Pharmacological Inhibition of the G9a/GLP Histone Methyltransferase and Anxiety-related Behaviour in the Mouse

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology and Toxicology
University of Toronto

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ABSTRACT

Anxiety disorders are the most prevalent category of psychiatric diagnosis and are one of the leading causes of disability in developed countries. Several existing antidepressants with anti-anxiety properties have been shown to downregulate G9a, a methyltransferase that methylates lysine 9 of histone H3 (H3K9). Histone methylation regulates epigenetic responses to environmental stress. Thus we sought to investigate the role of G9a/GLP in regulating anxiety behaviour during adulthood and neurodevelopment. C57BL/6 mice were treated with G9a/GLP inhibitors, UNC0642 and A-366, at doses of 1mg/kg, 2mg/kg or 4mg/kg. In adult mice, both drugs decreased anxiety-related behaviours. Inhibition of G9a/GLP in utero increased anxiety-like behaviours and decreased social interaction in adulthood. These findings suggest that G9a/GLP inhibition has the potential to alleviate anxiety symptoms and could be pursued as a novel anti-anxiety drug target. Our data also provide evidence that epigenetic mechanisms can contribute to the development of anxiety disorders.
ACKNOWLEDGEMENTS

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Contents
ABSTRACT ......................................................................................................................... ii
ACKNOWLEDGEMENTS ...................................................................................................... iii
LIST OF ABBREVIATIONS ................................................................................................. vii
LIST OF TABLES ................................................................................................................ x
LIST OF FIGURES .............................................................................................................. xi
CHAPTER ONE – INTRODUCTION .................................................................................... 1
  1.1 - Statement of Problem ............................................................................................... 1
  1.2 - Objectives .................................................................................................................. 2
  1.3 - Hypotheses ................................................................................................................. 3
  1.4 - Clinical Features of Anxiety ..................................................................................... 4
  1.5 - Epidemiology of Anxiety .......................................................................................... 5
  1.6 - Mechanism of Anxiety ............................................................................................. 5
    1.6.1 - Low and High Road for Anxiety ....................................................................... 6
    1.6.2 – Amygdala ............................................................................................................ 6
    1.6.3 - Medial Pre-frontal Cortex ............................................................................... 7
    1.6.4 – Hippocampus ..................................................................................................... 8
    1.6.5 - Current Model of Anxiety ................................................................................. 8
  1.7 - Treatment of Anxiety .............................................................................................. 9
    1.7.1 – Psychotherapy .................................................................................................. 9
    1.7.2 – Benzodiazepines ............................................................................................ 10
    1.7.3 - Antidepressants ...............................................................................................10
  1.8 - Epigenetic Mechanisms ..........................................................................................12
    1.8.1 - DNA Methylation ............................................................................................13
    1.8.2 - Histone Modifications ....................................................................................16
  1.9 - Epigenetic Effect of Current Drugs Used in Psychiatry ..........................................20
  1.10 - G9a/GLP ..................................................................................................................21
    1.10.1 - G9a/GLP Has Diverse Biological Functions ............................................... 21
    1.10.2 - G9a/GLP Plays a Role in Anxiety ................................................................. 23
  1.11 - Drug Inhibitors of G9a/GLP ..................................................................................24
  1.12 - Animal Models ......................................................................................................25
    1.12.1 - Validities of Animal Models ......................................................................... 25
    1.12.3 - Types of Anxiety Models ..............................................................................27
CHAPTER TWO - MATERIALS AND METHODS ........................................................................37
2.1 - Animals ..................................................................................................................37
2.2 - Drugs and formulations .........................................................................................37
2.3 - Injection Schedule .................................................................................................37
2.4 - Behavioural Studies ..............................................................................................38
   2.4.1 - Elevated Zero Maze (EZM) .............................................................................38
   2.4.2 - Marble Burying ...............................................................................................39
   2.4.3 - Novelty-suppressed Feeding (NSF) .................................................................39
   2.4.4 - Crawley’s Three-chambered Social Interaction Test ......................................40
   2.4.5 - Effect of UNC0642 and A-366 on histone methylation ..................................40
   2.4.6 - Statistical Analysis .........................................................................................41

CHAPTER THREE – RESULTS..............................................................................................44
3.1 - The Effect of Chronic UNC0642 Injections on Anxiety-related Behaviours in Adult Mice ..........................................................44
   3.1.1 - Time Spent in the Open EZM Areas .................................................................44
   3.1.2 – Number of Marbles Buried ..............................................................................45
   3.1.3 - Latency to Feed in the Novel Environment after Food Deprivation ..............46
   3.1.4 – Whole Brain H3K9me2 Levels .....................................................................47
3.2 – The Effect of Chronic A-366 Injection on Anxiety-Related Behaviours in Adult Mice .................................................................49
   3.2.1 - Time Spent in the Open EZM Areas .................................................................49
   3.2.2 – Number of Marbles Buried ..............................................................................50
   3.2.3 - Latency to Feed in Novel Environment ...........................................................51
   3.2.4 – Whole Brain H3K9me2 Levels .....................................................................52
3.3 – The Effect of In Utero Exposure to UNC0642 on Anxiety-related Behaviour in Adulthood .................................................................53
   3.3.1 - Time Spent in the Open EZM Areas .................................................................53
   3.3.2 - Number of Marbles Buried ..............................................................................54
   3.3.3 - Crawley’s Three-Chambered Social Interaction Test ......................................55
   3.3.4 – The Whole Brain H3K9me2 Levels ..................................................................56
3.4 The effect of G9a/GLP Inhibition on Locomotion ....................................................58
CHAPTER FOUR – DISCUSSION ........................................................................................................61
4.1 Effect of G9a/GLP Inhibition on Anxiety-related Behaviours in Adulthood ..................61
  4.1.1 - G9a/GLP is Modulated by Chronic Social Defeat ..............................................63
  4.1.2 - Genome-wide Analysis of Histone Methylation Changes ...............................64
  4.1.3 - Potential Mechanisms for Unconditioned Anxiety ...........................................68
  4.1.4. - Potential Mechanisms for Conditioned Anxiety ..............................................68
  4.1.5 - The Effect of Venlafaxine on H3K9me2 .............................................................69
4.2 - *In Utero* Exposure of UNC0642 in Mice Increased Anxiety-Related Behaviours in Later life ..........................................................69
  4.2.1 - Alternative Interpretations .................................................................................70
4.3 - Future Experiments .................................................................................................72
  4.3.1 - Conditioned Models of Anxiety .........................................................................72
  4.3.2 – Molecular Studies ..............................................................................................72
  4.3.3 - *In Utero* Exposure to UNC0642 ....................................................................73
4.4 - Limitations ..............................................................................................................73
4.5 - Summary and Conclusion ......................................................................................75
REFERENCES .....................................................................................................................77
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5hmC</td>
<td>5'- hydroxymethylcytosine</td>
</tr>
<tr>
<td>5mC</td>
<td>5' Methylated Cytosine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
</tr>
<tr>
<td>CAMH</td>
<td>Centre for Addiction and Mental Health</td>
</tr>
<tr>
<td>CBT</td>
<td>Cognitive Behaviour Therapy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA Methyltransferase</td>
</tr>
<tr>
<td>DSM-V</td>
<td>Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition</td>
</tr>
<tr>
<td>E9.5</td>
<td>Embryonic day 9.5</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal–Regulated Kinases</td>
</tr>
<tr>
<td>EZM</td>
<td>Elevated Zero Maze</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Generalized Anxiety Disorder</td>
</tr>
<tr>
<td>GLP</td>
<td>G9a-Like Protein</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Histone 3 Lysine 27 Trimethylation</td>
</tr>
<tr>
<td>H3K36me3</td>
<td>Histone 3 Lysine 36 Trimethylation</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Histone 3 Lysine 4 Trimethylation</td>
</tr>
<tr>
<td>H3K9</td>
<td>Histone 3 Lysine 9</td>
</tr>
</tbody>
</table>
H3K9me2  Histone 3 Lysine 9 Dimethylation
HAT     Histone Acetyltransferases
HDAC    Histone Deacetylases
HMT     Histone Methyltransferases
HPA     Hypothalamus-Pituitary Axis
LG      Licking and Grooming
MAOI    Monoamine Oxidase Inhibitor
MAPK    Mitogen-Activated Protein Kinases
mPFC    medial Pre-Frontal Cortex
NAc     Nucleus Accumbens
NIH     National Institute of Health
PFC     Pre-Frontal Cortex
PTSD    Post-Traumatic Stress Disorder
RCT     Randomized Control Trial
REST    RE1-Silencing Transcription
SAD     social anxiety disorder
SAM     Sadenosylmethionine
SERT    Serotonin Transporter
SNRI    Serotonin Norepinephrine Reuptake Inhibitor
SP      Specific Phobia
SSRI    Selective Serotonin Reuptake Inhibitor
TCA     Tricyclic Antidepressant
TET     Ten-Eleven-Translocation
LIST OF TABLES

**Table 1.1**  Physiological Symptoms of Anxiety

**Table 1.2**  The Effect of Histone Methylation on Gene Expression

**Table 1.3**  Behavioural Tests for Measuring Anxiety-related Behaviours in Mice

**Table 4.1**  Genes with the Same H3K9me2 Changes after Chronic Social Defeat and Social Isolation

**Table 4.2**  Summary of Main Findings
LIST OF FIGURES

Figure 1.1  Chromosome Organization and Possible Sites for Epigenetic Modifications
Figure 1.2  Multiple Biological Roles of G9a/GLP
Figure 1.3  Chemical Structures of UNC0642 and A-366
Figure 1.4  Apparatus of the Elevated Mazes
Figure 1.5  Apparatus of the Marble Burying Test
Figure 1.6  Apparatus for Novelty-suppressed Feeding Test
Figure 1.7  Apparatus of Crawley’s Three-chambered Social Interaction Test
Figure 2.1  Experiment Flow Chart for Chronic G9a/GLP Inhibitors Injection in Adult Mice
Figure 2.2  Experiment Flow Chart for In Utero UNC0642 Exposure
Figure 3.1  Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Mice Anxiety-related Behaviour in the EZM
Figure 3.2  Chronic Venlafaxine at 16mg/kg, UNC0642 at 2mg/kg and 4mg/kg Decreased Mice Anxiety-related Behaviour in the Marble Burying Test
Figure 3.3  Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Mice Anxiety-related Behaviour in the novelty suppressed feeding test
Figure 3.4  Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Global H3K9me2 levels in Mice brain
Figure 3.5 Chronic Venlafaxine at 16mg/kg and A-366 at 2mg/kg
Decreased Mice Anxiety-related Behaviour in the EZM

Figure 3.6 Chronic Venlafaxine at 16mg/kg, and A-366 at 2mg/kg
Decreased Mice Anxiety-related Behaviour in the Marble Burying Test

Figure 3.7 Chronic Venlafaxine at 16mg/kg and A-366 at 2mg/kg
Decreased Mice Anxiety-related Behaviour in the NSF test

Figure 3.8 Chronic Venlafaxine at 16mg/kg, A-366 at 2mg/kg and
4mg/kg Decreased Global H3K9me2 levels in Mice brain

Figure 3.9 *In Utero* Exposure to UNC0642 at 4mg/kg increased Mice Anxiety-related Behaviour in the EZM

Figure 3.10 *In Utero* Exposure to UNC0642 at 4mg/kg increased Mice Anxiety-related Behaviour in the Marble Burying Test

Figure 3.11 *In Utero* Exposure to UNC0642 at 4mg/kg Decreased Mice sociability-related Behaviour in the Crawely’s Three-chambered Social Interaction Test

Figure 3.12 *In Utero* Exposure of UNC0642 did not Alter H3K9me2 levels

Figure 3.13 Chronic adult UNC0642 and A-366 and *In Utero* UNC0642 did not Alter Locomotor Activity.
CHAPTER ONE – INTRODUCTION

1.1 - Statement of Problem

Three million Canadians are affected by anxiety disorders, which cause substantial functional impairment and decreased productivity. Anxiety disorders have become one of the leading causes of disability in developed economies such as Canada (Üstün et al. 2004). The current treatment options for anxiety disorders include drug classes such as benzodiazepines and selective serotonin reuptake inhibitors (SSRIs). However, these treatments are associated with unwanted side effects and moderate efficacy (Murrough et al. 2015). Chronic use of benzodiazepine is associated with dependence, addiction, tolerance and withdrawal (Ashton 2005). Post-traumatic stress disorders (PTSD), generalized anxiety disorders (GAD) and social anxiety disorders (SAD) are only partially responsive to SSRIs (Stein et al. 2006; Kapczinski et al. 2003). Despite these shortcomings of current treatments, no novel drug for the treatment of anxiety disorders has come to market in the last two decades (Murrough et al. 2015). Thus, there is a need in the field of psychiatry for the identification of novel drug targets for anxiety disorders.

An emerging concept in psychiatry is that epigenetic mechanisms can regulate behaviour. Epigenetic mechanisms are dynamic processes that produce stable and long lasting changes to gene expression without altering DNA sequences. They can do so through the acetylation and methylation of histone proteins which can enhance or inhibit gene expression (Szyf 2015). Some epigenetic regulators that are involved with anxiety-related behaviours are the histone methyltransferases G9a and G9a-like-protein (GLP) (Tsankova et al. 2007). G9a and GLP form a heterodimer which is responsible for the
mono-and dimethylation of histone 3 lysine 9 (Shinkai and Tachibana 2011). Mice with postnatal conditional knockout of G9a/GLP in the forebrain exhibited decreased exploratory and anxiety-like behaviours (Schaefer et al. 2009). In addition, paroxetine, an antidepressant with known anti-anxiety properties, decreased levels of G9a (Zimmermann et al. 2012). Together, these data suggest that G9a/GLP is potentially involved in anxiety-related behaviours and could be a pharmacological target.

G9a/GLP also plays an important role in neurodevelopment, ultimately impacting anxiety-related behaviours. Mice that lack a functional copy of GLP have increased anxiety and altered social behaviours (Balemans et al. 2010). In humans, the lack of functional GLP results in Kleefstra syndrome which is characterized by an increased level of anxiety, mental retardation and autistic-like behaviours (Kleefstra et al. 2005; Balan et al. 2014).

