The Role of Histone Demethylases in Learning and Memory in the Mushroom Body of Drosophila melanogaster

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Abstract

Intellectual disability (ID) is a neurodevelopmental disorder associated with many epigenetic regulators and chromatin modifying enzymes like histone lysine methyltransferases (KMTs) and demethylases (KDMs). Here, I systematically investigate the role of 7 KDMs: Su(var)3-3, KDM2, Lid, CG2982, UTX, KDM4B, JHDM2, and 1 KMT: trr in the context of learning and memory using Drosophila melanogaster. Genetic knockdown of each gene in the mushroom body (MB) of flies are tested for short- and long-term memory impairment using courtship conditioning. Knockdown of 6 KDMs and trr resulted in memory loss. MB morphology was analyzed to determine potential cause of memory loss. However, no gross morphological defects were observed following knockdown. This suggests the cause of memory loss is not due to structural deformities to the MB but may be due to defects in memory-dependent transcriptional activation or cell identity. These findings will help uncover the roles of KDMs in regulated neuronal processes and Drosophila memory.

Keywords:

Drosophila melanogaster, epigenetics, memory, histone lysine demethylase (KDM), histone lysine methyltransferase (KMT), courtship conditioning, mushroom body, intellectual disability
Lay Summary

Intellectual disability (ID) is a neurodevelopmental disorder characterized by limited intellectual function and adaptive behaviour before the age of 18. ID is associated with many enzymes that regulate gene transcription. Currently, there are over 350 known dominant ID genes with many of these associated with post-translational histone modifications (PTMs). These modifications alter the physical structure of DNA to determine how cells “read” genes. These PTMs have roles in defining gene expression patterns in different cell types and have also been strongly implicated in the regulation of higher brain functions, like learning and memory. There are many types of PTMs, one being histone methylation which is known to be dynamically regulated in the context of learning and memory but the function of histone demethylases in the brain is not well described. Here, I will systematically investigate the roles of several histone lysine demethylases in the context of learning and memory using the model organism, *Drosophila melanogaster*. Genetic knockdown of these genes in the memory center of the fly brain called the mushroom body (MB), were tested for short- and long-term memory defects using courtship conditioning. This memory assay utilizes the innate mating behaviour exhibited by males in an attempt to copulate with an unresponsive female. A learning defect is determined if males fail to respond to the rejection by reducing the amount of courting or a reduced memory index compared to the corresponding control. Knockdown of several of these KDMs resulted in loss of both short- and long-term memory suggesting that these genes may play a role regulating memory dependent pathways in the memory center of fly brains. To determine if these defects are caused by MB defects, we also analyzed MB morphological defects following knockdown of these genes and observed no obvious defects. Therefore, these genes do not cause a structural defect but rather may affect neuronal – cell identity or transcriptional activation.
Statement of Co-Authorship

This study was conducted under the supervision of Dr. Jamie M. Kramer. This project was devised by Dr. Kramer and organised and designed in cooperation with Dr. Kramer. All experiments were performed by the author with the exception of the RT-qPCR data which was completed by Taylor Lyons. Courtship video analysis was completed with assistance from the following work-study students and laboratory volunteers: Mohammed Sarikahya, Alycia Crooks, Jun Li Wang, Shengjie Ying and Subhrodeep Ghose.
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- Knockdown of KDM2 in the MB Impairs Short- and Long-Term Memory
- Knockdown of Lid in the MB Impairs Short- and Long-Term Memory
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<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<tr>
<td>BDSC</td>
<td>Bloomington Drosophila Stock Centre</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium&lt;sup&gt;2+&lt;/sup&gt;</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CREB binding protein</td>
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<td>Courtship index</td>
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<td>Complex associated with Set1</td>
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<td>CREB</td>
<td>cAMP element binding protein</td>
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<td>Cis-vaccenyl acetate</td>
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<td>FAD</td>
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<td>GABA</td>
<td>Gamma aminobutyric acid</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GO</td>
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<td>G&lt;sub&gt;i&lt;/sub&gt; protein alpha subunit</td>
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<td>KD</td>
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<td>Histone methyltransferase 2C</td>
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<tr>
<td>KMT2D</td>
<td>Histone methyltransferase 2D</td>
</tr>
<tr>
<td>Lid</td>
<td>Little imaginal disc</td>
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<tr>
<td>LSD1</td>
<td>Lysine specific demethylase 1</td>
</tr>
<tr>
<td>LTM</td>
<td>Long term memory</td>
</tr>
<tr>
<td>MB</td>
<td>Mushroom body</td>
</tr>
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<td>MI</td>
<td>Memory index</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO66</td>
<td>Nucleolar Protein 66</td>
</tr>
<tr>
<td>NS-XLMR</td>
<td>Non-syndromic X-linked mental retardation</td>
</tr>
<tr>
<td>NSD1</td>
<td>Nuclear Receptor Binding SET Domain Protein 1</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>PHF8</td>
<td>PHD Finger Protein 8</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PMF</td>
<td>Premated female</td>
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<tr>
<td>PTM</td>
<td>Post translational modification</td>
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<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>rut</td>
<td>Rutabaga</td>
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<tr>
<td>SAM</td>
<td>S-Adenosyl methionine</td>
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<td>SET</td>
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<td>siRNA</td>
<td>Short interfering RNA</td>
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<td>STM</td>
<td>Short term memory</td>
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<td>Su(var)3-3</td>
<td>Suppression of variegation 3-3</td>
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<td>SWIRM</td>
<td>Swi3p, Rsc8p, Moira</td>
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<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose Non-Fermentable</td>
</tr>
<tr>
<td>TRiP</td>
<td>Transgenic RNAi Project</td>
</tr>
<tr>
<td>trr</td>
<td>Trithorax related</td>
</tr>
<tr>
<td>trx</td>
<td>Trithorax</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
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<tr>
<td>UTX</td>
<td>Ubiquitously transcribed tetratricopeptide repeat, X chromosome</td>
</tr>
<tr>
<td>VDRC</td>
<td>Vienna Drosophila Resource Centre</td>
</tr>
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<td>--------</td>
<td>----------------------------------</td>
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<tr>
<td>XLID</td>
<td>X-linked Intellectual Disability</td>
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Chapter 1

1 INTRODUCTION

The formation and maintenance of memory has intrigued the neuroscience community for decades. Memory, as the basis of human behaviour, is the ability to encode, retain and retrieve information. It allows us to learn which is the acquisition of knowledge and adapt from environmental stimuli that we encounter in our day to day lives. Hence, deficiencies in this ability can inhibit individuals from functioning independently in society. As such, there has been growing interest in understanding the cellular and molecular mechanisms that underly learning and memory.

