 inhibition reduces EtOH consumption led us to investigate an additional phenotype known to be altered by EtOH administration, locomotor behavior. While BECs are increasing, EtOH has been shown to elicit a stimulatory effect, which is thought to be rewarding/pleasurable (Liljequist and Engel 1982; Blednov et al. 2004; Spear and Varlinskaya 2010). Conversely, as BECs are decreasing, EtOH

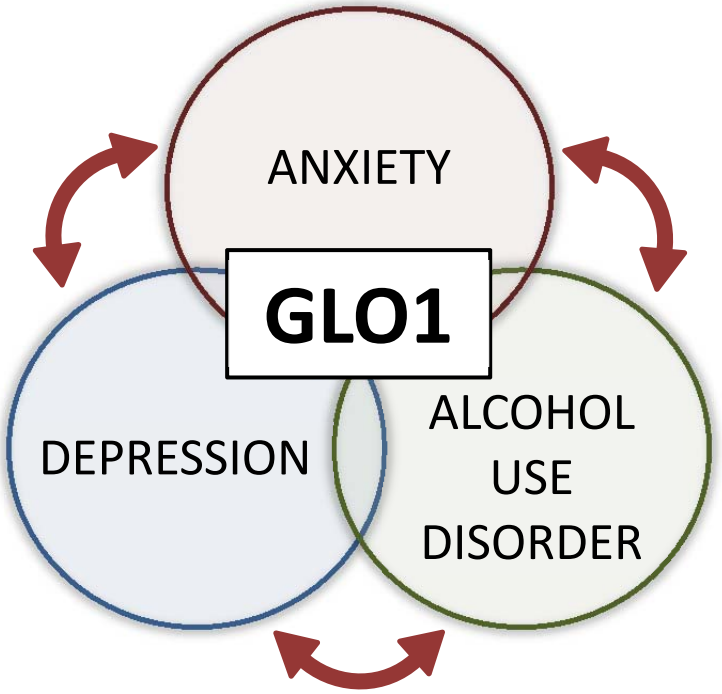

Figure 5.1. GLO1 is associated with anxiety, depression and alcohol use disorders. In addition to the shared role of GLO1 in all three disorders, these disorders are highly associated with each other. There are high incidences of comorbidity between any two disorders or all three together. There may be similar underlying pathology as the result of shared genetic liability and associated risk factors. Additionally, each disorder interacts with the other and symptoms of one disorder can potentially exacerbate symptoms of the other leading to relapse.

elicits a sedative effect which is thought to be more aversive (Spear and Varlinskaya 2010). People who experience a greater stimulatory effect from EtOH consumption at their first EtOH experience are at higher risk for later developing AUDs and insensitivity to the more adverse effects of EtOH is associated with increased EtOH consumption in rats (Doremus-Fitzwater et al. 2010; Spear and Varlinskaya 2010). In mice, EtOH induces a stimulatory effect on locomotor behavior at lower doses of EtOH administration (1-2g/kg), while higher doses of EtOH administration result in locomotor depression (2.5+g/kg) (Liljequist and Engel 1982; Blednov et al. 2004). EtOH is thought to mediate these effects through actions at GABA-A receptors (Liljequist and Engel 1982). As MG is a competitive partial agonist at GABA-A receptors, one lingering question following the studies investigating the regulation of EtOH consumption by GLO1 was whether pBBG interacted with EtOH to alter locomotor behavior in the OFT.

Two independent follow up studies were performed to investigate this interaction. First, mice were pretreated with either VEH, 6.25 or 50mg/kg pBBG 2 hours before testing and were then injected with either saline or 2g/kg EtOH and immediately placed into the OFT for 50 minutes. There was a significant interaction between pBBG pretreatment and EtOH treatment (**Figure 5.2a**; $F(2,74)=6.985$, $p<0.01$). While there were no significant effects of pBBG treatment in saline treated mice, both 6.25 and 50mg/kg pBBG were significantly less active compared to VEH treatment in EtOH treated mice (6.26 mg/kg $p<0.05$, 50mg/kg $p<0.01$). There was a non-significant trend towards an increase in activity in 50mg/kg pBBG-treated mice receiving saline (50 pBBG) compared to 50mg/kg pBBG-treated mice receiving EtOH (50 pBBG + 2 EtOH). These data suggest that pBBG blocks the stimulatory effects of 2g/kg EtOH.