New small molecule inhibitors of G9a/GLP have recently become available. We use these new small molecule compounds to explore a novel potential target for treating anxiety symptoms, and to better understand the role of this histone methyltransferase on brain development and behaviour (Vedadi et al. 2011). UNC0642 (Liu et al. 2013) and A-366 (Sweis et al. 2014) are chemical inhibitors of G9a/GLP with 100-fold selectivity over other epigenetic regulators. UNC0642 and A-366 are equipotent and both demonstrate effective reduction of H3K9 dimethylation after chronic treatment in vitro.

1.2 - Objectives

To date, the effect of pharmacological manipulation of epigenetic enzymes on animal behaviours has not been investigated in depth. Thus, our primary objective was
to examine the effect of the histone methyltransferase inhibitors UNC0642 and A-366 on anxiety-related behaviours in the adult mouse and on early neurodevelopment. These experiments are of potential significance because they could improve our understanding of how epigenetic mechanisms affect behaviour and could identify new molecular targets for treatment anxiety disorders.

1.3 - Hypotheses

We hypothesize that UNC0642 and A-366 will have anxiolytic-like effects in the mouse. More specifically, we hypothesize that these experimental drugs will: 1) increase time spent in the open areas of the elevated zero maze; 2) decrease the number of marbles buried in marble burying test; and 3) decrease latency to feed in novelty-suppressed feeding test. In addition, we also hypothesize that in utero exposure to UNC0642 will produce deficits in social interaction and increase anxiety. More specifically, G91/GLP inhibition during early brain development will: 1) decrease time spent exploring the stranger mouse in the three-chamber social interaction test; 2) decrease time spent in the open areas of the elevated zero maze; and 3) increase the number of marbles buried in the marble burying test. Finally, we hypothesize that chronic UNC0642 and A-366 treatment decrease H3K9me2 levels and in utero exposure to UNC0642 also decrease H3K9me2.

Thus, the primary endpoint for our study was change in anxiety-related behaviours in mice after G9a/GLP inhibition with the test drugs UNC0642 and A-366. The secondary endpoint was altered H3K9me2 levels.
1.4 - Clinical Features of Anxiety

Anxiety disorders are the most prevalent category of psychiatric diagnosis, with 15-20% of the population meeting diagnostic criteria during their lifetime (Pelissolo et al. 2001). The Diagnostic and Statistical Manual of Mental Disorders -- Fifth Edition (DSM-V) recognizes the following anxiety disorders: separation anxiety disorder, selective mutism, specific phobia, SAD, panic disorder, agoraphobia, GAD, substance/medication-induced anxiety disorder and anxiety disorder due to another medication condition (Association and Others 2013). Anxiety may impede normal functioning through autonomic hyperarousal, subjective nervousness, or avoidant behaviour (Roy-Byrne and Wagner 2004). Table 1.1 below outlines some of the physiological symptoms of anxiety.

<table>
<thead>
<tr>
<th>Area</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Uncomfortable awareness of the heart (palpitations), tachycardia, chest pain</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Lump in the throat, difficulty swallowing, nausea, emesis, diarrhea, stomach cramps</td>
</tr>
<tr>
<td>General</td>
<td>Tension, nervousness, fatigue, hyperarousal, insomnia or hypersomnolence, hot or cold sensations</td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>Muscle pains, tremor, spasms</td>
</tr>
<tr>
<td>Neurological</td>
<td>Vertigo, lightheadedness, deficits in attention and concentration, blurred vision, numbness or tingling in mouth or extremities, tinnitus, disequilibrium</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Shortness of breath, increased frequency of respiration, choking sensation</td>
</tr>
</tbody>
</table>
1.5 - Epidemiology of Anxiety

In Canada, three million Canadians were affected by anxiety disorders in the past year. Females are two times more likely to be affected by anxiety than males (Huber and Henrich 2003). Onset of anxiety disorders often occurs in early adolescence and young adulthood, and they often manifest before mood or substance use disorders (Kessler et al. 2005). When left untreated, anxiety disorders also lead to impairment in social functioning and decreased work productivity (Mendlowicz and Stein 2000). Consequently, they have become one of the leading causes of disability in developed countries (Üstün et al. 2004). Patients with anxiety disorders are three to five times more likely to seek medical care and six times more likely to be hospitalized for a psychiatric disorder (Lépine 2002; McNaughton and Gray 2000). Anxiety disorders have high concurrence with other psychiatric disorders: in particular depression, with comorbidity of 60% (Hirschfeld 2001) and autism spectrum disorder, with a comorbidity of 70% (Nadeau et al. 2011). Such a high rate of comorbidity suggests a shared underlying neurological mechanism. Further studies have shown that patient suffering from anxiety disorders often do not receive adequate treatment (Pollack et al. 2004). Thus, there is a need in psychiatry for a more effective treatment for anxiety disorders.

1.6 - Mechanism of Anxiety

Anxiety is a label applied to a psychological state of arousal, similar to fear, that includes a collection of both physical and mental symptoms. Anxiety responses prepare organisms for potential danger in their environment (Yerkes and Dodson 1908). However, anxiety can also be maladaptive when triggered inappropriately. Prolonged anxiety can become disruptive and affect function in many domains of life (Etkin 2010).
This section aims to provide a brief literature review on the neural mechanisms underlying both normal and pathological forms of anxiety.

1.6.1 - Low and High Road for Anxiety

LeDoux proposed that emotional stimuli reach the amygdala, the primary region for anxiety response, through two different but interacting pathways, which he labelled with the vernacular terms: the low road and the high road (LeDoux 1998). The “low road” refers to the direct pathway from the thalamus to the amygdala. This pathway bypasses the cortex, allowing a fast but non-specific representation of the stimuli to reach the amygdala, preparing the organism to defend against potential danger in a general way (LeDoux 2003). The “high road” includes the cerebral cortex, and allows animals to cognitively appraise stimuli in the environment and adjust their responses accordingly. The cortex can identify the fear-triggering stimulus. This identification process differentiates between harmful and harmless stimuli, and helps to calibrate the appropriate response. The cortex is capable of suppressing amygdala activity if the initial trigger is deemed not to be a threat. It has been hypothesized that dysfunction of the high road removes the normal suppression of the low road, and could be a contributing factor to pathological anxiety (LeDoux 2000).

1.6.2 – Amygdala

The amygdala is the major brain region responsible for anxiety and the fear response. The basolateral amygdala (BLA) and the central nucleus, two of subnuclei in amygdala, are integral for anxiety (Olmos and Heimer 1999). In rodents, the BLA is responsible for evaluating the threat value of stimuli, while the central nucleus is responsible for initiating species-specific fear defense mechanisms (Davis and Whalen
The BLA is the primary structure in the amygdala that receives information from the thalamus and other associated sensory cortices (Amaral et al. 1992). The central nucleus is an output region which projects to the brain stem, the forebrain and the hypothalamus. Lesions to the central nucleus terminate responses to the fearful stimuli in rodents (Paxinos and Mai 2004). The human amygdala can be activated by emotionally negative stimuli in neuroimaging studies (Phan et al. 2002; Wager et al. 2003). Lesions to the amygdala results in deficiencies in labeling fearful facial expression and encoding fear-based memories (Adolphs et al. 1994). Hence, the function of amygdala is conserved between species. Herry and colleagues (2007) exposed mice and humans to a series of predictable and unpredictable neutral tones. In both groups, amygdala activation was increased by the unpredictable neutral tones, which caused respective species-specific anxiety behaviours in both groups.

1.6.3 - Medial Pre-frontal Cortex

The medial pre-frontal cortex (mPFC) is consistently activated in anxiety, and plays an important role in appraising and regulating the anxiety response (Etkin and Wager 2007). Dorsomedial PFC appraises and monitors emotion (Ghashghaei et al. 2007). Kalisch et al (2006) induced anxiety in healthy subjects by telling them that they would receive an electric shock during the trial. Neuroimaging results showed an increased activation in the dorsomedial PFC. In later trials, the subjects were distracted with challenging working memory tests and the dorsomedial PFC activation decreased. The results suggested that dorsomedial PFC was involved in higher level emotional appraisal, which could be disrupted when attentional load was high. The ventromedial PFC may regulate emotional response to anxiety. Phelps et al (2004) found increases in
ventromedial PFC activity decreased amygdala activity in human subjects undergoing fear extinction. This result is consistent with rodent data, where lesions in the ventromedial PFC impaired extinction of conditioned fear in rats (Morgan et al. 1993).

1.6.4 – Hippocampus

The hippocampus is known for its role in learning and memory, but emerging evidence has implicated its involvement in the anxiety mechanisms as well. In animals, hippocampal lesions reduce anxiety-related behaviours in ethological unconditioned paradigms such as the elevated plus maze (Deacon et al. 2002; Treit and Menard 1997). These paradigms test innate behaviours that have no explicit role for prior learning. In the elevated plus maze, the animal is conflicted between the choice to explore the new environment and the innate fear of open spaces where they are vulnerable to predators (Sousa et al. 2006). An emerging hypothesis is that the hippocampus is activated in response to these conflicting desires. Its activation increases arousal and modulates attentional processes to help animals respond to salient stimuli (Bannerman et al. 2014; Gray and McNaughton 2003).

1.6.5 - Current Model of Anxiety

The current model suggests that the amygdala registers and reacts to negative emotional stimuli based on the information received from the thalamus and sensory cortex. In turn, the amygdala stimulates and directs regions such as the hypothalamus and brain stem. The amygdala also sends information to the dorsomedial PFC for further monitoring and evaluating of stimuli. The dorsomedial PFC sends information about stimuli to the ventromedial PFC, which in turn sends feedback to the amygdala.
resulting in context-depend regulation. This could mean suppressing amygdala activity. In conjunction with mPFC, the hippocampus helps to mediate the anxiety response by modulating attentional processes (Etkin 2010).

In patient populations with PTSD, SAD, and specific phobias, the amygdala was shown to be hyperactivated. Furthermore, in patient populations with PTSD and generalized anxiety disorder, there is a hypoactivation of mPFC regions (Etkin and Wager 2007). Taken together, the evidence suggests that anxiety disorders involve a deficiency in mPFC in regulating a hyperactivated amygdala.

1.7 - Treatment of Anxiety

Treatments for anxiety include both medication and psychotherapy (Hammond 2005). Cognitive psychotherapy techniques aim to reduce overall anxiety by teaching the patients cognitive techniques to stop anxiety-inducing thoughts. Behavioural treatments focus on relieving anxiety through mindfulness or through repeated exposure to the anxiety-provoking stimulus to promote extinction of the fear response. The goal of medication is to reduce physiological symptoms associated with anxiety (Burijon 2007). The classes of anti-anxiety medication includes benzodiazepines, and antidepressants.

1.7.1 – Psychotherapy

Psychotherapy includes individual or group cognitive behaviour therapy (CBT) (Stahl and Moore 2013). The treatment begins by teaching the patients about anxiety disorders (Crawley et al. 2008). Then the therapist can help the patient to identify anxiety-provoking stimuli and replace negative thoughts with more rational and encouraging thoughts (Garcia-Lopez et al. 2006). Finally, and most importantly, patients are gradually exposed to anxiety-provoking stimulus through stimulation and in vivo,
using the learned skills to cope with anxiety (Hayward et al. 2000). While CBT is highly effective, it requires trained therapists, significant patient engagement and considerable resources (McManus et al. 2012).

1.7.2 – Benzodiazepines

Benzodiazepines were developed in 1960s as anxiolytic agents (Shorter 2005) that bind to GABA<sub>A</sub> receptors. Benzodiazepines act as positive allosteric modulators of GABA<sub>A</sub> by enhancing the conductance of chloride ions across neural membrane. This hyperpolarizes the neural membrane and decreases the probability of neuron firing through action potentials (Brady and Siegel 2012). Benzodiazepines have effective and immediate anxiolytic response. However, benzodiazepines have several serious side effects, including cognitive impairment, addiction, withdrawal and tolerance (Onyett 1989). Due to these side effects, benzodiazepines are recommended for acute but not chronic treatment of anxiety disorders (Cloos and Ferreira 2009).

1.7.3 - Antidepressants

Most current anti-anxiety treatments are SSRIs or serotonin norepinephrine reuptake inhibitors (SNRIs) (Murrough et al. 2015). Randomized control trials (RCT) generally reported that 60 to 75% of anxiety disorder patients responded to SSRIs treatment compared to response rates between 40 and 60% for placebo (Baldwin et al. 2011).

Other classes of antidepressants used for anti-anxiety treatments are tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) (Stein et al. 2006). Both increase synaptic serotonin and norepinephrine. While TCAs do so by inhibiting
serotonin transporter (SERT) and the norepinephrine transporter (Tatsumi et al. 1997), MAOIs prevent monoamine oxidase from breaking down serotonin and norepinephrine (Feighner 1999). TCAs and MAOIs have demonstrated reasonable efficacy in anxiety disorders. However, they cause serious side effects such as cognitive impairment and delirium. Furthermore, the medications have a narrow therapeutic window and can be fatal in overdose due to cardiac effects (Geddes et al. 2012). Because of this, TCAs are usually reserved for second-line treatment. Overall, the efficacy of antidepressants as a class is quite limited. One-third of anxiety patients do not respond to these medications, even after treatment with multiple antidepressants (Rush et al. 2006).