1.1 Chromatin Regulators in Intellectual Disability

Intellectual disability (ID) is a neurodevelopmental disorder that affects roughly 1-3% of the world population. It is characterized by significant limitations in cognitive function and adaptive behaviour before the age of 18. Limited cognitive function is defined by an IQ of less than 70. Limitations in adaptive behaviour are associated with deficits in conceptual, social and practical skills used and learned by individuals to function in their day to day lives. Currently, there are over 1000 genes that have been implicated in ID. Recent advances suggest that dominant \textit{de novo} mutations are the most common cause of ID. In fact, a study found that dominant \textit{de novo} copy number variations (CNVs) and single nucleotide polymorphisms cause roughly 60% of all ID cases while rare inherited forms of ID only account for only 2% of all cases (Gilissen et al., 2014). Presently, there are over 450 known dominant ID genes. The cellular components that are enriched in ID genes were assessed using Gene Ontology (GO) enrichment analysis on the known dominant ID genes. Two main categories were identified through this analysis, chromatin regulation and neuronal component. While it is understandable that neuronal components would play a large part in a neurodevelopment disorder, the connection between chromatin regulation and ID is less straightforward (Figure 1).

To begin understanding how chromatin regulators cause ID, it is important to apprehend the basic structure of how DNA is packaged and organized. Since each cell contains roughly 2 metres of DNA, a highly regulated and complex packaging system is required to ensure the DNA is accessible while inside a 5 μm nucleus. As such, roughly 147 base pairs of DNA is wrapped around a histone octamer forming a nucleosome subunit (Cutter & Hayes, 2015). These highly conserved
histone octamers are composed of two H2A-H2B dimers and one H3-H4 tetramer (Luger et al., 1997). A linker histone (H1) is used to connect the core octamers forming a structure that resembles beads on a string (Hergeth & Schneider, 2015). Chromatin is therefore defined as a complex formation of DNA and proteins found in eukaryotic cells (Kornberg, 1977). Chromatin accessibility is important in regulating gene expression and plays an essential role in establishing and maintaining cellular identity. Gene expression is dynamically regulated across the genome based on a network of permissible physical interactions of enhancers, promoters, insulators and chromatin-binding factors and chromatin accessibility plays an important part in this regulation (Klemm et al., 2019).

One major mechanism that controls the accessibility of DNA is post translational modifications (PTMs) to histone tails. These modifications are covalently bound to the exposed amino-terminal of histone tails and can be modified to alter the charge of the histone and its binding properties. One mark that has been an important focus in regulation of gene expression in ID is histone methylation (Faundes et al., 2018; Kim et al., 2017). This particular PTM is dynamically regulated by two types of enzymes, histone methyltransferase (HMT) and histone demethylase (HDM). Indeed, several HMTs and HDMs have been implicated in ID including the following histone lysine methyltransferases (KMTs): EHMT1, KMT2A, KMT2B, KMT2C, KMT2D, KMT2E, KMT5B, SETD1A, SETD1B, SETD2, NSD1, EZH2, ASH1L and demethylases (KDMs): KDM1A, KDM3B, KDM5A, KDM5B, KDM5C, KDM6A, KDM6B, PHF8 (Faundes et al., 2018; Kim et al., 2017; Parkel et al., 2013). Some examples of ID disorders that these genes are associated with include, the intragenic euchromatin histone methyltransferase 1 (EHMT1) mutations known to cause Kleefstra syndrome (KS) (Kleefstra et al., 2009). Another KMT that is associated with KS is the histone methyltransferase, KMT2C (Koemans, Kleefstra, et al., 2017). In addition, a truncating mutation in the KMT, NSD1, has been identified in 77% of patients with Sotos syndrome, a disorder commonly associated with ID (Kurotaki et al., 2002). The histone lysine demethylase, KDM6A, a known cause for Kabuki syndrome which is an ID disorder with autistic behaviour and developmental delays (Bögershausen & Wollnik, 2013; Miyake et al., 2013), JARID1C, also known as KDM5C, a histone lysine demethylase associated with non-syndromic X-linked mental retardation (NS-XLMR) (Jensen et al., 2005) and finally a recent unnamed neurodevelopmental disorder (OMIM #616728) that features facial dysmorphisms distinctive to
ID has been linked to the very first KDM discovered, *LSD1/KDM1A* (Chong et al., 2016; Rauch et al., 2012; Tunovic et al., 2014).

While there is still much to be uncovered, there is growing evidence supporting the importance of chromatin regulation through post translational histone modifications, in particular KMTs and KDMs, in regulating gene expression in neurodevelopmental disorders like ID. While genetic information is largely identical in every eukaryotic cell, different cell types can have widely different gene expression patterns. Inappropriate regulation and balance of gene expression patterns in response to developmental and environmental changes can lead to disorders like ID. Therefore, proper stability and dynamics in chromatin state influenced by histone modifications is thought to be crucial for proper gene expression important in cognitive function (Mirabella et al., 2016). While several KMTs and KDMs have been associated with ID, a large part of why ID remains without treatment is due to our lack of understanding in the role of ID genes in cognitive development. Many animal models have been developed to study the *in vivo* effects of ID genes including the use of rats, mice and flies. Here, I look at KDMs, a relatively unexplored enzyme that catalyzes the removal of methyl marks on histone proteins, to determine if KDMs play a role in chromatin regulation that influences memory formation using *Drosophila melanogaster.*
Figure 1. Gene Ontology Enrichment Analysis for Cellular Components of Dominant ID Genes.

Gene Ontology enrichment analysis (cellular components) for the 453 dominant ID genes (https://sysid.cmbi.umcn.nl/). GO enrichment analyses function by quantifying the annotated GO terms on a subset of input genes, the 453 dominant ID genes in this case, and compares their prevalence to a random sample of genes. GO terms that are over-represented in a gene set are therefore considered enriched. Bar graph represents the top 40 most highly enriched values in terms of cellular components. Terms relating to chromatin regulation are highlighted in purple.