In the second study, we wanted to assess the interaction between 6.25mg/kg pBBG and either 1g/kg or 2.5 g/kg EtOH to establish a dose response curve. Mice received preinjections of

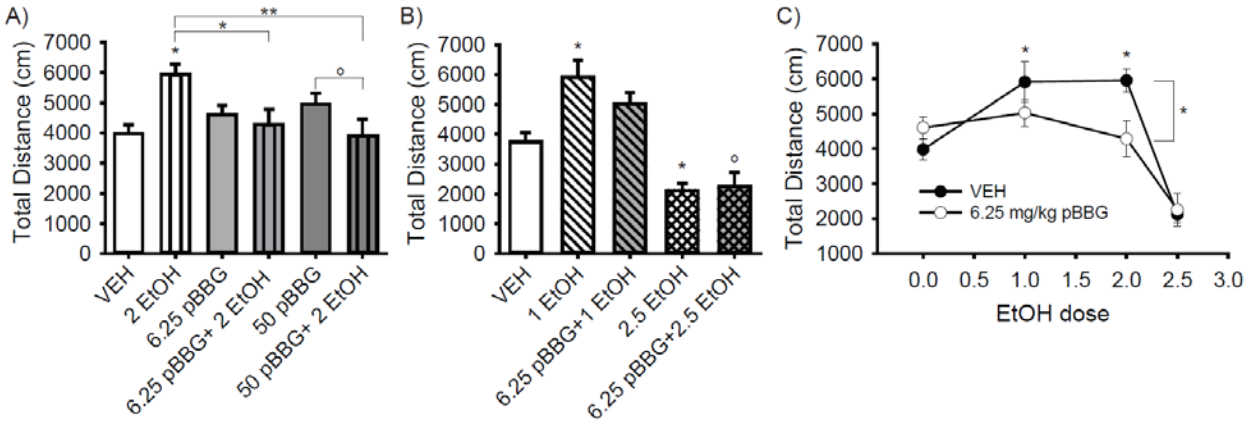

Figure 5.2 pBBG interacts with EtOH to blunt the stimulatory effects of low doses of EtOH, but shows no effect on the locomotor depressant effects of high doses of EtOH. (A) both 6.25mg/kg pBBG and 50 mg/kg pBBG 2 hours before testing prevent the stimulatory effect of 2g/kg EtOH. (B) 6.25 mg/kg pBBG is also able to blunt the stimulatory effect of 1g/kg EtOH, but was not able to reduce the locomotor depressant effect of 2.5g/kg EtOH. (C) Locomotor data from animals receiving either VEH or 6.25 mg/kg pBBG from panels 'a' and 'b' were plotted as a function of increasing EtOH dose to illustrate that pBBG alters the dose response curve. Locomotor activity is reduced in EtOH + pBBG treated mice relative to EtOH + VEH treated mice at EtOH doses that are stimulatory, but not depressant.

either VEH or 6.25 mg/kg pBBG. Then mice received injections of saline, 1g/kg EtOH or 2.5 g/kg EtOH immediately prior to placement in the OFT for 50 minutes. As we previously saw no effect of 6.25mg/kg pBBG pretreatment on locomotor behavior, we did not have a group receiving 6.25mg/kg pBBG and no EtOH. There was an overall effect of treatment on locomotor behavior (**Figure 5.2b**; $F(4,57)=15.944$, $p<0.001$). Posthoc tests revealed that 1g/kg EtOH significantly increased locomotor behavior compared to saline, while there was no significant difference between saline and 6.25 mg/kg pBBG + 1 g/kg EtOH. Conversely, the larger dose of EtOH (2.5g/kg) significantly reduced locomotor behavior ($p<0.05$), while pBBG plus the larger dose of EtOH (6.25 mg/kg pBBG + 2.5 g/kg EtOH) showed a non-significant trend towards decreasing locomotor behavior compared to saline ($p=0.053$). These data again suggested that pBBG can block the stimulatory effect of EtOH, but that there is no effect of pBBG on the locomotor depressant effects of EtOH.

Figure 5.2c illustrates the difference in dose response curves when 6.25mg/kg pBBG is given in conjunction with increasing EtOH doses compared to increasing EtOH dose alone. pBBG administration seems to blunt the stimulating effects of EtOH administration, while pBBG seems to have no effect on the locomotor depressant effects of higher doses of EtOH. These studies begin to point to a mechanism by which GLO1 inhibition reduces EtOH consumption, though future studies will be needed to better elucidate and clarify these differences.

In chapter 4, GLO1 inhibitors showed antidepressant-like effects in multiple models of depression-like behavior. We hypothesize that the mechanisms by which GLO1 inhibition reduces depression-like behavior rely on subsequent increases in MG concentrations and the actions of MG on GABA-A receptors. However, further studies are needed to elucidate the particular effects of GLO1 inhibitors and MG on GABA-A receptors that lead to antidepressant-

like effects because the molecular pharmacology is relatively uncharacterized due to the novelty of these results. It may be important to assess additional molecular markers of antidepressant onset, such as GSK3B or mTOR or to investigate downstream effects on the regulation of AMPA, NMDA or GABA-A receptors in particular neuroanatomical regions that may indicate effects on excitatory or inhibitory drive (Machado-Vieira et al. 2008a; Willner et al. 2013; Martinowich et al. 2013; Russo and Nestler 2013).