Despite the shortcomings of current antidepressants, most antidepressant and anti-anxiety medications in development are still designed to increase the amount of norepinephrine and/or serotonin in the synapse. They do so via different mechanisms: either by blocking reuptake or inhibiting degradation of monoamine neurotransmitters (Berton and Nestler 2006). However, there is increasing evidence to suggest that the anti-anxiety effects of antidepressants are likely mediated through more than just the monoamine neurotransmitter systems. Some hypothesize that antidepressants exert their actions by increasing brain-derived neurotrophic factor (BDNF), which increases cortical synaptogenesis and neurogenesis in the hippocampus (aan het Rot et al. 2009). The antidepressants potentially upregulate BDNF via epigenetic mechanisms which will be discussed in more detail in the next section.
1.8 - Epigenetic Mechanisms

**Figure 1.1. Chromosome Organization and Possible Sites for Epigenetic Modifications.** In eukaryotic cells, nucleosomes facilitate the packing of DNA into chromatin. Individual nucleosome is composed of an octamer containing four histone dimers, one dimer each of histones H2A, H2B, H3, and H4. 147 bp of DNA wrap around the octamer in approximately two superhelical turns. Histone modifications alter the accessibility of DNA to the transcriptional machinery in a regulated fashion. These modifications occur on the exposed N-terminal tails of histones. In general histone acetylation is often associated with increased gene expression. The effect of histone methylation on gene expression will depend on the location and the number of methyl groups transferred to the histone. Lastly, cytosine bases of DNA can be methylated and results in gene repression.

In general, epigenetic mechanisms produce stable changes in gene expression by modifying histones and/or DNA bases without changing the DNA sequence (Mahgoub and Monteggia 2013). In the brain, these modifications mediate changes in diverse aspects of neuronal functions including learning, memory (Korzus et al. 2004; Levenson et al. 2004; Levenson and Sweatt 2005), social behaviour and maternal care (Champagne et al. 2006; Garfield et al. 2011). Epigenetic modifications may also contribute to the long lasting behavioral abnormalities seen in psychiatric disorders such as drug addiction (Maze et al. 2010; Robison and Nestler 2011), depression (Covington et al. 2009) and PTSD (Chertkow-Deutsher et al. 2010).
Gene-environment interactions are important in most complex disorders, including brain disorders such as anxiety (McEwen 2000). For instance, environmental factors such as stress are often chronic in nature and extend over significant periods of time (Nestler et al. 2002). There is growing evidence suggesting that epigenetic mechanisms induce stable and lasting changes in gene expression in response to these types of environmental factors (McEwen 2000). In fact, epigenetic mechanisms might partly explain the difficulty in identifying the specific genetic variations that contribute to these disorders, despite data from twin and other types of family studies showing that anxiety disorders are heritable (Feinberg 2007; Mill and Petronis 2007). Thus, the study of epigenetic mechanisms could provide new perspectives to understanding normal behavioral traits as well as disease etiology in humans.

The following section will briefly summarize epigenetic mechanisms such as DNA methylation, histone acetylation, and histone methylation (Figure 1.1). Furthermore, the section will discuss how these mechanisms mediate changes in the brain to respond to stress.

1.8.1 - DNA Methylation

DNA methylation is a dynamic process that can affect neurogenesis (Ma et al. 2009), regulate synaptic function (Feng et al. 2010) and facilitate memory formation (Miller and Sweatt 2007). 5’ methylated cytosine (5mC) bases are the most commonly-reported form of DNA methylation. The methyl group projects into the major groove of DNA but it does not interfere with the normal hydrogen bonding with guanine bases (Newell-Price et al. 2000). In mammals, methylation of cytosine bases predominantly occurs in the palindromic sequence 5’-CpG-3’. Approximately 3% of all cytosine bases
in the human genome are methylated (Nafee et al. 2008). The majority of methylated cytosine bases are found in the promoters of 50-60% human genes, resulting in repression of gene transcription (Wang and Leung 2004). Proper cytosine methylation is required for cell differentiation, genetic imprinting, suppression of repetitive elements, and X-chromosomal inactivation (Bird 2008).

1.8.1.1 - Mechanisms of DNA Methylation

DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs): DNMT1, DNMT2, DNMT3a, and DNMT3b (Weber and Schübeler 2007). DNMT1 maintains methylation patterns during DNA replication, while DNMT3a and DNMT3b appear to catalyze de novo methylation of previously unmethylated double-strand DNA (Newell-Price et al. 2000; Kim et al. 2009). DNMTs can directly interact with transcription factors, presumably allowing the methylation of promoter regions at specific locations. In a recent study, nearly 80 transcription factors were found to interact with DNMTs (Hervouet et al. 2009).

There are several proposed DNA demethylation pathways. Recently, ten-eleven-translocation (TET) hydroxylase-induced oxidation-deamination has been proposed as a new candidate for DNA demethylase in the brain. TET oxidizes 5mC into 5'-hydroxymethylcytosine (5hmC), which can be further oxidized into 5-formolcystosine and 5-carboxylcystosine (Tahiliani et al. 2009; Kriaucionis and Heintz 2009; Ito et al. 2011; He et al. 2011). All three forms of 5mC derivatives seem to be enriched in brain, and 5hmC displays a developmentally programmed acquisition in neuronal cells, further
implicating TET as a player in the epigenetic regulation of the brain (Szulwach et al. 2011).

1.8.1.2 - DNA Methylation Changes in Response to Stress

One paradigm to study the effect of stress in rodents is the predator exposure model. In the acute version, rodents are immobilized during a cat exposure to maximize the expression of an intense fear response and helplessness (Zoladz et al. 2008). In the chronic version, the rodents are placed in a cage scented with predator odor for five or more consecutive days (Lim et al. 2016).

Stress increases DNA methylation and subsequently decreases Bdnf expression in the brain. Stress activates the hypothalamus-pituitary axis (HPA) and causes the release of glucocorticoids, the main stress hormones (Smith and Vale 2006). One brain region that they target is the hippocampus because it has a high density of glucocorticoids receptors (Nishi et al. 2007). One hypothesis suggests that the activation of glucocorticoids receptors in the hippocampus ultimately leads to the repression of Bdnf (Schmidt and Duman 2007). Roth et al. found that after predator exposure stress, rats had decreased Bdnf expression and increased methylation of Bdnf promoter in the hippocampus (Roth et al. 2011). These mechanisms could contribute to the morphological changes and neuronal impairments reported after chronic stress in animal models and in depressed humans (Sapolsky 1996; Gould et al. 1997).
1.8.2 - Histone Modifications

In eukaryotic cells, nucleosomes facilitate the packing of DNA into chromatin (Alberts 1989). Individual nucleosome is composed of an octamer containing four histone dimers, one dimer each of histones H2A, H2B, H3, and H4. 147 bp of DNA wrap around the octamer in approximately two superhelical turns (Borrelli et al. 2008). The strength of interaction between the DNA and nucleosomes can be affected by post-translational, reversible covalent modifications of histones (Berger 2007). Histone modifications alter the accessibility of DNA to the transcriptional machinery in a regulated fashion. These modifications occur on the exposed N-terminal tails of histones resulting in two states: euchromatin and heterochromatin. In the euchromatin state, DNAs are lightly packed and available for transcription. In contrast, DNAs are densely packed and not available for transcription in the heterochromatin state (Strahl and Allis 2000).

1.8.2.1 - Histone Acetylation

Histone acetylation negates the positive charge of lysine residues in the histone tail. This weakens the interaction between the negatively charged DNA and the histone and is associated with transcriptional activation. In contrast, lack of histone acetylation correlates with gene repression (Cheung et al. 2000).

1.8.2.1.1 - Mechanisms of Histone Acetylation

Histone acetyltransferases (HATs) acetylate histone with acetyl coenzyme A as a co-substrate. HATs contain a large family of proteins which acetylate multiple lysine
residues in the tails of both H3 and H4 (Berndsen and Denu 2008). Histones are deacetylated by histone deacetylases (HDACs). HDACs can be divided into three classes. In the brain, Classes I and II appear to regulate histone deacetylation at most genes, while Class III enzymes deacetylate numerous nuclear and cytoplasmic substrates in addition to histones and are implicated in numerous cellular functions such as circadian rhythm (Nakahata et al. 2009; Sassone-Corsi 2011) and metabolism (Bellet and Sassone-Corsi 2010).

1.8.2.1.2 - Histone Acetylation Changes in Response to Stress

Chronic social defeat is another behavioural paradigm used to study the effect of stress in rodents. In brief, the test subject is exposed to an aggressive partner over an extended period of time (Golden et al. 2011). After mice experienced chronic social defeat, there is an increase in H3 acetylation in nucleus accumbens (NAc) for at least 10 days. This increase in histone acetylation is associated with decreased HDAC2 expression in the region (Covington et al. 2009). Furthermore, local inhibition of HDAC2 resulted in antidepressant like effects (Uchida et al. 2011). Finally, in depressed humans, increased H3 acetylation and decreased HDAC2 levels are also seen in the NAc (Sun et al. 2013).

1.8.2.2 - Histone Methylation

Histone methylation can result in both transcriptional activation and repression depending on the particular residue modified and the extent of methylation. Unlike acetylation, methylation does not substantially alter the charge of the histones residue,
but can dramatically change the steric profile and potential molecular interactions via multivalent addition of mono-, di-, or tri-methyl groups (Maze et al. 2010).

1.8.2.2.1 - Mechanisms of Histone Methylation

Histone methyltransferases (HMTs) can methylate both lysine and arginine residues using Sadenosylmethionine (SAM) as a cosubstrate. Lysines are demethylated by lysine demethylases while deaminases convert methylated arginine residues to citrulline (Rice and Allis 2001).

1.8.2.2.2 - Histone Methylation Changes in Response to Stress

The regulation of histone methylation in response to stress is dynamic and complicated. Trimethylation of lysine 4 of H3 (H3K4me3) is associated with transcriptional initiation, while di- and trimethylation of lysines 9 and 27 of H3 (H3K9me2/3 and H3K27me2/3) are associated with transcriptional repression (Maze et al. 2010). In contrast, H3K36me3 is associated with transcription elongation. The table below summarizes the effect of various histone lysine and arginine methylation on gene expression (Barski et al. 2007). Table 1.2 below lists some known effect of histone methylation on gene expression.
### Table 1.2. The Effect of Histone Methylation on Gene Expression

<table>
<thead>
<tr>
<th>Histone</th>
<th>Lysine (K)/Arginine (R)</th>
<th>Mono-/Di-/Tri Methylation (Me1/Me2/Me3)</th>
<th>Effect on Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone 3</td>
<td>K4</td>
<td>Me1 Me2 Me3</td>
<td>Increase Gene Expression</td>
</tr>
<tr>
<td></td>
<td>K9</td>
<td>Me1 Me2 Me3</td>
<td>Increase Gene Expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Me2 Me3</td>
<td>Decrease Gene Expression</td>
</tr>
<tr>
<td>K27</td>
<td>Me1</td>
<td>Increase Gene Expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Me2 Me3</td>
<td>Decrease Gene Expression</td>
<td></td>
</tr>
<tr>
<td>K36</td>
<td>Me1 Me3</td>
<td>Increase Gene Expression</td>
<td></td>
</tr>
<tr>
<td>K79</td>
<td>Me1 Me2</td>
<td>No Effect</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>Me1 Me2</td>
<td>No Effect</td>
<td></td>
</tr>
</tbody>
</table>
### Table

<table>
<thead>
<tr>
<th>Histone</th>
<th>Lysine (K)/Arginine (R)</th>
<th>Mono-/Di-/Tri Methylation (Me1/Me2/Me3)</th>
<th>Effect on Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone 4</td>
<td>K20</td>
<td>Me1</td>
<td>Increase Gene Expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Me3</td>
<td>No Effect</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>Me2</td>
<td>No Effect</td>
</tr>
<tr>
<td>Histone 2B</td>
<td>K5</td>
<td>Me1</td>
<td>Increased Gene Expression</td>
</tr>
</tbody>
</table>

After undergoing chronic social defeat, stress increased H3K27me3 levels at the Bdnf gene promoter in hippocampus resulting in its repression (Tsankova et al. 2006). This result is consistent with predator exposure study discussed above where stress is also seen to decrease Bdnf expression in the hippocampus, these studies provide strong evidence that epigenetic mechanism mediate the response to stress and could contribute to pathologies of psychiatric disorders such as anxiety (Roth et al. 2011).

### 1.9 - Epigenetic Effect of Current Drugs Used in Psychiatry

Epigenetic mechanisms are also important in the actions of psychiatric drugs. For example, the mood stabilizer valproic acid is a histone deacetylase inhibitor that improves cognitive function in animal models of dementia (Fischer et al, 2007; Kilgore et al, 2010; Qing et al, 2008). The antipsychotics risperidone and clozapine target the histone deacetylase HDAC2 (Vedadi et al, 2011). The antidepressant imipramine downregulates HDAC5, leading to increased histone acetylation at the Bdnf promoter and higher levels of Bdnf (Krishnan and Nestler, 2008; Tsankova et al, 2006). Conversely, more targeted histone deacetylase inhibition can have antidepressant
effects (Covington et al, 2009). The tricyclic antidepressants amitriptyline, imipramine as well as the selective serotonin reuptake inhibitor paroxetine inhibit DNMT1 activity by decreasing levels of G9a, which is a known stimulator of DNMT1 (Zimmermann et al, 2012a). Amitriptyline, imipramine and paroxetine all have anti-anxiety effects. Thus we hypothesized that selective targeting of G9a would alleviate anxiety phenotypes.

1.10 - G9a/GLP

G9a and G9a-like-protein (GLP) are histone methyltransferases (HKMT) that are responsible for mono- and dimethylation of histone 3 lysine 9 (Shankar et al, 2013). The two proteins share very similar in vitro, G9a and GLP share the same histone substrate specificities and can independently exert HKMT activity. While G9a and GLP can both form homodimers, the G9a-GLP heterodimer complex is the functional form of H3K9 HKMT in vivo. Knockout of either G9a or GLP can drastically reduce the levels of H3K9me1 and H3K9me2. Furthermore, the G9a and GLP double knockout does not further reduce H3K9me1 and H3K9me2 levels (Tachibana et al. 2005). Thus, G9a generally cannot compensate for the loss of GLP HKMT function in vivo, and vice versa. This further supports the notion that the G9a-GLP heterodimer complex is essential for biological functions.