1.2 Molecular Mechanisms of Memory

Learning is often considered as the early phase of information acquisition. The information stored and then retrieved for later use is then referred to as memory. Memory can be temporally classified into two main types, short-term memory (STM) which can be formed after brief training periods and long-term memory (LTM) which can be formed after longer and more persistent training. It is commonly accepted that LTM requires gene transcription and de novo protein synthesis while STM does not (Bourtchouladze et al., 1998; Flood et al., 1975; Igaz et al., 2002). STM formation is thought to be associated with activation of receptors and intracellular signaling cascades of secondary messengers (Androschuk et al., 2015). However, at the molecular level, both STM and LTM formation in neurons occur through the cyclic adenosine monophosphate (cAMP) pathway (Blum et al., 2009).
Much of what we know now about the cellular and molecular mechanisms of associative long-term memory started with the sea slug, *Aplysia californica*, and the fruit fly, *Drosophila melanogaster* (Brunelli et al., 1976; Quinn & Dudai, 1976). Indeed, many learning and memory paradigms teach approach or avoidance by pairing two individual stimuli together, a conditioned stimulus (CS) and an unconditioned stimulus (US) (Figure 2). Significant stimulation from the environment converge at the molecular level onto adenylyl cyclase (AC) to initiate associative memory. In *Drosophila*, STM requires cAMP signaling in mushroom body γ lobes (Zars et al., 2000). The pathway is initiated when a ligand binds to cell surface G-protein coupled receptors (GPCR). The binding of the ligand subsequently releases the α subunit of the G protein (Gαs) that encodes a GTPase that hydrolyzes GTP to GDP. The α subunit is then free to interact with *rutabaga* (*rut*) adenylyl cyclase. This interaction is terminated when the α subunit hydrolyzes GTP to GDP. Interestingly, constitutive activation of Gαs in intrinsic neurons of the MB produces learning and memory defects in *Drosophila* (Connolly et al., 1996). Gαs modulates cAMP signaling by activating AC. However, adenyl cyclase is also dependent on Ca²⁺/calmodulin to regulate cAMP levels. An influx of Ca²⁺ into neurons occurs when glutamate binds to NMDA- and AMPA-type receptors. The Ca²⁺ in the neuron will then bind to the secondary messenger, calmodulin, leading to the activation of AC and therefore increased cAMP synthesis. The cAMP secondary messenger then activates Protein Kinase A (PKA), which is an enzyme that phosphorylates protein targets found downstream of the pathway. STM is thought to involve elevations in PKA activity which in turn impacts trafficking and PTMs of synaptic proteins and ion channels (Blum et al., 2009). Homeostasis of cAMP production is maintained by the activity of cAMP phosphodiesterases (PDE) encoded by *dunce*, which degrades cAMP into adenosine monophosphate (AMP) (Dudai et al., 1976; Livingstone et al., 1984).

Like STM, LTM also requires cAMP driven PKA activity, however it requires longer bursts of PKA activity than STM (Müller, 2000). For robust LTM formation, extended PKA activity is required (T. Tully et al., 1994). The longer PKA activity can then phosphorylate the cAMP responsive element binding protein (CREB) in the nucleus to induce transcription which is required for LTM formation (Bourtchuladze et al., 1994). To initiate transcription, the transcription factor, CREB, binds to cAMP responsive element (CRE) and recruits a number of coactivators including CREB-binding protein (CBP). This binding protein is a histone acetyltransferase which highlights the importance of epigenetics in memory (Hirano et al., 2016). Although CREB is well
understood as a transcription factor involved in LTM regulation it is not the only transcription factor that is activated by learning (Alberini, 2009). A recent study has found that following courtship conditioning, the MB of trained male flies upregulates many genes that are involved in LTM formation (Jones et al., 2018). With that being said, there is still a great deal to uncover about how the process of memory is initiated and maintained especially in terms of transcriptional regulation required for memory formation.

**Figure 2. Molecular Mechanisms of Drosophila Short- and Long-Term Associative Memory.**

A simplified diagram depicting the mechanisms of short- and long-term associative memory with a particular focus placed on the cAMP signaling pathway. Signals from the environment, unconditioned stimulus (US) and conditioned stimulus (CS), are required to initiate associative memory. Activation of *rutabaga* adenyl cyclase begins with a ligand binding (L) to cell surface GPCR and an influx of Ca$\textsuperscript{2+}$ binding to calmodulin. Once intracellular cAMP is high enough, protein kinase A (PKA) will be activated. *Dunce* encoded phosphodiesterase (PDE) prevents cAMP accumulation and thus PKA inactivation. STM formation requires phosphorylation of ion channels mediated by active PKA. LTM formation requires consistent activation of PKA to the nucleus to induce transcription by phosphorylating cAMP responsive element binding protein (CREB).
1.3 Mechanisms of Histone Methylation and Demethylation

Histone methylation is largely considered to be a stable mark and is highly site specific, meaning that distinct HMTs modify a single residue often to a certain degree of methylation (Soares et al., 2017). The stability of this histone methylation is mostly due to the high thermodynamic stability of the N-CH$_3$ bond in addition to its relatively long half-life. Unlike other histone modifications that influence net charge of the residue they modify, histone methylation works by acting as a recognition site for effector proteins that can change the chromatin environment between repressive and active transcription (Taverna et al., 2007). This change is dependent on the number of methyl groups on the specific residue. Histone methylation is mediated by histone methyltransferases and these marks are removed by histone demethylases. While there are two other residues reported, arginine and histidine methylation, the focus of this thesis will be on the methylation of lysine residues (Greer & Shi, 2012). Histone lysine methyltransferases can be subdivided into two domains, the SET [suppressor of position-effect variegation 3-9 (Su(var)3-9), enhancer of the eye colour mutant zeste (En(zeste)), and the homeotic gene regulator Trithorax] containing domain and non-SET containing domain (Black et al., 2012; Cheng, 2014). Lysine residues can be unmethylated, mono- (me1), di- (me2), or tri-methylated (me3) on their ε amine group. Methylation on lysine residues use S-Adenosyl methionine (SAM) as a cofactor and methyl donor group (Black et al., 2012). Indeed, many studies have shown that histone lysine methyltransferases tend to have a high degree of enzymatic specificity, for example KMT1A/B tri-methylates histone 3 lysine 9 (H3K9me3) from a monomethylated state (H3K9me1) (Peters et al., 2002). On the other hand, KMT1C (also known as G9a) methylates to a di-methylate (H3K9me2) preferentially from a mono-methylated state (Tachibana et al., 2002).