GLO1 inhibitors are likely to show a very distinct pharmacological profile. This is first because the effects of GLO1 inhibition will be regulated by the specific neuroanatomical distribution of MG production that is likely to vary by regional activity. Another distinguishing feature of MG is that MG is cell permeable, and as such, it is possible that MG preferentially acts at extrasynaptic GABA-A receptors where concentrations of GABA are low. As a competitive partial agonist, MG may also show a particularly unique pharmacological profile that is dependent on both the concentration of MG and other agonists or modulators of GABA-A receptors. For example, if concentrations of MG are high and concentrations of GABA are low, MG may act primarily as an agonist and result in increased hyperpolarization. In contrast, if concentrations of both MG and GABA are high then MG will compete with GABA and result in a reduced hyperpolarization of the cell. Therefore, MG may increase or decrease inhibitory drive in a region-specific and concentration-dependent manner.

Future studies are needed to better elucidate the mechanisms by which GLO1 and MG alter behavior. First, it is particularly important to develop a reliable assay to measure MG concentrations in brain. In our hands, HPLC assays have shown high variability and while relative differences in tissue concentrations were measurable, absolute concentrations were more difficult to ascertain (Distler et al. 2012b). Yet in order to better understand the role of MG in

both normal and diseased states, the ability to quantify MG is very important. For example, as MG is a byproduct of glycolysis, it is likely that concentrations of MG increase following consumption of sugars (Brownlee 2001; Ahmed and Thornalley 2007). Food intake induces a hedonic or satiated state that is associated with reduced anxiety and thought to be involved in the development and maintenance of obesity (Sarker et al. 2013). While many complex factors contribute to these effects, it is possible that increases in MG following food intake could also contribute to this hedonic response. Thus, quantifying increases in MG following sugar intake and determining the neuroanatomical distribution of these increases could elucidate a role of MG in eating behaviors. This may be particularly interesting in the context of diabetes, as diabetes is associated with high levels of plasma MG, but also a high rate of anxiety disorders (Brownlee 2001; Matafome et al. 2012; Skapare et al. 2013; Ducat et al. 2014) that could point to a dysfunctional regulation of this system.

It will also be interesting to assess alterations in MG concentrations within different regions in the brain following chronic GLO1 inhibitor treatment, EtOH administration or concurrent EtOH and pBBG administration. In studies using chronic or subchronic GLO1 inhibitor treatment, determining the level of increase in MG concentration in discrete neuroanatomical regions could add to our understanding of the neurobiology of depression by highlighting regions of interest. As discussed in chapter 3, MG is found in many alcoholic beverages and may increase following EtOH consumption through either endogenous production of exogenous ingestion (Nemet et al. 2006; Angeloni et al. 2014; Ojeda et al. 2014). Therefore, increases in MG concentrations following EtOH consumption may provide negative feedback on alcohol consumption. Quantifying MG following EtOH consumption or administration could determine whether concentrations of MG reach pharmacological levels that could alter further

EtOH consumption. Quantifying MG following co-administration of EtOH and pBBG could also add to our understanding of the mechanisms by which pBBG alters EtOH consumption. More specifically, our recent findings discussed within this chapter suggest that pBBG blunts the stimulatory effects of low doses of EtOH, but not the depressant-like effects (sedation/ataxia) of higher doses of EtOH. These results could be the result of pBBG increasing MG concentrations to different levels within different anatomical regions. It may therefore be important to quantify concentrations of MG in regions associated with these effects (e.g. striatum or cerebellum) to elucidate the mechanisms by which pBBG reduces EtOH consumption.

Neuroanatomical regions and neuronal populations differ in the distribution and incidence of GABA-A receptor subunit conformations which may explain the distinct behavioral effects of subunit specific GABA-A receptor modulators (Marowsky et al. 2004; Möhler 2012a; Blednov et al. 2014). Thus, differences in the sensitivity or response to MG by specific GABA-A receptor subtypes will contribute to MG's particular pharmacological profile. Further electrophysiological studies are therefore needed to determine the subunit specificity of MG. Additional studies could determine whether specific subunits are required for the effects of GLO1 inhibitors on depression-like, anxiety-like or EtOH consumption by using subunit specific knockdown mice to determine interactions between genotypes and treatment. In addition to adding to our understanding of mechanism by which MG alters behavior, this information may be particularly helpful in assessing the therapeutic efficacy of GLO1 inhibitors by predicting putative side effects of increases in MG following chronic pBBG administration. For example, $\alpha 1$ containing-receptors are sedating, but $\alpha 2/\alpha 3$ partial agonists at GABA-A receptors are non-sedating and involved in the regulation of dendritic maturation and adult neurogenesis that has been associated with antidepressant efficacy (Malberg et al. 2000; Earnheart et al. 2007; Luscher et al.