1.10.1 - G9a/GLP Has Diverse Biological Functions

G9a/GLP plays an important role in various biological processes such as embryonic development (Figure 1.2, Shinkai and Tachibana, 2011). In mice, G9a and GLP knockout is lethal at embryonic day 9.5 (E9.5) due to severe growth defects
Tachibana et al. 2002, 2005). G9a and GLP mutants have a significantly lower level of H3K9 methylation. Furthermore, in utero inactivation of G9a HKMT is also embryonic lethal, similar to G9a knockout mice (Shinkai and Tachibana, 2011). Overall, the data suggest that G9a/GLP-mediated H3K9 methylation is critical for mouse development.

**Embryo Development**

![Embryo Development Diagram](image)

**Figure 1.2. Multiple Biological Roles of G9a/GLP.** Based on studies of knockout mice, G9a/G9a-like protein (GLP) has been shown to affect biological processes such as embryo development, immune response, brain functions and cell growth. More recently, based the postnatal knockout of G9a in the forebrain of mice, G9a has been implicated in the anxiety response processes. Modified from Shinkai and Tachibana, 2011.

G9a/GLP is also known to be involved in drug response. Previous studies have found that repeated cocaine exposure decreased G9a level in NAc neurons in mice. The changes in G9a level were associated with decreased H3K9 methylation. Furthermore, mice with decreased H3K9 methylation were more susceptible to chronic social defeat stress. Overexpression of G9a in the NAc was able to rescue the cocaine-induced changes in neuron morphology and behavioral. Finally, inhibition of G9a in the NAc resulted in behavioural and physiological changes similar to those induced by chronic cocaine exposure.
1.10.2 - G9a/GLP Plays a Role in Anxiety

In addition to the roles of G9a/GLP discussed above, recent studies have suggested that G9a/GLP is involved in anxiety. Mice with postnatal conditional knockout of G9a/GLP in the forebrain exhibited decreased exploratory and anxiety-like behaviours as well as altered social behaviour (Schaefer et al, 2009). G9a and GLP participate in the RE1-silencing transcription factor (REST) complex, which plays a critical role in embryonic brain development and in learning (Roopra et al, 2004; Tahiliani et al, 2007). Mice that lack a functional copy of GLP have increased anxiety and decreased social behaviours (Balemans et al, 2010). In humans, the lack of functional GLP results in Kleefstra syndrome, which is characterized by an increased level of anxiety, mental retardation and autistic-like behaviours (Kleefstra et al, 2005). Several variants of the G9a and GLP genes are also associated with autism in some patients (Balan et al, 2014). Given the complexity and dynamic role of G9a/GLP have in multiple biological process, we sought to further examine the role of G9a/GLP in anxiety-related behaviours.
1.11 - Drug Inhibitors of G9a/GLP

![Chemical Structures of UNC0642 and A-366](image)

**Figure 1.3. Chemical Structures of UNC0642 and A-366.** When studied in cell lines, the two compounds are equipotent at inhibiting G9a/G9a-like-protein (GLP) function after 72 hours of incubation.

New small molecule inhibitors of G9a/GLP have recently become available. We use these new drug compounds to investigate the potential for G9a/GLP as a novel potential target for treating anxiety symptoms, and to better understand the role of this histone methyltransferase on brain development and behaviour (Vedadi et al., 2011). UNC0642 (Liu et al., 2013) and A-366 (Sweis et al., 2014) are chemical inhibitors of G9a/GLP with 100 fold selectivity over 15 other methyltransferases (figure 1.3). UNC0642 and A-366 are equipotent and both demonstrate effective reduction of H3K9 dimethylation after chronic treatment *in vitro*. 5mg/kg UNC0642 was shown to have Cmax of 68ng/mL in the brain and was well tolerated (Liu et al., 2013). UNC0642 was tested against the National Institute of Mental Health psychoactive drug screen program selectivity panel which contains a selection of GPCRs, transporters and ion channels such as serotonin and dopamine receptors. It was also tested against 50 representative kinases such as ERK and GSK. With the exception of the H3 histamine receptor, UNC0642 has 300 fold selectivity for G9a/GLP over a variety of kinases, GPCR, ion...
channels and transporters (Liu et al., 2013). There were no such data for A-366. These chemical inhibitors allow us to examine the effect of G9a/GLP on anxiety-related behaviours during adulthood and neurodevelopment. Overall, the experiments allow us to determine if pharmacological inhibition of G9a/GLP might be a potential anxiety treatment and investigate critical periods in neurodevelopment that could affect the pathogenesis of anxiety and other symptoms relevant to human brain disorders.

1.12 - Animal Models

Modelling psychiatric disorders in animals is challenging because of the complexity and heterogeneity of many psychiatric disorders (Richardson-Jones et al., 2010). Furthermore, the current diagnosis relies on the self-reported internal state of the patients, which is impractical to achieve with animals (Fava et al. 2008). Thus, rather than recapitulating a psychiatric disorder in its entirety, most current animal models focus on the physical features and behaviours that can be assessed in animals and that are related in some way to psychiatric disorders in humans (Gottesman and Gould 2003). Animal models have advanced our understanding of psychiatric disorders by providing insights into neurotransmitter systems and brain circuitry, and guiding the search for therapeutics and novel drug targets (Frazer and Morilak 2005; Lapiz-Bluhm et al. 2008). This section will review animal models for anxiety disorders and behaviour paradigms for assessing anxiety-related abnormalities.

1.12.1 - Validities of Animal Models

Given the complexity and heterogeneity of many psychiatric disorders, stringent parameters are required to validate animal models for aspects of psychiatric conditions.
There are three types of validities applied to the animal models: face, construct and predictive validity (Bloom and Kupfer 1995).

Face validity refers to the overt features of the animal model and how closely these resemble symptoms of the human psychiatric disorder (McKinney 1984). For instance, in the chronic mild stress model of rodent depression, one outcome is the deterioration of the fur coat (Willner 1997). In clinical settings, some depressed patients display decreased personal hygiene and self-care. Thus, the similarity between the coat state in mice and personal grooming in humans contributes to the face validity of the mild chronic stress model (Willner and Mitchell 2002).

Construct validity refers to how well the animal model can recapitulate hypothesized mechanisms of human psychiatric disorders (Bloom and Kupfer 1995). As discussed above, it has been observed that H3 acetylation increased and HDAC2 levels decreased in the NAc of depressed patients (Sun et al. 2013). In the chronic social defeat model, rodents have similar changes in their NAc (Covington et al. 2009). Thus, chronic social defeat has construct validity for modelling stress responses that are relevant to understanding mechanisms for depression in humans.

Finally, predictive validity is evaluated by how well the animal model can predict response to a treatment consistent with what is seen in human clinical populations (Bloom and Kupfer 1995). For example, all existing pharmacological treatments for human depression increase swimming time in the forced swim test. This is largely because the forced swim test has commonly been used to screen potential antidepressant drugs in pre-clinical development. However, there are no existing animal tests that are perfectly predictive of human therapeutic effects.
The different types of model validities can be independent of each other. Each of the three validities, though important, has shortcomings (van der Staay 2006). Face validity can be too stringent and overly subjective, focusing on what a model should “look like” (Willner and Mitchell 2002). Construct validity can be overly broad, especially when the mechanism of the disorder is unknown, which is the case for most psychiatric disorders. A stringent predictive validity may select against treatments that act through novel mechanisms (Bloom and Kupfer 1995). An ideal animal model should model more than one aspect of the target disorder and be valid on multiple levels (Richardson-Jones et al, 2010).

1.12.3 - Types of Anxiety Models

The animal models of anxiety-related behaviours can be divided into two broad categories: learned (conditioned) and innate (unconditioned) (Kumar et al. 2013). The majority of learned behavioural models are based on variations of fear conditioning. In this paradigm, the animals are classically conditioned to an aversive stimulus (Kalueff et al. 2007). While the conditioned models of anxiety are important tool in neuroscience research, the goal of the thesis is to focus on the innate behaviours that are detected by the unconditioned models of anxiety. The unconditioned models of anxiety try to recapitulate defensive behaviours in response to a real or perceived threat (Blanchard et al. 2001). In the animal models, this is achieved by creating internal tensions between two conflicting desires (Sousa et al. 2006). For instance, in the elevated plus maze test, the internal tension is created between the animal’s desire to explore and the avoidance of open area (Walf and Frye 2007). In the following section, there will be more in-depth discussion about the elevated plus/zero maze, marble burying, novelty-suppressed
feeding and Crawley’s three chamber social interaction test. Table 1.3 below provides a brief overlook at some of the unconditioned anxiety-related behaviour test.

Table 1.3. Behavioural Tests for Measuring Anxiety-related Behaviours in Mice

| Behavioural Test                          | Paradigm                                                                 | Index of Anxiety                                                                 |
|------------------------------------------|------------------------------------------------------------------------|
| Elevated zero maze                      | Create a conflict between rodents’ innate aversion to open areas and the opposing drive to explore novel environments | The amount of time spent in the open areas of the apparatus.                  |
| Marble burying                          | Digging is an innate behaviour in rodents and they will spontaneously dig when the bedding is deep enough | The number of marbles buried at the end of the test                          |
| Novelty-suppressed feeding               | Create a conflict between the food-deprived rodents need to feed and the fear of open novel areas | The latency for the animal to feed in the novel environment                  |
| Crawley’s three-chambered social interaction | The social interaction test utilizes the innate tendency to investigate an unfamiliar conspecific through following, sniffing and grooming | The amount of time the test animal spent investigating the stranger mice in the sociability phase |
1.12.4 - Elevated Plus/Zero Maze

Figure 1.4. Apparatus of the Elevated Mazes. (a) Apparatus of the elevated plus maze (b) Apparatus of the elevated zero maze. They both contain regions of open areas and close areas. The main index of anxiety is the amount of time spent in the open areas of the apparatus. For the elevated plus maze, the intersection between the four arms represents a region of ambiguity. It was difficult to determine if time in the intersection should be counted towards time spent on the open arms or closed arms. The continuous tracks design of the elevated zero maze eliminates the ambiguity associated with the elevated zero maze.

In the wild, mice are the prey of many predators such as eagles, owls and snakes (Walf and Frye, 2009). Thus, mice have the natural tendency to avoid open areas where there is no shelter. The elevated plus maze is developed based on the conflict between the aversion to open areas and the opposing drive to explore novel environments (Walf and Frye 2007). More detailed descriptions of the apparatus and procedure can be found in the methods section. In brief, the test contains two types of areas: the open and closed arms. The test animal has equal access to both regions.
(Figure 1.4a). The time spent in the open vs. closed arms is used as the marker of anxiety-like behaviour. Less anxious animals spend more time in the open arms.

The elevated plus maze has become a common laboratory tool to assess unconditioned anxiety-related behaviours in mice (Flint et al. 1995). The elevated plus maze has strong face and predictive validity. For example, clinically used anti-anxiety medications such as chlordiazepoxide, diazepam, phenobarbitone, sodium pentobarbital and ethanol increase time spent on the open arms. On the other hand, known anxiogenic drugs such as yohimbine, pentylenetetrazole, caffeine and amphetamine, reduce time spent in the open arms in rats and NIH Swiss mice (Pellow et al. 1985; Lister 1987). Furthermore, the elevated plus maze also has a strong construct validity, since the GABA<sub>A</sub> receptor that is targeted by benzodiazepines is implicated in modulating anxiety. As well, the glutamate system has been implicated in the pathophysiology of anxiety disorders (Labrie et al. 2009). Glutamine receptor ligands are shown to exert anxiolytic effect by increasing time spent on the open arms in both mice and rats (Spooren and Gasparini 2004; Pałucha et al. 2004; Poleszak et al. 2008).

The elevated plus maze relies on the novelty of the environment, so the test-retest reliability has been an important debate for the paradigm. Currently, there is no consensus within the field. Some studies reported decreased activity on the open arms of the maze in animals that have been previously exposed to the elevated plus maze (Bertoglio and Carobrez 2000; Bertoglio and Carobrez 2002). Another study reported no difference between mice that were tested on three occasions in the elevated plus maze, whether it was once per week or on three consecutive days (Pellow et al. 1985; Lister
However, in general, the field agrees that for greater reliability, the animals should be tested on the elevated plus maze at two time points that are three weeks apart, and in different rooms (Adamec et al. 2006; Adamec et al. 2004).

The elevated zero maze is a variation of the elevated plus maze (Figure 1.4b). In the past, there was ambiguity when the animal stayed at the intersection of the four arms of the elevated plus maze. It was difficult to determine if time in the center zone should be counted towards time spent on the open arms or closed arms (Shepherd et al. 1994). Because the elevated zero maze is a continuous track without a center area, this eliminates the ambiguity associated with the elevated plus maze. It has been demonstrated that the elevated zero maze has the same validity as the elevated plus maze (Braun et al. 2011).

1.12.5 - Marble Burying

*Figure 1.5. Apparatus of the Marble Burying Test.* Digging is an innate behaviour in rodents and they will spontaneously dig when the bedding is deep enough. The main parameter for measurement of anxiety-related behaviours is the number of marbles buried. Relative to the mouse on the left side, the mouse on the right demonstrates greater anxiety-related behaviour. Modified from Deacon, 2009.

Digging is a species-typical behaviour in mice that is sensitive to genetic strain, brain lesions and pharmacological treatments (Dudek et al. 1983; Deacon and Rawlins 1987).
Mice dig in the ground to find food, to hoard food, to create a refuge from predators or cold and to make a safe nursery area for the young (Fertig and Edmonds 1969). In the laboratory, mice dig vigorously when provided with sufficiently deep bedding (Contet et al. 2001). Digging behaviour can be quantified by measuring how many marbles are buried in a testing arena (Figure 1.5). In this test, the animal is not intentionally burying the marbles but rather the marbles are being buried as a by-product of the animal’s digging activity. The greater the digging activity, the more marbles are buried, which is an indirect index of anxiety.

The marble burying test has predictive validity. Marble burying activity is reduced by anxiolytic agents such as the benzodiazepine drugs (Broekkamp et al. 1986). Furthermore, antidepressant drugs, especially those with known anti-anxiety effects, also reduce marble burying (Njung’e and Handley 1991). There is no consensus about the face and construct validity of the test. One of the main reasons is that digging is a behaviour specific for rodents; thus, it is difficult to find a human equivalent (Deacon 2006). Some have hypothesized that marble burying/digging behaviours will be affected by agents influencing hippocampal function (Gray and McNaughton 2003). As discussed above, the hippocampus plays a part in the anxiety response by increasing arousal and modulating attentional processes (Bannerman et al. 2014; Gray and McNaughton 2003). Thus, it could potentially provide some support to the construct validity of the marble burying test.