It wasn’t until 2004 when the first histone lysine demethylase, Lysine Specific Demethylase 1 (LSD1) was discovered (Shi et al., 2004). With the initial discovery of LSD1, decades of debate over the reversibility of histone methylation ended and our understanding of the homeostatic regulation of histone methylation began. Since then, over 20 different KDMs have been identified and characterized (Table 1). The LSD family was the first to be discovered and contains a flavin adenine dinucleotide (FAD) monoamine oxidase domain that demethylates H3K4me2 and H3K4me1 (Yujiang Shi et al., 2004). Therefore, LSD1/KDM1A is only able to demethylate mono- (me1) and di-methylated (me2) lysine residues. The demethylase works by oxidative cleavage of
the α-carbon bond of the methylated lysine to form an imine intermediate that will then hydrolyze to form formaldehyde, releasing the demethylated lysine as well as one molecule of H$_2$O$_2$ (Yujiang Shi et al., 2004). LSD1 is comprised of SWIRM (derived from Swi3p, Rsc8p, and Moira) and nicotinamide adenine dinucleotide – binding (NAD-binding) domains (Chen et al., 2006; Tochio et al., 2006). Since the LSD family is unable to demethylate tri-methylated lysine residues, researchers began to look for other classes of HDMs and discovered the evolutionarily conserved protein group known as the Jumonji (JmjC) family (Klose et al., 2006). Indeed, it has been suggested that the JmjC histone demethylases favour trimethylated substrates (Cloos et al., 2008). The JmjC domain is used to catalyze demethylation through the oxidative methyl groups. The JmjC demethylases rely on α-ketoglutarate, O$_2$ and Fe(II) as cofactors for demethylation (Yang Shi & Whetstine, 2007). Similarly, to KMTs, KDMs also display high specificity for both the site and degree of methylation. For example, KDM4A-KDM4D can remove H3K9me3/H3K9me2, H3k36me3/H3K36me2, and H1.4K26me3/H1.4K26me2 but are unable to remove H3K9me1 or H3K36me1 (Cloos et al., 2008; Klose et al., 2006; Trojer et al., 2009; Whetstine et al., 2006).

1.4 Biology of Histone Lysine Methylation

The dynamic process of histone methylation requires proper function of both histone methyltransferase and demethylase. The importance of these marks in chromatin regulation is highlighted by the fact that these enzymatic products are highly conserved (Table 1). One particular example is methylation of H3K4. This specific modification is catalyzed by a highly conserved complex called the COMPASS (Complex Proteins Associated with Set1) complex (Miller et al., 2001). The SET domain genes are a highly conserved gene family that encodes proteins with chromatin based transcriptional activities that have been uncovered from yeast to humans. Initial discovery in yeast identified only one COMPASS H3K4 methyltransferase, Set1 (Nislow et al., 1997). In Drosophila, the COMPASS complex is divided into three family members, dSet1/ COMPASS which is the direct descendent of the yeast Set1 complex and two COMPASS-like complexes, Trithorax (trx) and Trithorax-related-containing (trr) complex (Mohan et al., 2011). Mammals, with higher corresponding complexity have six COMPASS family members, KMT2A, KMT2B (homologs of trx), KMT2C, KMT2D (homologs of trr) and KMT2E and KMT2F (homologs of dSet1) (Collins et al., 2019). All of these enzymes are
responsible for H3K4 mono-, di- and trimethylation with non-redundant functions (Shilatifard, 2012). While H3K4 is generally considered an active mark the degree of H3K4 methylation also corresponds with different activities. For example, H3K4me1 is most abundant toward the end of genes (Pokholok et al., 2005) and enhancers (Rada-Iglesias, 2018), H3K4me2 is enriched in intragenic regions and can also mark enhancer regions (He et al., 2010), and finally H3K4me3 is highly enriched near the transcription start site (TSS) of active genes (Barski et al., 2007). Another well studied mark is H3K9 which is commonly considered a repressive mark, specifically H3K9me2/me3. This is due to their colocalization with heterochromatin and enrichment at inactive genes (Hathaway et al., 2012; Peters et al., 2002). While, H3K9 methylation has been implicated in gene silencing, a large-scale analysis found that H3K9me3 is enriched in many active promoters (Squazzo et al., 2006). Finally, H3K27 methylation has been traditionally considered to be a repressive mark however genomic studies found that H3K27me3 can colocalize with H3K4me3 at bivalent promoters which drive low expression levels (Bernstein et al., 2006). In addition, recent studies in Drosophila mutants show that H3K27 methylation is essential for Polycomb-mediated gene repression (Pengelly et al., 2013). In some cases, histone methylation may also play a role in nucleosome stability as well as a regulatory function. In fact, some studies suggest that transcriptional regulation is not the primary role of some HMTs like H3K36 methyltransferase Set2 (Lenstra et al., 2011). For example, in gene bodies, H3K36me3 associates with the chromodomain protein Eaf3 found in the conserved Rpd3S lysine deacetylase complex. In yeast, deletion of Eaf3 or the H3K36 methyltransferase Set2 increases histone acetylation in gene bodies. This suggests that H3K36me3 is responsible for recruitment of Rpd3S to gene bodies but was later shown that loss of H3K26me3 or Eaf3 chromodomain protein does not affect Rpd3S localization suggesting that H3K36me3 played a role in regulating the catalytic activity of Rpd3S instead (Carrozza et al., 2005; Joshi & Struhl, 2005; Keogh et al., 2005; B. Li et al., 2007)

Amongst the various other histone modifications, methyl marks have been implicated in many roles in development and pathological processes due to their stability (Barski et al., 2007). Cognitive ability and disorders like ID are thought to result from changes in brain transcriptomes. Histone modification patterns provide insight on chromatin state and thus gene transcription which are important in cognitive function. Generally, H3K4, H3K36 and H3K79 methylations are considered to correspond with active transcription, whereas H3K9, H3K27 and H4K20 methylations are thought to be associated with repressed transcription (Black et al., 2012). These
genome-wide analyses provide insight on how histone modifications and other genomic elements are regulated and serve as a foundation for future research in genome structure and function as well as help better understand the role of chromatin regulators.