2011; Möhler 2012).

Although mounting evidence shows that GLO1 inhibitors may have applications in the treatment of anxiety, depression and alcohol use disorders, a negative correlation was observed between *Glo1* copy number and sensitivity to neuropathic pain in diabetic mice (Jack et al. 2011; Jack et al. 2012). Subsequent mechanistic studies demonstrated that overexpression of human GLO1 reduced hyperalgesia in diabetic mice (Bierhaus et al. 2012). Although a correlation between a SNP in *Glo1* and diabetic neuropathy among type 2 diabetics has been reported in humans, the effect was not statistically significant when corrected for multiple comparisons (Groener et al. 2013). However, recent work has demonstrated decreased GLO1 activity in patients with painful diabetic neuropathy as compared to those with painless diabetic neuropathy, suggesting a role for GLO1 in pain (Skapare et al. 2013). The mechanism of MG-induced hyperalgesia has been attributed to protein modification and activation of TRPA1 receptors (Bierhaus et al. 2012; Andersson et al. 2013). Such studies underscore the need to assess the potential cytotoxic consequences of GLO1 inhibition and suggest that GLO1 inhibitors may be contraindicated in diabetic patients (Bierhaus et al. 2012; Andersson et al. 2013).

Current GLO1 inhibitors, such as pBBG, have most frequently been based on the glutathione scaffold and have been patented for a variety of disorders (Vince et al. 1971; Lo and Thornalley 1992; Murthy et al. 1994; Hamilton et al. 1999; More and Vince 2009). MeGFN, flavonoids, curcumin and other non-peptidic reagents have also been evaluated for their GLO1 inhibitory activity (Thornalley et al. 1996; Santel et al. 2008; Takasawa et al. 2008; Kawatani et al. 2008; Shehzad et al. 2010; Chiba et al. 2012; Kanoh et al. 2013). Although these compounds generally inhibit GLO1 activity with therapeutically useful K_i, utilizing native structures such as glutathione as a scaffold *a priori* increases the risk of interaction with other signaling pathways

and could result in undesired off-target effects or limited bioavailability (Takasawa et al. 2008; Shehzad et al. 2010; Gupta et al. 2013). Poor cell permeability has also hampered the utility of some glutathione analogs and flavonoids *in vitro*, while poor absorption and bioavailability have limited the success of curcumin in human trials (Thornalley et al. 1996; Shehzad et al. 2010; Gupta et al. 2013). Further, many existing inhibitors of GLO1 were intended as anti-tumor agents and as such, have frequently been evaluated *in vitro* for their ability to inhibit cellular proliferation and induce apoptosis in tumor cells at high concentrations (Thornalley et al. 1996; Santel et al. 2008; Takasawa et al. 2008; Chiba et al. 2012). Thus, a key question is whether doses of GLO1 inhibitors can be identified produce therapeutic effects without also producing undesired effects such as increases in neuropathic pain. Identification or synthesis of novel GLO1 inhibitors could address the limitations of current inhibitors by reducing off target effects and minimizing side effects. Ultimately, the therapeutic viability of GLO1 inhibitors requires the identification of an inhibitor with excellent oral availability, a favorable pharmacokinetics and dynamics and negligible toxicity after chronic treatment.

5.6 Overview and Conclusions

The chapters within this thesis have implicated a role for GLO1 and MG in anxiety, depression and alcohol use disorders. Further, they have supported a strong therapeutic potential of GLO1 inhibitors for the treatment of these disorders. While effective in many cases, current drug therapies for neuropsychiatric disorders such as anxiety, depression and alcohol use disorders are plagued by confounding off-target effects and often carry a risk for addiction in patients, generating the need for novel pharmaceuticals to treat these debilitating disorders. Therapeutic treatment by GLO1 inhibition/MG accumulation would provide a pharmacological avenue for anxiety, depression and alcohol use disorders that is fundamentally distinct from the

current pharmacopeia, such as positive allosteric modulators of GABA-A receptors.

Thus, GLO1 inhibition has the potential to improve efficacy, reduce side effects and ultimately treat multiple highly comorbid disorders. While evidence in mice suggests that GLO1 inhibition alters behavior, concerns about neuropathic pain and cytotoxicity mandate further exploration and characterization of lead compounds to properly evaluate the therapeutic efficacy of GLO1 inhibitors for the treatment of neuropsychiatric disorders.

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