In addition, compared to the elevated plus maze test discussed above, the marble burying test can be administered continuously and does not suffer from test
decay (Deacon, 2009). This is why the marble burying test was chosen as part of the battery of anxiety tests for the study.

1.12.6 – Novelty-Suppressed Feeding

![Figure 1.6. Apparatus for Novelty-suppressed Feeding Test.](image)

**Figure 1.6. Apparatus for Novelty-suppressed Feeding Test.** The test animal is food deprived for 24 hours and is placed in a novel open arena with a food pellet in the center. The main measurement of anxiety-related behaviour in the time that take a food deprived animal to eat the food pellet placed in the centre. Modified from Samuel and Hen, 2011.

Novelty-suppressed feeding (hyponeophagia) is a feeding-based test. The test animal, deprived of food for 24 hours, faces a choice between approaching and consuming a piece of food in the center of a brightly lit novel open arena, or staying to the side and avoiding the center of this anxiogenic environment. The main measure of the test is the latency to eat. This is defined as the amount of time it takes for the animal to bite the food pellet, situated in the center of the arena, with use of forepaws while sitting on its haunches (Figure 1.6). The more "anxious" animal has a significantly longer latency to eat than the control animals.

Novelty-suppressed feeding has predictive validity. Drugs with anxiolytic properties, such as benzodiazepines, barbiturates, azapirones, and b-adrenergic
antagonists, decrease hyponeophagia in rodents (Dulawa and Hen 2005; Shephard and Broadhurst 1982; Britton and Britton 1981). Novelty-suppressed feeding also has strong face validity with respect to testing new antidepressants. The positive effect of antidepressant is seen after chronic injection in the novelty-suppressed feeding test, similar to how known antidepressants require chronic treatment to yield beneficial effects in humans (Merali et al. 2003). Chronic treatment with various antidepressants has been shown to decrease latency to eat, including selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (prozac) (David et al. 2009) and tricyclics (TCAs) such as imipramine and desipramine (Santarelli et al. 2003; Surget et al. 2008). Anxiety and depression have a high comorbidity with co-occurrence rates up to 60% (Gorman 1996; Leonardo and Hen 2006), suggesting some shared underlying mechanisms. Therefore, the use of novelty-suppressed feeding to study the effects of chronic G9a/GLP inhibition mediating the anti-anxiety response is potentially relevant to our knowledge of both anxiety and depression mechanisms.

1.12.7 – Crawley’s Three-chambered Social Interaction Test

The social interaction test utilizes the innate tendency to investigate an unfamiliar conspecific through following, sniffing and grooming (Blanchard and Blanchard 2003). The paradigm consists of two phases: 1) a sociability phase that investigates social approach and avoidance-related motivation; 2) a social novelty phase that assesses social memory and the ability to discriminate a socially novel stimulus (i.e. a new other mouse) (Labrie et al. 2008; Moy et al. 2004,). While the test was initially used to study the etiology of human mood and anxiety disorders, it has become increasingly used in
studies of autism spectrum disorders (Crawley 2004). During the sociability phase, the amount of time the test mouse spends with the stranger mouse is a measurement for social anxiety-related behaviours (Figure 1.7). A more anxious mouse spends less time with the stranger mouse.

Behaviours related to sociability are influenced by genetic factors. Moy et al (2007) examined sociability-related behaviours in ten inbred strains of mice. C57BL/6J, C57L/J, DBA/2J, FVB/NJ, C3H/HeJ, and AKR/J, tended to be more social, while A/J, BALB/cByJ, BTBR T (+) tf/J, and 129S1/SvImJ were less so. Furthermore, A/J, BALB/cByJ, and 129S1/SvImJ strains showed high levels of anxiety-related behaviours in the elevated plus maze. Thus, the low level of sociability was attributed to the high level of anxiety in these strains (Moy et al. 2007). This study further supports the validity of social behaviour as a component of measuring anxiety in mice.

Pharmacological intervention can affect social behaviour. Acute administration of anxiogenic drugs, such as amphetamine and caffeine, reduced social interaction whereas chronic administration of benzodiazepines increased social interaction (File and Seth 2003; File and Hyde 1979). As discussed above, the basolateral nucleus plays an integral role in the anxiety response by receiving sensory information from the thalamus. Local injection of neuropeptide Y, an endogenous known for its anxiolytic-like effect, into the basolateral nucleus of the amygdala increased social interaction (Wu et al. 2011). These data provide further support for the construct validity of this test for social anxiety-related behaviour.
Figure 1.7. Apparatus of Crawley’s Three-chambered Social Interaction Test. In the habituation phase, the animal is allowed to explore the apparatus. In the sociability phase, a stranger mouse is introduced in one of the chambers under the cage. This phase investigates social approach and avoidance-related motivation. In the social novelty phase, another stranger mouse is introduced. This phase assesses social memory and the ability to discriminate a socially novel stimulus (i.e. a new other mouse). The main measurement for anxiety-related behaviours is the amount of time the test animal spent investigating the stranger mice in the sociability phase. Modified from Kuti and Page, 2011 and Kaidanovich-Beilin et al, 2011.
CHAPTER TWO - MATERIALS AND METHODS

2.1 - Animals

Wild-type male and female C57BL/6 mice were purchased from Charles River Laboratories Inc. (Toronto, ON Canada). Male mice were housed in pairs and female mice were housed in groups of six to ten. All mice were held in a temperature-controlled (23 ± 1 °C) and humidity-controlled (50 ± 10%) room with a 12-hour artificial light/dark cycle (lights on at 0700h). Mice had ad libitum access to standard laboratory rodent chow and water. All protocols were approved by the Centre for Addiction and Mental Health (CAMH) Animal Care Committee.

2.2 - Drugs and formulations

All drugs were administered at a volume of 4mL/kg. 16 mg/kg venlafaxine (Tocris, Canada) was dissolved in 0.9% saline solution. UNC0642 was a gift from Dr. Jian Jin’s Lab at Mount Sinai Hospital, NY, USA. A-366 was purchased from Tocris Canada. 1-2µL of HCl was added to enhance UNC0642 and A-366 solubility in 0.1M sodium citrate buffer. UNC0642 and A-366 were diluted to the concentration used for injection at a pH of 7.4. Chemical structures for both drugs are shown in Figure 1.3.

2.3 - Injection Schedule

For chronic treatment, UNC0642 or A-366 at 1, 2, or 4 mg/kg was injected daily for 14 days. The doses were selected based on the fact that UNC0642 at 5mg/kg was the highest tolerated dose in mice for acute administration (Liu et al 2013). Thus, to avoid toxicity in chronic injections, UNC0642 at 4mg/kg was chosen. In vivo pharmacokinetic data for A-366 was limited. However, given that UNC0642 and A-366
are equipotent *in vitro* G9a/GLP inhibition, we chose a dose of A-366 at 4mg/kg as the maximum. Figure 2.1 is a flowchart of the experimental design for chronic UNC0642 or A-366 i.p. injection in adult mice.

Venlafaxine is a popular antidepressant that is also used to treat anxiety symptoms in humans. Venlafaxine was chosen as a positive control because others have reported that it reduces anxiety-like behaviour in mice (Dhir and Kulkarni, 2008; Thase, 2006). On the day of the experiment, the animals also receive the appropriate injection thirty minutes before the behavioural tests. Each treatment group initially had 15 mice. This was based on the group size in previous studies using venlafaxine (Dhir and Kulkarni, 2008; Thase, 2006) and the potential for losing some animals during testing due to unexpected reasons such as fighting between cage mates.

For treatment during pregnancy, UNC0642 at 2 or 4mg/kg was injected daily starting at E9.5 until birth. All injections took place between 1000-1600h. Given the potential environmental influences on neurodevelopment, all the females used in the study were impregnated within 3-4 days of each other. Two plugged females were randomly assigned for each group with the expectation that the average litter size would be 6 pups. Figure 2.2 is a flowchart of the experimental design for this study. 0.1M citrate buffer was used as vehicle control for all behavioural experiments.

**2.4 - Behavioural Studies**

All behavioural studies were carried out between 1000-1700h.

**2.4.1 - Elevated Zero Maze (EZM)**

The EZM consisted of a 5cm wide circular track with an inner diameter of 40cm. The maze had four quadrants: two opposing open quadrants and two opposing closed
quadrants. The closed quadrants had walls 15cm high. The maze was elevated 40cm above the ground in a brightly-lit room with six, 32-Watt fluorescent overhead lights (2,800 lumens each). The test mouse was placed in an open quadrant facing a closed quadrant, and allowed to explore the maze for five minutes. If the mouse fell off the maze, it was immediately put back at the location from which it fell. Each trial was video recorded and analyzed using Ethovision XT software v10 (Noldus Information Technology, The Netherlands). Between trials, the apparatus was cleaned with virox and 70% ethanol to sanitize and remove odor.

2.4.2 - Marble Burying

A plastic cage, approximately 20 × 30cm, was filled with 5cm of corn knob bedding which was lightly pressed down to make a flat even surface. Twenty glass marbles were evenly spaced in five rows of four on the flat surface. The test animal was placed in the cage for 30 minutes and recorded using Ethovision. At the end of the trial, the number of marbles at least two-thirds buried below the corn knob bedding were counted.

2.4.3 - Novelty-suppressed Feeding (NSF)

Mice were food deprived for 16 hours prior to the test, and weighed before and after testing. On the test day, mice were temporarily transferred to a holding cage. During the trial, the test animal was placed in an open field (40cmx40cmx40cm) with a solid floor. A food pellet was placed in the center of the box, and the time for the mouse to begin eating was recorded. If the test mouse did not eat in the open field for 10 minutes, a latency of 600s to eat in the novel environment was recorded. The mouse
was then returned to the home cage with a pre-weighed food pellet and allowed to eat for 5 minutes, after which the weight of the food pellet was recorded.

**2.4.4 - Crawley’s Three-chambered Social Interaction Test**

Social interaction was assessed in a rectangular, three chamber glass box. Each chamber was 19 x 45 cm. Adjacent chambers were separated by a clear Plexiglas wall with an open middle section, allowing free movement between chambers. Stranger mice were contained in cylindrical wire cages (10 x 10 cm), with one cage in each side chamber. The test animals were habituated to the testing room for one hour before each trial. The trial consisted of three phases: habituation, social affiliation, and social novelty. For the habituation phase, the test animal was allowed to explore the middle chamber for 5 minutes. For the social affiliation phase, a stranger mouse (inside the mesh cage) was introduced into one of the side chambers. The movement of the test mouse in all three chambers was recorded for 10 minutes using Ethovision. For the social novelty phase, another stranger mouse was introduced into the other, previously vacant side chamber. Again, the activity of the test mouse was recorded as it explored all three chambers. After each trial, the apparatus was cleaned with 70% ethanol and virox.

**2.4.5 - Effect of UNC0642 and A-366 on histone methylation**

Western blotting was performed with an SDS-PAGE electrophoresis system as described previously (Rumbaugh and Miller, 2011). Briefly, 24μg protein samples were resuspended in a reduced sample buffer, and then electrophoresed on a 1.5M Tris gel with Tris running buffer. The proteins were transferred to a nitrocellulose membrane and probed either with primary antibody against all histone 3 proteins (Abcam, ab1791) or
with an antibody against the histone 3 protein dimethylated at lysine 9 (Abcam, ab1220). A horseradish peroxidase-conjugated goat anti-mouse antibody was then added, and secondary antibodies were detected through enhanced chemiluminescence (ECL Plus, General Electric Healthcare, Milwaukee, WI). The total amount of histone 3 protein was used as the loading control. The amount of dimethylated histone 3 protein relative to total histone 3 protein was calculated and compared between treatment groups.

2.4.6 - Statistical Analysis

All data were analyzed using SPSS 21.0 (IBM). All behavioural data were tested for normality using the Shapiro-Wilk test. The non-parametric ANOVA (Kruskal-Wallis test) was used when the data were not normally distributed. Dunn’s post hoc test was used for non-parametric data. Otherwise, one-way ANOVA was used to analyze the results with Dunn’s post hoc test. All data are expressed as mean ± SEM, and the minimum level of statistical significance was set at p < 0.05.
In brief, mice were randomly assigned to five different treatment groups: vehicle (saline), venlafaxine at 16mg/kg, UNC0642 or A366 at 1mg/kg, 2mg/kg and 4mg/kg (N=15). After 14 days of i.p. injections, mice were put through a battery of anxiety-related behaviour tests: the elevated zero maze, the marble burying test and the novelty-suppressed feeding. After the completion of the tests, mice were killed via cervical dislocation. The brain was collected in addition to the heart, liver, lungs, kidneys, intestine, and spleen.
Figure 2.2. Experiment Flow Chart for *In Utero* UNC0642 Exposure. In brief, mice were randomly assigned to three different treatment groups: vehicle (saline), UNC0642 at 2mg/kg and 4mg/kg. Pregnant mice were given i.p. injections starting on embryonic day 9.5 until the birth of the pups. When the offspring reached 8 weeks of age, they were put through a battery of anxiety-related behavior tests: the elevated zero maze, the marble burying test and the three-chambered social interaction test. After the completion of the tests, mice were killed via cervical dislocation. The brain was collected in addition to the heart, liver, lungs, kidneys, intestine, and spleen.
CHAPTER THREE – RESULTS

3.1 - The Effect of Chronic UNC0642 Injections on Anxiety-related Behaviours in Adult Mice

3.1.1 - Time Spent in the Open EZM Areas

Figure 3.1. Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Anxiety-related Behaviour in the EZM. Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=14-15 per group). Drug treatment had a significant effect on the time mice spent in the open EZM areas (F4,66=4.228, p=0.004). Venlafaxine at 16mg/kg (53.79±14.63s, p=0.017) and UNC0642 at 4mg/kg (54.77±11.39s, p=0.017) increased the time mice spent in the open areas. *p<0.05

One-way ANOVA revealed a significant main effect of drug treatment (F4,66=4.228, p=0.004) on time spent in the open EZM area (Figure 3.1). A Dunnett’s post hoc test revealed that the amount of time mice spent in the open areas was statistically significantly higher after venlafaxine at 16mg/kg (53.79±14.63s, p=0.017) and UNC0642 at 4mg/kg (54.77±11.39s, p=0.017) compared to the vehicle injection
(16.04±4.2 s). UNC0642 at 1mg/kg (17.19±5.16s, p=1.00) and UNC0642 at 2mg/kg (30.8±6.92s, p=0.618) did not statistically significantly increase the time spent in the open areas compared to vehicle treatment.