Table 1. Conservation of Histone Lysine Demethylases from *Drosophila* to Humans.

<table>
<thead>
<tr>
<th>Histone Demethylase Activity</th>
<th><em>Drosophila</em> KDM</th>
<th>Human KDM Ortholog</th>
<th>Predicted Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4</td>
<td>*Su(var)*3-3</td>
<td>KDM1A/LSD1</td>
<td>H3K4me1/2</td>
</tr>
<tr>
<td></td>
<td><em>Kdm2</em></td>
<td>KDM2A, KDM2B</td>
<td>H3K4me3, H3K36me1/2</td>
</tr>
<tr>
<td></td>
<td><em>Lid</em></td>
<td>KDM5A, KDM5B, KDM5C, KDM5D</td>
<td>H3K4me2/3</td>
</tr>
<tr>
<td></td>
<td>NO66 (CG2982)</td>
<td>NO66, MINA53</td>
<td>H3K4me2/3</td>
</tr>
<tr>
<td>H3K9</td>
<td>KDM4A</td>
<td>KDM4A, KDM4B, KDM4C, KDM4D, KDM4E</td>
<td>H3K9me2/3, H3K36me2/3</td>
</tr>
<tr>
<td></td>
<td>KDM4B</td>
<td>KDM4A, KDM4B, KDM4C, KDM4D, KDM4E</td>
<td>H3K9me2/3, H3K36me2/3</td>
</tr>
<tr>
<td></td>
<td>JHDM2/ KDM3</td>
<td>KDM3A, KDM3B, KDM3C</td>
<td>H3K9me2</td>
</tr>
<tr>
<td>H3K27</td>
<td><em>UTX</em></td>
<td>KDM6A, KDM6B, UTY</td>
<td>H3K27me2/3</td>
</tr>
<tr>
<td></td>
<td><em>Jarid2</em></td>
<td>Jarid2</td>
<td>No histone demethylase activity (Sanulli et al., 2015).</td>
</tr>
</tbody>
</table>

1.5 Histone Methylation and Demethylation in Neurons

A critical component of neuronal function is the dynamic regulation of transcription by chromatin regulation (Borrelli et al., 2008). Through environmental stimuli, neurons continuously adapt their gene expression patterns making them a good substrate to study the function of chromatin regulators like HMTs and HDMs (Swahari & West, 2019). Indeed, several HMTs and HDMs have been studied in neuronal function and have found crucial roles in development, cell fate and disease. Although the function of these enzymes is not limited to neurons, it will be the main focus of this thesis. Whilst methylation is largely considered a stable mark, a study looking at acute and chronic stress suggested that methyl marks may be subject to rapid change. Acute and chronic stress were able to influence changes in H3K9me3 and H3K27me3 in the hippocampus highlighting an effect of chromatin modifications in normal cognitive processes (Hunter et al.,
Studies in mice found that neuronal ablation of the H3K4 methyltransferase, \textit{KMT2A/Mixed-lineage leukemia 1 (Mll1)} in the postnatal forebrain and adult prefrontal cortex neurons in mice is associated with increase anxiety, cognitive deficits and locomotor dysfunction (Jakovcevski et al., 2015). Another study looking at mice lacking the \textit{KMT2B/MLL2} gene in adult forebrain neurons found deficits in STM and LTM (Kerimoglu et al., 2013). Another study looking at rats found deficiency in \textit{MLL1} displayed memory defects in contextual fear conditioning (Gupta et al., 2010). In humans, mutations in \textit{KMT2A} and \textit{KMT2B} are associated with Weidmann-Steiner syndrome and Dystonia 28, respectively, and both disorders are associated with ID (Collins et al., 2019). One particular mark, H3K4me3, near gene promoters has been correlated with high levels of transcriptional activity (Barski et al., 2007, Santos-Rosa et al., 2002). In fact, several studies have looked at H3K4me3 as a regulator of memory formation (Collins et al., 2019). Therefore, it isn’t surprising that all of the known H3K4 methyltransferases and 4/6 H3K4 demethylases have been associated with impaired cognitive function (Collins et al., 2019). In fact, several KDMs have gene regulatory functions in neurons including \textit{LSD1/KDM1A}, \textit{KDM6B} and \textit{KDM5C} (Swahari & West, 2019). In adult mice, loss of \textit{LSD1/KDM1A} resulted in paralysis, widespread neuronal death in the hippocampus and cortex as well as learning and memory defects (Christopher et al., 2017). Memory in the adult mice were assessed using the Morris water maze and fear conditioning assays prior to the onset of motor defects (Christopher et al., 2017). This suggests that continuous expression of \textit{LSD1} in adult mice brains are required for the maintenance of proper neuronal function. Mutations in \textit{LSD1}’s demethylase function in human brain development has been associated with ID (Pilotto et al., 2016). The results of this study found three missense point mutations mapped on \textit{LSD1} associated with a variety of pathological conditions including neurological disorders like ID (Pilotto et al., 2016). Another KDM that has shown to play an important role in neuronal function is \textit{KDM6B}. Specifically, \textit{KDM6B} acts in postmitotic neurons to regulate synaptic function. Loss of \textit{KDM6B} function resulted in impaired late upregulation of GABA and glutamate receptors upon synaptic function (Wijayatunge et al., 2018). Finally, familial mutations in \textit{KDM5C} has been identified as one of the more frequent causes of X-linked ID (XLID) (Jensen et al., 2005). \textit{KDM5C} knockout mice offer a model to study the neurological effects of \textit{KDM5C} disruption since the model exhibits many cognitive and social abnormalities seen in patients with the mutation. At a cellular level, neurons of the knockout mice have dendritic branching defects (Iwase et al., 2016). In addition, studies looking at \textit{KDM5C} knockout mice found...
an upregulation of a large set of genes suggesting that $KDM5C$ acts as a transcriptional repressor (Iwase et al., 2016; Scandaglia et al., 2017). While most ID-associated mutations in $KDM5C$ disrupt the enzymatic function, a point mutation was identified that neither disrupts protein stability or enzymatic function suggesting a non-histone demethylase function of $KDM5C$ that contributes to brain development (Vallianatos et al., 2018). These are just a few studies highlighting the importance of histone methyltransferases and demethylases in cellular development and function. While the function of these enzymes remains to be fully explored it is important to understand the roles these enzymes play in neurons to help develop therapeutics for disorders like ID.