3.1.2 – Number of Marbles Buried

Figure 3.2. Chronic Venlafaxine at 16mg/kg, UNC0642 at 2mg/kg and 4mg/kg Decreased Anxiety-related Behaviour in the Marble Burying Test. Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=14-15 per group). Drug treatment had a significant effect on the time mice spent in open-areas (F4,66 = 17.361, p<0.001). Venlafaxine at 16mg/kg (2.57±0.49, p<0.001), UNC0642 at 2mg/kg (7.86±0.70, p=0.039) and UNC0642 at 4mg/kg (7.14±0.69, p=0.005) decreased the number of marbles buried in the marble burying test. *p<0.05, **p<0.01, ***p<0.001

One-way ANOVA showed a significant main effect of drug treatment (F4,66=17.361, p<0.001) on the number of marbles buried in the marble burying test (Figure 3.2). A Dunnett’s post hoc test revealed that the number of marbles buried was statistically significantly lower after venlafaxine at 16mg/kg (2.57±0.49, p<0.001), UNC0642 at 2mg/kg (7.86±0.70, p=0.039) and UNC0642 at 4mg/kg (7.14±0.69,
p=0.005) compared to the vehicle injection (10.4±0.88). UNC0642 at 1mg/kg (8.43±0.63, p=0.149) did not statistically significantly decrease the number of marbles buried relative to vehicle treatment.

3.1.3 - Latency to Feed in the Novel Environment after Food Deprivation

![Graph showing latency to feed in the novel environment](image)

**Figure 3.3. Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Anxiety-related Behaviour in the Novelty-suppressed Feeding Test.** Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=11-13 per group). Drug treatment had a significant effect on the latency to feed in the novel environment after food deprivation ($\chi^2(4)=10.94, p=0.027$). Venlafaxine at 16mg/kg (73.17±9.59s, $p=0.022$), and UNC0642 at 4mg/kg (84.82±20.81s, $p=0.02$) decreased the latency to feed. *$p<0.05$

Some animals could not complete the test due to fighting between cage mates, thus decreasing the sample size. Data was tested for normality using the Shapiro-Wilk test, which showed the data was not normally distributed. The Krustal-Wallis, a non-parametric alternative to one-way ANOVA, was used. The Kruskal-Wallis test showed
that there was a statistically significant difference in the latency to feed in the novel environment between the different drug treatments, $\chi^2(4) = 10.94, p = 0.027$ (Figure 3.3). 

*Post hoc* analysis showed that venlafaxine at 16mg/kg (73.17±9.59 s, p = 0.025), and UNC0642 at 4mg/kg (84.82±20.81 s, p = 0.023) had a significant effect on the latency to feed in the novel environment compared to the vehicle treatment (118.62±20.48).

UNC0642 at 1mg/kg (128.54±22.58 s, p = 0.991) and UNC0642 at 2mg/kg (87.46±15.49 s, p = 0.059) did not affect the latency to feed.

### 3.1.4 – Whole Brain H3K9me2 Levels

![Bar graph showing H3K9me2 levels](#)

*Figure 3.4. Chronic Venlafaxine at 16mg/kg and UNC0642 at 4mg/kg Decreased Global H3K9me2 Levels in Mouse Brain.* Mice were injected for 14 days. Mice received the treatment 30 minutes before being killed by cervical dislocation (N = 4 per group). Drug treatment had a significant effect on the H3K9me2 ($F_{4,15} = 4.858, p = 0.021$). Venlafaxine at 16mg/kg (0.35±0.15, p < 0.008) and UNC0642 at 4mg/kg (0.47±0.11, p = 0.03) decreased H3K9me2 level. *p < 0.05, **p < 0.01*
One-way ANOVA showed a significant main effect of drug treatment (F4,15=4.858, p=0.0103) on the global histone 3 methylation levels (Figure 3.4). A Dunnett’s post hoc test revealed the H3K9me2 levels were significantly lower after venlafaxine at 16mg/kg (0.35±0.15, p<0.008) and UNC0642 at 4mg/kg (0.47±0.11, p=0.03) compared to the vehicle injection (1±0.1). UNC0642 at 1mg/kg (0.75±0.07, p=0.396) and UNC0642 at 2mg/kg (0.63±0.12, p=0.114) did not change histone 3 methylation levels.
3.2 – The Effect of Chronic A-366 Injection on Anxiety-Related Behaviours in Adult Mice

3.2.1 - Time Spent in the Open EZM Areas

Figure 3.5. Chronic Venlafaxine at 16mg/kg and A-366 at 2mg/kg Decreased Anxiety-related Behaviours in the EZM. Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=13-14 per group). Drug treatment had a significant effect on the time mice spent in open areas (F4,63=5.453, p=0.001). Venlafaxine at 16mg/kg (60.66±7.58s, p=0.006) and A-366 at 2mg/kg (56.25±5.98s, p=0.029) increased the time mice spent in open areas. *p<0.05; **p<0.01

One-way ANOVA showed a significant main effect of drug treatment (F4,63=5.453, p=0.001) on time spent in the open areas of the elevated zero maze (Figure 3.5). A Dunnett’s post hoc test revealed that the amount of time mice spent in the open areas was statistically significantly higher after venlafaxine at 16mg/kg (60.66±7.58s, p=0.006) and A-366 at 2mg/kg (56.25±5.98s, p=0.029) compared to the vehicle injection (31.87±4.64s). A-366 at 1mg/kg (29.38±5.39s, p=0.995) and A-366 at
4mg/kg (36.88±6.72s, p=0.943) did not statistically significantly increase the time spent in open EZM areas compared to vehicle treatment.

3.2.2 – Number of Marbles Buried

![Bar Graph](image)

**Figure 3.6. Chronic Venlafaxine at 16mg/kg, and A-366 at 2mg/kg Decreased Anxiety-related Behaviour in the Marble Burying Test.** Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=13-14 per group). Drug treatment had a significant effect on the number of marbles buried (F4,63=9.865, p<0.001). Venlafaxine at 16mg/kg (3.79±0.67, p<0.001), and A-366 at 2mg/kg (6.15±0.82, p=0.001) decreased the number of marble buried in the marble burying test. ***p<0.001

One-way ANOVA showed a significant main effect of drug treatment (F4,63=9.865, p<0.001) on the number of marbles buried in the marble burying test (Figure 3.6). A Dunnett’s *post hoc* test revealed that the number of marbles buried was statistically significantly lower after venlafaxine at 16mg/kg (3.79±0.67, p<0.001), and A-366 at 2mg/kg (6.15±0.82, p=0.001) compared to the vehicle injection (10.77±0.91). A-
366 at 1mg/kg (8.29±0.79, p=0.127) and A-366 at 4mg/kg (8.43±0.96, p=0.167) did not statistically significantly decrease the number of marbles buried to vehicle treatment.

3.2.3 - Latency to Feed in Novel Environment

Figure 3.7. Chronic Venlafaxine at 16mg/kg and A-366 at 2mg/kg Decreased Anxiety-related Behaviour in the NSF Test. Mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=13-14 per group). Drug treatment had a significant effect on the latency for the mice to feed in the novel environment (F3,48=7.02, p<0.001). Venlafaxine at 16mg/kg (70.15±8.36s, p<0.001) and A-366 at 2mg/kg (85.5±7.36s, p=0.009) decreased the time mice took before feed in the novel environment. **p<0.01; ***p<0.001

One-way ANOVA showed a significant main effect of drug treatment (F3,48=7.02, p<0.001) on the latency to feed in the novel environment (Figure 3.7). A Dunnett’s post hoc test revealed that the latency to feed was statistically significantly lower after venlafaxine at 16mg/kg (70.15±8.36s, p<0.001) and A-366 at 2mg/kg (85.5±7.36s, p=0.009) compared to the vehicle injection (124.08±7.36s). A-366 at
1mg/kg (101.5±9.69s, p=0.156) did not statistically significantly decrease the latency to feed compared to vehicle treatment. The i.p. injections of A-366 at 4mg/kg 30 minutes before the test resulted in death. Thus, no animals given A-366 at 4mg/kg were tested. Immediately after the injection, the animals showed hunched backs and squinted eyes. Then 2-5 minutes later, a seizure-like state ensued and the limbs were moving uncontrollably. The seizure-like state lasted 1-2 minutes until the limbs stopped moving and the animals died.

3.2.4 – Whole Brain H3K9me2 Levels

![Bar chart showing H3K9me2 levels with vehicle, 16mg/kg Venlafaxine, 1mg/kg A-366, 2mg/kg A-366, and 4mg/kg A-366 treatments.]

Figure 3.8. Chronic Venlafaxine at 16mg/kg, A-366 at 2mg/kg and A-366 at 4mg/kg Decreased Global H3K9me2 Levels in Whole Mouse Brain. Mice were injected daily with the drug shown for 14 days. Mice received the treatment 30 minutes before the sacrifice via cervical dislocation (N = 5 per group). Drug treatment had a significant effect on H3K9me2 levels (F4,18=4.3, p=0.013). Venlafaxine at 16mg/kg (0.33±0.09, p=0.03), A-366 at 2mg/kg (0.32±0.1,
p=0.028) and A-366 at 4mg/kg (0.11±0.04, p=0.004) decreased H3K9me2 level. *p<0.05; **p<0.01

One-way ANOVA showed a significant main effect of drug treatment (F4,18=4.3, p=0.013) on the global histone 3 methylation levels (Figure 3.8). A Dunnett’s post hoc test revealed the H3K9me2 levels were significantly lower after venlafaxine at 16mg/kg (0.33±0.09, p=0.03), A-366 at 2mg/kg (0.32±0.1, p=0.028), and A-366 at 4mg/kg (0.11±0.04, p=0.004) compared to the vehicle injection (1±0.001). A-366 at 1mg/kg (0.61±0.27, p=0.272) did not change the histone 3 methylation levels.

3.3 – The Effect of In Utero Exposure to UNC0642 on Anxiety-related Behaviour in Adulthood

3.3.1 - Time Spent in the Open EZM Areas

Figure 3.9. In Utero Exposure to UNC0642 at 4mg/kg Increased Adult Anxiety-related Behaviour in the EZM. Pregnant mice were injected starting E9.5 until the birth of the pups which were tested after eight weeks (N = 6-8 per group). Drug treatment had a significant effect
on the time mice spent in the open area (F2,19=4.898, p=0.0193). UNC0642 at 4mg/kg (4.280±1.104s, p<0.05) decreased the time mice spent in the open area. *p<0.05

One-way ANOVA showed a significant main effect of in utero exposure to UNC0642 (F2,19=4.898, p=0.0193) on time spent in the open areas of the EZM (Figure 3.9). A Dunnett’s post hoc test revealed that the amount of time mice spent in the open areas was statistically significantly lower after UNC0642 at 4mg/kg during pregnancy (4.280±1.104s, p<0.05) compared to the vehicle injection (24.62±4.26s). UNC0642 at 2mg/kg (15.9±4.76s, p>0.05) did not statistically significantly decrease the time spent in the open areas compared to vehicle treatment.

3.3.2 - Number of Marbles Buried

**Figure 3.10. In utero exposure to UNC0642 at 4mg/kg Increased Anxiety-related Behaviour in the Marble Burying Test.** Pregnant mice were injected starting E9.5 until the birth of the pups which were tested after eight weeks (N = 6-8 per group). Drug treatment had a significant effect on the time mice spent in the open area (F2,23=10.65, p=0.0005). UNC0642 at 2mg/kg (6.429±0.6851, p<0.001) and UNC0642 at 4mg/kg (5.429±1.251s, p<0.01) increased the number of marbles buried in the marble burying test. **p<0.01; ***p<0.001
One-way ANOVA showed a significant main effect of in utero exposure to UNC0642 (F2,19=4.898, p=0.0193) on the number of marbles buried in the marble burying test (Figure 3.10). A Dunnett’s post hoc test revealed that the number of marbles buried was statistically significantly higher after UNC0642 at 2mg/kg (6.429±0.6851, p<0.001) and UNC0642 at 4mg/kg during pregnancy (5.429±1.251s, p<0.01) compared to the vehicle treatment (2±0.444). UNC0642 at 2mg/kg (15.9±4.76s, p>0.05) did not statistically significantly decrease the time spent in the open area compared to vehicle treatment.

### 3.3.3 - Crawley’s Three-Chambered Social Interaction Test

![Graph showing percentage time](image)

Figure 3.11. In Utero Exposure to UNC0642 at 4mg/kg Decreased Sociability-related Behaviour in the Crawley’s Three-chambered Social Interaction Test. Pregnant mice were injected starting E9.5 until the birth of the pups which were tested after eight weeks (N= 6-8 per group). Drug treatment had a significant effect on the time mice spent in the chambers with stranger mice (F2,20=3.85, p=0.0383). UNC0642 at 4mg/kg (40.86±3.303, p<0.05) decreased the time spent in the stranger mouse chamber. *p<0.05
One-way ANOVA showed a significant main effect of *in utero* exposure to UNC0642 (F2,20=3.85, p=0.0383) on the time spent in the stranger mouse chamber (Figure 3.11). A Dunnett’s *post hoc* test revealed that the time spent was statistically significantly lower after UNC0642 at 4mg/kg during pregnancy (40.86±3.303, p<0.05) compared to the vehicle injection (50±1.79). In UNC0642 at 4mg/kg treated mice, there was no statistical difference between time spent in the chamber with stranger mice and time spent in the empty chamber (p=0.675). UNC0642 at 2mg/kg (47.57±2.163s, p>0.05) did not statistically significantly decrease the time spent in the stranger mouse chamber to vehicle treatment.

### 3.3.4 – The Whole Brain H3K9me2 Levels

![Figure 3.12. In Utero Exposure of UNC0642 did not Alter H3K9me2 levels.](image)

Mice were injected for 14 days. Pregnant mice were injected starting E9.5 until the birth of the pups which were tested after eight weeks (N=3-4 per group). Drug treatment during embryonic development did not have a significant effect on H3K9me2 levels in adults (F2,8=0.009, p=0.64).
One-way ANOVA did not show a significant main effect of *in utero* exposure to UNC0642 (F2,8=0.009, p=0.64) on global histone 3 methylation levels in adult mice (Figure 3.12). The level of histone 3 methylation was measured nine weeks after drug exposure.
3.4 The effect of G9a/GLP Inhibition on Locomotion

**Distance Travelled**

- **a) Chronic UNC0642**
- **b) Chronic A-366**
- **c) In Utero Exposure to UNC0642**

**Velocity**
Figure 3.13. Chronic UNC0642 and A-366 and in utero treatment with UNC0642 did not alter locomotor activity. For chronic treatment, mice were injected daily with the drug shown for 14 days. On the day of the trial, mice received the treatment 30 minutes before the test (N=13-14 per group). a) Chronic UNC0642 did not have significant effect on total distance travelled (F4,61=0.58, p=0.68) or velocity (F4,61=0.38, p=0.83) in adult mice. b) Chronic A-366 did not have significant effect on total distance travelled (F4,45=1.06, p=0.39) or velocity (F4,45=1.1, p=0.35) in adult mice. For the in utero exposure group, pregnant mice were injected starting E9.5 until the birth of the pups, which were later tested at eight weeks of age (N=6-8 per group). c) Drug treatment during embryonic development did not have a significant effect on total distance travelled (F2,26=1.58, p=0.22) or velocity (F2,26=1.64, p=0.22) in adult mice.

To ensure that the results of the previous behavioral tests, designed to measure anxiety-related behaviours, were not the result of non-specific effects of the compounds, such as sedation or motor impairment, we examined locomotor activity. One-way ANOVA did not show a significant main effect of UNC0642 treatment on total distance travelled (F4,61=0.58, p=0.68) or velocity (F4,61=0.38, p=0.83) in adult mice (Figure 3.13a). One-way ANOVA did not show a significant main effect of A-366 treatment on total distance travelled (F4,45=1.06, p=0.39) and velocity (F4,45=1.1, p=0.35) in adult mice (Figure 3.13b). One-way ANOVA did not show a significant main effect of in utero exposure to UNC0642 on total distance travelled (F2,26=1.58, p=0.22) and velocity (F2,26=1.64, p=0.22) in adult mice (Figure 3.13c).
### 3.4 Summary of Results

**Table 3.1 Summary of Main Findings**

<table>
<thead>
<tr>
<th></th>
<th>Chronic UNC0642</th>
<th>Chronic A-366</th>
<th>In utero UNC0642</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Behavioural Studies - effect of treatment on anxiety-related behaviours</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated Zero Maze</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Marble Burying Test</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>Novelty-suppressed Feeding</td>
<td>Decreased</td>
<td>Decreased</td>
<td>N/A</td>
</tr>
<tr>
<td>Social Interaction</td>
<td>N/A</td>
<td>N/A</td>
<td>Increased</td>
</tr>
<tr>
<td><strong>Molecular Studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Brain H3K9me2 Levels</td>
<td>Decreased</td>
<td>Decreased</td>
<td>No Change</td>
</tr>
</tbody>
</table>
CHAPTER FOUR – DISCUSSION

Up to 30% of the anxiety disorder patients do not respond to currently available drug treatments (Ballenger 2000). Despite of this, no mechanistically novel drug for the treatment of anxiety disorders has come to market in the last two decades (Murrough et al. 2015). Thus, the aim of this project is to investigate novel potential drug targets for treating anxiety disorders.

Our project is guided by previous findings that some antidepressants with anti-anxiety effects inhibit G9a/GLP (Zimmermann et al. 2012). In addition knockout mice models by Schaefer et al (2009) and Baleman et al (2010) have implicated G9a/GLP in modulating anxiety. Based on these findings, we hypothesized that inhibiting pharmacologically G9a/GLP \textit{in vivo} would have anti-anxiety effects in mice.

4.1 Effect of G9a/GLP Inhibition on Anxiety-related Behaviours in Adulthood

We found that the G9a/GLP inhibitors UNC0642 and A-366 had anxiolytic-like effects in three separate behavioural tests in adult mice. These effects were comparable to studies showing reduced anxiety-like behaviour in mice with post-natal knockout of G9a in the forebrain (Schaefer \textit{et al}, 2009) and was not caused by deficiencies in locomotor activities. Furthermore, decreased anxiety-related behaviours were associated with decreased global H3K9me2 levels in the brain. As these inhibitors have quite different chemical structures (Figure 1.2), we reason that they are unlikely to both have the same off-target effects. Therefore, our findings suggest that the anxiolytic-like effects of UNC0642 and A-366 are mediated through the inhibition of G9a/GLP, which
secondarily produced global changes in brain histone methylation. Therefore, G9a/GLP could be a useful target for the development of new medications to treat anxiety.

There are some limitations to the behavioral tests used in this thesis. In the marble burying test, chronic administration of UNC0642 and A-366 resulted in mice burying fewer marbles relative to the vehicle control but more than the venlafaxine at 16mg/kg treatment group, the positive control drug. Furthermore, in the elevated zero maze and the novelty suppressed feeding test, UNC0642 and A-366 caused a similar magnitude of change as venlafaxine at 16mg/kg. In the marble burying test, the main index of anxiety-like behaviour is the number of marbles buried at the end of the test. Unlike the time spent in the open areas in the elevated zero maze and the latency to feed in the novelty suppressed feeding test, this parameter is not a continuous variable and thus, some information about behaviour is not captured. Sometimes the marbles may be partially buried but not to the 2/3 threshold for counting this test. However, if the digging behaviour was continuously scored during the entire test, the results would likely show a similar pattern to that of the elevated zero maze and the novelty suppressed feeding test. The marble burying test was selected because of its predicative validity for drugs that are clinically effective for anxiety. For example, benzodiazepines decrease the number of marbles buried (Broekkamp et al. 1986, Njung'e and Handley 1991). Given that the aim of the project was to evaluate a potential novel target for anxiety treatment, the marble burying test was useful in this regard.

Both UNC0642 and A-366 treatment caused dose-dependent decreases in global brain H3K9me2 levels, while only the UNC0642 treatment group exhibited dose-dependent reductions in anxiety-like behaviour. This can be explained by the toxicity of
A-366 at the highest dose (4mg/kg). Animals died shortly after A-366 injection while they were food deprived for the novelty-suppressed feeding test. The lack of a dose-dependent response for A-366 may be due to toxicity preventing the assessment of anxiolytic-like effects at higher doses.

The mechanism of A-366 toxicity remains unclear. Given the fact that both UNC0642 and A-366 have similar potency for inhibiting G9a/GLP, A-366 toxicity could be caused by off-target effects. This is supported by the lack of toxicity in the UNC0642 treated group. On the other hand, A-366 at 4mg/kg showed the most robust reduction of H3K9me2 levels and was the only group that suffered toxic effect. This suggests that G9a/GLP inhibition could contribute to the toxicity. It would also be important to know how much A-366 reaches the brain, and whether the toxicity arises from systemic or neural effects of G9a inhibition. One experiment that could help address these questions is to increase the dose given in the UNC0642 group. If they exhibit similar toxicity at higher doses, i.e. acute injection resulting in death after long period of food deprivation, it would suggest that A-366 toxicity was mediated through G9a/GLP inhibition.

4.1.1 - G9a/GLP is Modulated by Chronic Social Defeat

After undergoing chronic social defeat, mice that demonstrated a decrease in sociability-related behaviours had decreased G9a/GLP and H3K9me2 levels in the NAc. Interestingly, Covington et al (2011) suggest that these molecular adaptations are stress-induced pathological changes. They found that the absence of G9a function in the NAc, either by conditional knockout or local pharmacological inhibition, also caused a decrease in sociability-related behaviours. Furthermore, local NAc overexpression of
G9a was able to rescue the abnormal social interaction which is one of the measurements of anxiety-related behaviours used in this thesis project.

We found similar decreases in social interaction with adult mice after *in utero* inhibition of G9a/GLP with A-366. Although the design of our embryonic G9a/GLP inhibition experiment is very different than the social defeat experiment described above, in both cases a decrease in sociability is associated with lower G9a/GLP levels. These similarities reinforce the importance of G9a/GLP and H3K9 methylation in regulating behaviours related to anxiety and in modulating responses to stress that in turn affects anxiety behaviour. Chronic social defeat is often used to model a depression-like state (Levita et al, 2012), and thus the effect of this environmental stressor is also relevant to anxiety, which is often co-morbid with depression.

Interestingly, Hunter et al (2009, 2012) reported that acute restraint stress increased H3K9 methylation level in the hippocampus. Treatment with fluoxetine, an SSRI antidepressant that is also used to treat anxiety symptoms, decreased H3K9 methylation levels in the hippocampus. These results further emphasize that H3K9 methylation levels play a dynamic role in anxiety behaviours in response to stress. Of course different areas of the brain have very different roles in modulating anxiety and behaviour, so it would be an important future area of investigation to determine the region-specific changes in H3K9me2 and G9a/GLP levels in response to a variety of environmental stressors relevant to mood and anxiety disorders.

### 4.1.2 - Genome-wide Analysis of Histone Methylation Changes

Wilkinson et al (2009) conducted a chip-chip analysis (chromatin immunoprecipitation followed by genome-wide promoter arrays) for H3K9/K27me2 in
the NAc after mice went through chronic social defeat or chronic isolation. For the mice experiencing chronic social defeat, 1285 genes showed increased H3 methylation and 799 exhibited decreased methylation. For the mice experiencing isolation, 1448 genes showed increased methylation and 615 displayed decreased methylation. 436 genes were similarly affected in both chronic social defeat and isolation. This example further underscores the complexity of histone methylation changes in response to environmental psychosocial stressors. Different types of experimental stress paradigms produce histone methylation levels that differ from gene to gene. The table 4.1 below lists some of the common genes affected in both chronic social defeat and isolation.

While this study provides some insight into the genome-wide changes associated with H3K9me2 levels, it would be useful to examine other brain regions that are crucial for the fear response and for modulating anxiety, such as the amygdala, the hippocampus and the medial prefrontal cortex.

**Table 4.1. Genes with the Same H3K9me2 Changes after Chronic Social Defeat and Social Isolation**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes implicated in cellular inflammation, death, or redox reactions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aktip</em>(AKT Interacting Protein) / <em>Ft1</em>(Fuse toes 1)</td>
<td>General regulator of apoptosis via PKB/Akt pathway</td>
<td>Remy and Michnick 2004</td>
</tr>
<tr>
<td><em>Casp14</em> (cysteine-aspartic acid protease 14)</td>
<td>Expressed during ischemic neuronal cell death</td>
<td>Krajewska et al. 2004</td>
</tr>
<tr>
<td><em>CD84</em> (Cluster of Differentiation 84)</td>
<td>Enhances Ifn-γ secretion during inflammatory responses</td>
<td>Yan et al. 2007</td>
</tr>
<tr>
<td>Genes</td>
<td>Gene activity</td>
<td>Reference</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>Fcrlb</em> (<em>Fc receptor-like B</em>)</td>
<td>Involved in cell-mediated cytotoxicity, inflammation, and hypersensitivity</td>
<td>Daëron 1997</td>
</tr>
<tr>
<td><em>Gstm4</em> (<em>Glutathione S-Transferase Mu 4</em>)</td>
<td>Reduces oxidative stress damage by inactivating reactive intermediates</td>
<td>Hayes et al. 2005</td>
</tr>
</tbody>
</table>

**Genes implicated in cellular inflammation, death, or redox reactions**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ikbkb</em> (<em>inhibitor of kappa light polypeptide</em>)</td>
<td>Activity allows for activation NfkB-dependent pathways</td>
<td>Schmid and Birbach 2008</td>
</tr>
<tr>
<td><em>IL-16</em> (<em>Interleukin 16</em>)</td>
<td>Marker of microglial activation in inflammatory states</td>
<td>Guo et al. 2004</td>
</tr>
<tr>
<td><em>Il1ri1</em> (<em>Interleukin 1 Receptor-Like 1</em>)</td>
<td>Involved in induction and signaling in TNFα, IL-2, IL-6, IL-12, and IL-18 pathways</td>
<td>Subramaniam et al. 2004</td>
</tr>
<tr>
<td><em>IL8ra</em> (<em>Interleukin 8 Receptor Alpha</em>)</td>
<td>Increased expression following inflammatory stimuli (LPS)</td>
<td>Lee et al. 2002</td>
</tr>
<tr>
<td><em>NEDD9</em> (<em>Neural Precursor Cell Expressed, Developmentally Down-Regulated 9</em>)</td>
<td>Implicated in NfkB signaling through Tax</td>
<td>Singh et al. 2007</td>
</tr>
<tr>
<td><em>Sep-15</em> (<em>15 KDa Selenoprotein</em>)</td>
<td>Possesses anti-oxidant properties; dysregulation is implicated in neurodegenerative disorders</td>
<td>Chen and Berry 2003; Ashrafi et al. 2007</td>
</tr>
<tr>
<td><em>SLAM7</em></td>
<td>Involved in NK cytotoxicity and B-cell activation</td>
<td>Calpe et al. 2008</td>
</tr>
<tr>
<td><em>SLAM9</em></td>
<td>Unknown function; homology suggests similar function to SLAM7</td>
<td>Calpe et al. 2008</td>
</tr>
</tbody>
</table>