1.6 *Drosophila* as a Model to Study Learning and Memory

The fruit fly, *Drosophila melanogaster*, has been used as a model organism in genetic research for over a century beginning in 1901 with William Castle but undoubtedly “fathered” by T.H. Morgan in 1910 with his discovery of the white eyed fly (Morgan, 1910). Research using *Drosophila* is aided by a wide variety of sophisticated genetic and molecular tools available to the fly community. In comparison to the human genome, the fly genome is considerably smaller and is comprised of four chromosomes that encode around 120 million base pairs of DNA. Despite the large difference in genome size, approximately 75% of human disease genes are conserved in the fly (Reiter et al., 2001). Despite the divergence between humans and flies, the molecular mechanisms that underly learning and memory are conserved between the two. In addition, model organisms like the fly allow researchers to use reverse and forward genetics to provide insight between the link of gene mutation and cognitive phenotypes in a simpler model than humans. In fact, many genes that were first characterized in *Drosophila* have subsequently been identified and studied in higher order mammals like mice and humans. Initial olfactory conditioning assays by Seymour Benzer revealed the capacity of *Drosophila* memory by associating certain odours with foot shock punishment (Benzer, 1967). Benzer used forward genetics to investigate various behaviours like learning by inducing mutations in flies and then screening individuals for phenotypes (Benzer, 1967). Several genes have been identified for abnormal olfactory learning including *dunce* (Dudai et al., 1976) and *amnesiac* (Quinn & Dudai, 1976). Many genes that regulate memory were first elucidated in these genetic screens using *Drosophila*. This includes
many memory related genes like *rutabaga* a gene that encodes adenylyl cyclase and *dunce* a gene that encodes a cAMP-specific phosphodiesterase (Akalal et al., 2006; Tim Tully, 1996). Several paradigms have since been developed to study learning and memory in flies including aversive shock conditioning, appetitive olfactory conditioning and courtship conditioning (Pitman et al., 2009). These memory assays can be used as a phenotype to understand the genetic connection between cognition and certain cellular and molecular components. Indeed, many individuals with ID often have impaired memory making this intellectual ability a good behaviour to study. Furthermore, research using *Drosophila* offers a number of practical advantages including relatively low costs, short life cycles that roughly take ten to twelve days, and a sizable number of progeny per female thus making it easy to generate large numbers for an experimental approach (Jennings, 2011).

1.6.1 Histone Methylation and Demethylation in *Drosophila melanogaster*

Most studies that have been mentioned have involved humans or model organisms like rats and mice when studying histone methyltransferase and demethylase dysfunction in the brain. However, there are several other model organisms like the fruit fly that have been used to study chromatin regulators and neuronal function. Flies offer a small and robust model to study the functions of these chromatin regulators in post mitotic neurons. Furthermore, many of the cellular pathways that are important for learning and memory formation are conserved from flies to humans. For example, the *Drosophila* euchromatin histone methyltransferase (EHMT) is a conserved protein family that is responsible for the methylation of H3K9. Mutations in *EHMT1* has been known to cause Kleefstra Syndrome, a severe form of ID (Kramer et al., 2011). Another study in flies has identified that loss of *lid*, the fly ortholog for *KDM5C*, cause cognitive defects and reveals a role for this enzymatic function in gene activation (Zamurrad et al., 2018). Another study in flies has identified several JmjC mutants, including *NO66* and *KDM2*, play a role in modulating circadian rhythm (Shalaby et al., 2018). This finding suggests that rather than a developmental role, JmjC proteins like many KDMs, function as regulators of behaviour (Shalaby et al., 2018). These are just a few studies to highlight the importance of using fruit flies to study the *in vivo* effects of chromatin regulators like KMTs and KDMs.
1.6.2 The Mushroom Body

The olfactory learning and memory center of the *Drosophila* brain is found in a pair of symmetrical neuropil structures called the mushroom body (MB) (Heisenberg, 2003). These neuropil structures are comprised approximately 2500 densely packed intrinsic neurons called Kenyon cells (KC) (Johard et al., 2008). Several studies have shown evidence that the MB is critical for olfactory learning and memory (De Belle & Heisenberg, 1994; Heisenberg et al., 1985). In addition, many genes known to be important for olfactory learning and memory has shown to be preferentially expressed in the MB (Crittenden et al., 1998). In fact, many components of the CREB-pathway like *rutabaga* adenylyl cyclase has shown elevated levels in the MB (Han et al., 1992). The mushroom body receives olfactory information from the environment through the antennal lobe which then gets relayed to the calyx of the MB. The dendrites of the KC project into the calyx and axons through the peduncles into the central brain to form three different subtypes of five distinct lobes, α, β, α’, β’, and γ lobes (Aso et al., 2009; T. Lee et al., 1999). These lobes are considered to be the main output site of the KC. Throughout development, the MB neuroblasts continually divide to give rise to the 3 major classes of MB neurons (α/β, α’/β’, and γ) (Kurusu et al., 2002). The different MB neurons arise in sequential order beginning with the γ neurons. During the late embryonic and early larval stages of development, the γ neurons project their axons in both the dorsal and medial directions (Lee et al., 1999). Formation of the α’/β’ neurons follows at the late larval stage and finally the α/β form during the pupal stage (Lee et al., 1999). The MB presents a very prominent display of structural plasticity and continues to morph during development as shown by the pruning of the γ neurons back to the peduncle followed by the re-extension of their axons into the medial lobe during the pupal stage (Lee et al., 1999).