*NfkB* - nuclear factor kappa B; *TNFα* - tumor necrosis factor alpha; *NK* – natural killer cells
<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes implicated in cellular plasticity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fgd5 (FYVE, RhoGEF And PH Domain Containing 5)</em></td>
<td>RhoGEF predicted to be involved in actin remodeling via Rac-Pac pathways</td>
<td>Rossman et al. 2005; Buchsbaum 2007</td>
</tr>
<tr>
<td><strong>Genes implicated in cellular plasticity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sorbs3 (Sorbin And SH3 Domain Containing 3)</em>/vinexin</td>
<td>Involved in cell–cell adhesion and actin dynamics</td>
<td>Kioka et al. 1999</td>
</tr>
<tr>
<td><strong>Genes implicated in gene regulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Jdp2 (Jun dimerization protein 2)</em>/Junm2 (Mus musculus Jun dimerization protein 2)</td>
<td>Binding to c-jun causes transcriptional repression via binding to AP-1 sites; binding to ATF-2 causes transcriptional repression via effects on HDAC3</td>
<td>Eitoku et al. 2008</td>
</tr>
<tr>
<td><em>Mcm8 (Minichromosome Maintenance 8 Homologous Recombination Repair Factor)</em></td>
<td>Required for eukaryotic genome replication</td>
<td>Maiorano et al. 2006</td>
</tr>
<tr>
<td><em>Spic (Spi-C Transcription Factor)</em></td>
<td>Transcription factor similar to Pu0.1 involved in B-cell development; other transcriptional functions unclear</td>
<td>Bemark et al. 1999</td>
</tr>
<tr>
<td><em>TFIIF (Transcription factor IIIF)</em></td>
<td>Part of the required core of transcriptional initiation machinery</td>
<td>Thomas and Chiang 2006</td>
</tr>
</tbody>
</table>

AP-1 - activator protein 1; ATF-2 - activating transcription factor 2; HDAC3 - histone deacetylase 3
4.1.3 - Potential Mechanisms for Unconditioned Anxiety

The inhibition of G9a/GLP, and the subsequent changes in histone methylation levels, leads to a variety of changes in gene expression. Previous studies found that when G9a function was inhibited, Bdnf expression increased in the central nucleus of the amygdala, a major brain region involved in the anxiety response (Zhang et al. 2014). Bdnf has been implicated in both animal and human anxiety. Postnatal knockout of Bdnf generated greater anxiety-like behaviours (Rios et al. 2001) and GAD patients receiving duloxetine had increased Bdnf levels in the brain (Ball et al. 2013). In other studies, the absence of G9a/GLP function upregulated laminin family proteins, which plays an important role in cell-to-cell communication (Schéele et al. 2007). Finally, several secreted cell function regulators, such as Carboxylesterase 7, Beta-defensin 1 and Gastrokine-1, were also upregulated when G9a/GLP was inhibitied (Holmes et al. 2008; Oien et al. 2004; Niyonsaba et al. 2005). These proteins could potentially induce a variety of neuronal changes that ultimately lead to decreased anxiety-related behaviours.

4.1.4 - Potential Mechanisms for Conditioned Anxiety

Another study has found that in response to chronic social defeat, H3K9me2 levels decreased at the Ras gene and led to increases in expression level (Covington et al. 2011). Ras is a small GTPase involved in MAPK/ERK signalling, which is responsible for cell growth by increasing gene expression. In conditioned models of anxiety, activation of ERK/MAPK in the lateral amygdala is essential for the fear learning process (Di Benedetto et al. 2009).
4.1.5 - The Effect of Venlafaxine on H3K9me2

Venlafaxine was chosen as our positive control because of its clinical use in humans as a treatment for depression and anxiety, and because previous animal studies have reported strong effects in the behavioural tests we used (Dhir and Kulkarni, 2008; Thase, 2006). As shown in our results, venlafaxine decreased anxiety-related behaviours in all three behavioural tests.

Unexpectedly, we found that venlafaxine also decreased H3K9me2 levels in the mouse brain. Previous studies have documented that the tricyclic antidepressants imipramine and amitriptyline, and the serotonin reuptake inhibitor paroxetine inhibit G9a activity in vitro (Zimmermann et al, 2012b). Venlafaxine is a combined serotonin and norepinephrine reuptake inhibitor that is similar to tricyclic antidepressants. Our findings suggest that venlafaxine may also mediate its effects through targeting histone methylation enzymes. To determine if venlafaxine decreases H3K9me2 via G9a/GLP inhibition, transgenic animals lacking G9a or GLP could be given venlafaxine to assess behavioral effects. If chronic venlafaxine is unable to decrease anxiety-related behaviours in these transgenic mice, this lends support to the hypothesis that venlafaxine exerts anti-anxiety effects through G9a/GLP inhibition. In any case, the decreased H3K9me2 after venlafaxine treatment reinforces the potential for G9a/GLP as a novel anxiolytic drug target.

4.2 - In Utero Exposure of UNC0642 in Mice Increased Anxiety-Related Behaviours in Later life

We also found that inhibition of G9a/GLP near the start of cortical lamination at E9.5 results in increased anxiety-like behaviour later in adulthood. This is consistent
with previous findings showing that mice with a haplodeficiency of GLP had increased anxiety-like behaviour and deficits in social interaction (Balemans et al., 2010). We found that in utero exposure to UNC0642 was not associated with later changes in global H3K9Me2 levels. It is likely that the embryonic effects of UNC0642 on global H3K9Me2 levels did not persist until adulthood. The acute effects of G9a/GLP inhibition could have had affected neurodevelopment to produce stable alterations in anxiety set-point, while H3K9 methylation reverted to normal levels. Our results suggest that important neurodevelopmental processes regulating adult anxiety occur between E9.5 and birth, since inhibition of G9a/GLP during this time window was sufficient to alter anxiety-related behaviours.

4.2.1 - Alternative Interpretations

In rodents, maternal care can be quantified by the frequency of pup licking and grooming (LG) behavior, which is the major source of tactile stimulation for the newborns that regulates endocrine, cardiovascular, and behavioral responses (Meaney 2001). Ultimately, maternal care can have long term effects on neural systems that regulate learning and memory, neuroplasticity, and emotional and stress responses (Zhang and Meaney 2010). For instance, later in life, the offspring of low LG mothers have a lower level of glucocorticoid receptors in the hippocampus compared to the offspring of high LG mothers. This lower level of glucocorticoid receptors is caused by greater histone methylation levels at the glucocorticoid receptor gene. Low LG offspring shown a heightened response to fear and a greater ability to learn in conditioned fear models (Zhang and Meaney 2010).
Given the results from the experiments above, it is possible that the maternal style of care could be a confounding factor in our experiment. To date, the effect of G9a/GLP inhibition on maternal LG behaviour has not been studied. It could be that at the highest dose of UNC0642 (4mg/kg), the G9a/GLP inhibition decreased mothers’ LG behaviours which manifested as an increased anxiety-related behaviours in the elevated zero maze, marble burying and social interaction.

4.2.2 Apparent Discrepancies Between the In Utero and Adulthood Study

*In utero* exposure to UNC0642 increased anxiety-related behaviours while chronic UNC0642 treatment during adulthood decreased anxiety-related behaviours in mice. These results are consistent with transgenic mice created by Balemans *et al* (2012) and Schafer *et al* (2007) respectively. The opposite results between the two study cohorts could reflect different in the function and effect of G9a/GLP activity between embryonic development to adulthood. During early embryonic development, G9a/GLP function is essential for life as double knockout of G9a or GLP resulted in embryonic lethality at E9.5 due to abnormal morphology (Shinkai and Tachibana 2011). In contrast, conditional knockout of G9a in the forebrain of postnatal mice did not cause lethality.

G9a/GLP affects different genes during neurodevelopment and adulthood. During embryonic development, G9a/GLP plays a critical role in lineage differentiation by suppressing neuronal genes in non-neuronal tissues (Shinkai and Tachibana 2011). As listed in Table 4.1, G9a/GLP mediates changes in genes involved in cellular plasticity and redox reactions. Therefore the apparent opposite effect of our *in utero*
versus adult experiments could be attributed to the different biological roles of G9a/GLP at these different time points.

4.3 - Future Experiments

4.3.1 - Conditioned Models of Anxiety

One strength of our study is that we used a variety of unconditioned test for anxiety-related behaviours in mice. However, we did not use any tests involving induced models of anxiety. The previous work by Covington et al (2011) showed that H3K9me2 levels decreased after chronic social defeat in the NAc. Venlafaxine was known to rescue the anxiety-related behaviours caused by chronic social defeat (Venzala et al. 2012). Furthermore, from our study, we have found that venlafaxine and G9a/GLP inhibitors decreased H3K9me2 levels. Thus, it would be of interest to examine the effect of G9a/GLP inhibition in conditioned models of anxiety such as chronic mild stress, chronic social defeat or chronic isolation. Based on our results, G9a/GLP inhibition could potentially reverse the effects of these conditioned models of anxiety.

4.3.2 – Molecular Studies

The scope of this project with respect to molecular work is limited. The analysis of H3K9me2 levels were at the whole brain level. As discussed above, there may be differences in the changes of H3K9me2 levels that are specific to particular brain regions. Immunohistochemistry may help to visualize the changes of H3K9me2 levels across the brain regions. Based on involvement in the anxiety mechanism, we would expect to observe changes in H3K9me2 levels in the medial prefrontal cortex, amygdala, hippocampus and potentially in nucleus accumbens. Furthermore, a more
optimized histone extraction protocol could be for smaller brain regions such as the amygdala. This would allow a region-specific quantification of H3K9me2 levels within the brain. Finally, a genome-wide chip analysis could potentially identify other and novel candidate genes involved in the anxiety response mechanism.

4.3.3 - *In Utero* Exposure to UNC0642

To examine and control for the effect of maternal LG behaviour on the anxiety-related behaviours in the offspring, cross-fostering could be applied. For some litters, the mother mouse that received the vehicle treatment will be become the new adoptive mother of the offspring of the mother that received 4mg/kg UNC0642, *vice versa*. For the other litters, the mother remains with her biological offspring, as in our current experiment. The maternal LG behaviours could be tracked using the Ethovision (ver 10.1, Noldus, Netherlands) over a long period of time. Then the anxiety-related behaviours could be assessed in the behavioural tests as described in this thesis. If maternal LG behaviour has an effect on the anxiety-related behaviours later in life, then there might be a diminished UNC0642 treatment effect. Furthermore, the offspring that are brought up by low LG mothers would show a greater level of anxiety-related behaviours than the offspring that are brought up by high LG mothers.

4.4 - Limitations

The animal behavioural experiments, especially anxiety models, are known to be affected by laboratory settings and handling techniques (Champy et al. 2004; Wahlsten et al. 2003). For our experiments, all studies were conducted at CAMH, with me being the principal animal handler. However, some undergraduate students also took part of
the project. While all the handlers were trained by the staff at CAMH animal facility, the impact of different handlers on the animal anxiety-related behaviours remains unknown.

Timing of the experiments may also affect animal behaviours. All the behavioural experiments were conducted between November 2014 to May 2016 during the day between 1000h and 1700h. Seasonal and circadian influences are both known to affect animal behaviours (Valdar et al. 2006).

When creating a battery of anxiety-related behavioural tests, we adhered to the ordering principle of least to most stressful test. Furthermore, we allowed animal a day of rest between two tests. However, we are uncertain to what extent the previous testing experiences affect the animal's performance in subsequent tests. For instance, both the elevated zero maze and novelty-suppressed feeding depend on an element of novelty. While we tried to control for the room settings and equipment set up, we did not know how the previous testing experience with the elevated zero maze affected the animal's' behaviour in the novelty-suppressed feeding test.

Animals have a different baseline of anxiety-related behaviours despite being from the same source and having the same genetic background. C57BL/6J mice are known to be relatively more aggressive. In our studies, we observed several incidents with exceptionally aggressive dominant individual mice. As a result, we were forced to euthanize some badly injured animals. This also resulted in some dominant mice being singly housed, which is analogous to chronic isolation. In theory, these interventions required for animal welfare could have affected the animal’s level of anxiety and could have confounded the drug effects that we attempted to measure. However, we were still
able to observe a significant effect of drug treatment despite these potential confounding factors.

Our experiment only used male mice as a way of controlling for the effects of the estrous cycle on female animal behaviours (Meziane et al. 2007). However, in the clinical setting, females are more likely to be affected by anxiety disorders. Furthermore, sex may also impact anxiety-related behaviours in mice, thus limiting the potential generalizability of our results to both males and females.

Finally, with respect to the instrumentation, the open areas of the elevated zero maze would benefit from being wider or having ledges that prevent mice from falling off. According to our protocols, the mice that fell off the maze were put back to where they were before falling off the maze. However, falling off could be a traumatic experience for the animal and could deter them from further exploration.

4.5 - Summary and Conclusion

In summary, we show that G9a/GLP inhibition with the drugs UNC0642 and A-366 has anxiolytic-like effects in mice. In contrast, embryonic G9a inhibition from E9.5 to birth produces increased anxiety and decreased social interaction later in life, consistent with the effects of genetic knockout studies published previously. Our results provide a proof of principle that G9a/GLP inhibition could be a novel strategy for developing new medications to treat anxiety, and that there is a critical window in the second half of gestation that affects the development of later anxiety set-point.
Although it is a promising start, further drug development should proceed with caution. Our study also suggests that venlafaxine decreases H3K9me2 levels potentially via the inhibition of G9a/GLP. In conjunction to the previous study by Zimmermann et al (2012), our data suggest that antidepressants inhibit G9a/GLP activity. If it is confirmed that other antidepressants exert their anti-anxiety action through G9a/GLP inhibition, the direct inhibition of G9a/GLP could decrease side effects associated with current antidepressants, which have many other targets. However, given the ubiquity of epigenetic regulation, drugs targeting G9a/GLP may have other systemic side effects such as what we observed with A-366. The effects of G9a/GLP inhibition during early development demonstrate that these epigenetic enzymes are important in the origin of anxiety-related behaviours and further research into this area is also warranted.
REFERENCES