It is widely accepted that proper MB development is critical for proper olfactory learning and memory to occur (Heisenberg et al., 1985). In fact, different MB neurons could be supporting diverse functions by distinct transcriptional profiles found in the different MB neurons subtypes (Shih et al., 2019). Current research shows that the α/β neurons play a distinct role in LTM formation and are important for memory retrieval (Akalal et al., 2006; Huang et al., 2012). Indeed, an investigation on a mutant called α-lobe absent (*ala*), flies that lacked either an α or β lobes, found that when both α-lobes were missing flies lacked LTM at 24 hours (Pascual & Préat, 2001).
In addition, the α’/β’ lobes are required for memory stabilization in aversive and appetitive odour memory (Krashes et al., 2007) and to retrieve immediate memory (Wang et al., 2008). The γ lobes are thought to represent the main neuronal substrate for STM primarily supported by the fact that transgenic expression of *rut*+ in the γ lobes is able to rescue learning defects of *rutabaga* mutants (Zars et al., 2000). Therefore, looking at the broad phenotypic characterization using mushroom body specific transgenic techniques can be a starting point for understanding whether or not KDMs are important for MB development in post-mitotic neurons.

### 1.6.3 Courtship Conditioning as a Learning and Memory Paradigm

Courtship conditioning is a memory assay that is used in behavioural analysis. Utilizing a natural *Drosophila* behaviour, courtship conditioning allows for ethological observation in a laboratory setting (Kamyshev et al., 1999; Siegel & Hall, 1979). The assay utilizes successive training and functional learning and memory with previously mated females (PMF) to suppressed courting attempts from the males when paired with subsequent females. Males that have successfully learned maintain suppressed courtship attempts for hours to days depending on the length of training and the persistence of neuronal circuit activity. Research utilizing this assay has found that the MB is required for courtship memory and that MB ablation result in STM and LTM impairment (McBride et al., 1999). In fact, a study found that courtship conditioning not only requires the MB but also uses neuronal circuitry similar to those seen in appetitive memory (Keleman et al., 2012; Montague & Baker, 2016; Zhao et al., 2018).

Most learning and memory paradigms teach approach or avoidance by pairing two individual stimuli, for example, a classical conditioning experiment pairing an odour with an electric shock (Malik & Hodge, 2014). However, courtship conditioning utilizes a natural stimulus, another fly, to teach a complex form of learning through reduced courtship. In courtship conditioning, researchers observe innate male courtship behaviour through a number of easily identifiable moves including orientation towards the female, chasing the female, taping the female, extension of one of his wings and attempting copulation (Koemans, Oppitz, et al., 2017; Sokolowski Marla B., 2001). However, when males are paired with a previously mated female (PMF) the female is unresponsive to the courting attempts and will subsequently reject the male flies’ courtship efforts. In addition, a pheromone called cis-vaccenyl acetate (cVA) gets deposited...
on the female during copulation which inhibits other males from exhibiting courtship behaviour towards the PMF (Billeter & Levine, 2015; Koemans, Oppitz, et al., 2017).

Courtship conditioning is used to measure the time spent courting and compares this time between a trained male fly to a socially naïve male fly. This is used to quantify the capacity of the trained fly to learn and form memories of rejection and therefore suppress courting when being tested. By altering the duration of training and subsequent isolated rest period, courtship can be used to study both STM and LTM. Flies with dysfunctional memory will be unable to suppress courting behaviour and will continually court new PMFs despite previous training.

### 1.7 Rationale and Objective

A wide range of human disorders has been associated with the misregulation, mutation, amplification and deletion of histone modifications including many that affect cognitive function like ID (Black & Whetstine, 2013; Cloos et al., 2008). Although several KDMs and KMTs have already been implicated in the etiology of ID, the in vivo effects of these genes are not well characterized.

Previous studies in our lab has identified a role for individual components of the SWI/SNF complex, a chromatin remodeling complex, in Drosophila learning and memory. Specifically, Brm, Bap60, Snr1 and E(γ)3 are required for STM and LTM while osa was only required for LTM (Chubak et al., 2019). Furthermore, the study revealed that certain SWI/SNF components are required for axon pruning of the mushroom body γ lobes during γ neuron remodeling. GO analysis also revealed that the SWI/SNF complex is the most over-represented cellular component disrupted when it comes to ID (Figure 1). Another study in our lab found that the H3K4 histone methyltransferase, trx, was only required for long-term memory in flies (Raun, 2019). While other components like Set1 are required in both short- and long-term memory (Raun, 2019). Further research on H3K4 methyltransferases found that knockdown of trr resulted in STM loss however the LTM effects have yet to be studied (Koemans et al., 2017). Moreover, the H3K9 methyltransferase, G9a, was found to regulate habituation which is a form of non-associative memory and is required in courtship memory (Kramer et al., 2011). Through these studies we have gained novel insight into chromatin regulators and how they function in Drosophila memory. Since it is established that histone marks like H3K4 and H3K9 can be dynamically regulated in the brain through KMTs and KDMs we decided to conduct a broad screen on KDMs to determine
how they may mediate gene regulation in post-mitotic neuronal development and function. Considering the high level of conservation between human and flies in terms of KDMs, KMTs, and the conserved molecular mechanism of memory formation the results from this research should be broadly applicable to understanding memory biology. We rationalize that

As such, I used *Drosophila melanogaster* to investigate the role of 7 different KDMs and 1 KMT in their functional role in associative memory in the MB. The overall goal of this project is to use *Drosophila melanogaster* as a model organism to screen KDMs for potential effects on MB development and courtship memory. I hypothesize that some KDMs will be required to regulate *Drosophila* courtship memory since brain function is dynamically regulated and many KMTs have been associated with regulation of brain function. In this research project, I aimed to:

1) Use a systematic genetic knockdown in the MB for individual *Drosophila* KDMs to establish a role in short- and long-term memory using courtship conditioning
2) Determine if knockdowns of the KDMs affect gross MB morphology

This study is the first to investigate the roles of *Drosophila* KDMs and *trr* in post-mitotic neurons in a brain region relevant to memory formation. Therefore, this project will help further expand on the role of these KDMs and *trr* in memory and to help guide future research.
Chapter 2

2 METHODS

2.1 Fly Husbandry and Stocks

All fly stocks were maintained over a standard mixture of sugar, cornmeal, yeast and agar media at room temperature in 35mL plastic vials. All experimental flies were reared at 25°C and 70% humidity with a 12-hour light-dark cycle. Fly stocks used were either obtained from Bloomington Drosophila Stock Center (BDSC) (Bloomington, IN, USA) (Perkins et al., 2015) or Vienna Drosophila Resource Center (VDRC) (Vienna, Austria) (Dietzl et al., 2007). Female flies used for courtship conditioning were generated using Canton-S and Oregon-R mixed genetic background by J.M. Kramer.

Inducible RNAi knockdown flies obtained from BDSC were generated through the Transgenic RNAi Project (TRiP) by inserting the hairpin RNA using a VALIUM (Vermilion-AttB-LoxP-Intron-UAS-MCS) 1, 10, or 20 vector into the genomic landing site attP40 (chromosome 2) or attP2 (chromosome 3). TRiP lines utilizing the 1st generation VALIUM 1 and 10 vectors result in a long hairpin RNA which required co-expression of UAS-Dicer-2 to increase knockdown efficiency (Dietzl et al., 2007). Lines generated using 2nd generation VALIUM 20 utilize short hairpin RNA molecules and include a combination of second and third chromosome transgenes. The VDRC lines used were obtained from two different genetic libraries, KK and GD. The KK library from VDRC was generated using the φC31 mediated site-directed recombination at the VIE-260B landing site on the second chromosome (Green et al., 2014). The GD genetic library from VDRC was generated using a random P-element insertion (Dietzl et al., 2007).

All controls used in the experiment had the same genetic background as their respective transgenic RNAi constructs but without the P-element or transformation vector insertion. The exception to this is TRiP lines that used VALIUM20 insertions. Controls for the RNAi TRiP lines with VALIUM20 insertions used a hairpin stock targeting mCherry (mCherry-RNAi) instead of the attP2 genetic background stock due to the presence of scutoid [sc*] which is found on the VALIUM20 RNAi stocks’ X chromosome. Experimental flies from different RNAi lines and appropriate controls were crossed to flies from the same GAL4 driver stock therefore all
knockdown flies had the same genetic background as their respective controls. A list of all fly stocks used alongside a brief description can be found in (Appendix A).

2.2 Mushroom Body-Specific Knockdown of KDMs using the GAL4-UAS System

All experimental flies were reared at 25°C and 70% humidity with a 12-hour light-dark cycle. Knockdown of KDMs in the mushroom body (MB) was achieved using the GAL4-UAS system to induce RNA interference (RNAi) mediated knockdown. This bipartite system utilizes GAL4, a yeast transcription factor, that activates expressions of genes under the control of an Upstream Activating Sequence (UAS) enhancer (Brand & Perrimon, 1993). The GAL4-UAS system allows for tissue-specific gene expression to target knockdown in the learning and memory center of the fly brain. In addition, to focus on the learning and memory aspect of this experiment, the learning and memory part of the brain should be the only part affected. Therefore, knockdown was restricted to the MB using the transgenic \textit{R14H06-GAL4} “driver” construct which expresses GAL4 under the MB specific enhancer fragment from the \textit{rutabaga} gene (Jenett et al., 2012).

To generate knockdown flies, homozygous male \textit{R14H06-GAL4} (BL48667) “driver” flies were crossed with virgin homozygous female “responder” flies expressing \textit{UAS-RNAi} sequences specific to a \textit{Drosophila} KDM mRNA transcript as well as their corresponding controls (Figure 4) (Table 2). Gal4 induces expression of hairpin RNA (hpRNA) in the progeny of the parental crosses which then get processed into small interfering RNAs (siRNAs) by Dicer-2 triggering the formation of the RNA-induced silencing complex (RISC) to direct sequence-specific degradation of the target mRNA which results in knockdown of our gene of interest. Two types of hairpin transcripts can be transcribed, short and long hpRNA. While endogenous Dicer-2 is sufficiently effective at processing short-hairpin RNA, long-hairpin RNAi libraries (TRiP’s VALIUM 1 VALIUM10 collections and VDRC’s GD and KK libraries) required co expression of \textit{UAS-Dicer2}. Only the F1 heterozygous males from the \textit{R14H06-GAL4} and \textit{UAS-RNAi} crosses were collected and isolated at eclosion for 4 days before being used for courtship conditioning.

MB-specific knockdown of KDMs was conducted using at least two different RNAi stocks targeting the same genes but with different target sequences. The exception to this was \textit{JHDM2} and \textit{NO66} which displayed difficulty collecting male progeny for memory testing in one of the
lines used. When possible, different transgenic libraries were chosen to control for possible off-target effects and differences in genetic backgrounds.

Figure 3. The GAL4-UAS system allows for mushroom body specific knockdown of KDM gene expression.

MB-specific driver, R14H06-GAL4, drives expression of RNAi constructs under the control of Upstream Activation Sequence enhancer. Homozygous male R14H06-GAL4 drivers are crossed to homozygous UAS-RNAi female flies. Progeny of the cross result in heterozygous flies that have enabled GAL4/UAS binding and expression of our gene of interest in the mushroom body. The RNAi pathway is initiated by Dicer-2 which cleaves dsRNA into siRNA. The antisense strand of the siRNA binds to the RISC complex that guides the complex to the target mRNA to initiate target mRNA degradation.
Table 2. List of control and sample KDM knockdown genotypes used in both MB-specific knockdown.

<table>
<thead>
<tr>
<th>Lethality Assay Controls</th>
<th>Control Genotypes</th>
<th>Knockdown Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{Act5C - GAL4}{Y} ) UAS - mCherry^{RNAi} \ + \ + \ +</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{Act5C - GAL4}{Y} ) UAS - RNAi + + +</td>
</tr>
<tr>
<td></td>
<td>( \frac{y^1, v^1}{Y} ) attP40 +</td>
<td>( \frac{y^1, v^1}{Y} ) Act5C - GAL4 - +</td>
</tr>
<tr>
<td>attP2 (Dicer-2)</td>
<td>( \frac{y^1, v^1}{Y} ) Act5C - GAL4 attP2 +</td>
<td>( \frac{y^1, v^1}{Y} ) Act5C - GAL4 UAS - RNAi + +</td>
</tr>
<tr>
<td>GD (60000)</td>
<td>( \frac{w^{1118}}{Y} ) Act5C - GAL4 +</td>
<td>( \frac{w^{1118}}{Y} ) Act5C - GAL4 UAS - RNAi + +</td>
</tr>
<tr>
<td>KK (60100)</td>
<td>( \frac{y^1,w^{1118}}{Y} ) attP, ( y^+ ), ( w^{3^r} ) +</td>
<td>( \frac{y^1,w^{1118}}{Y} ) UAS - RNAi + +</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Courtship Controls</th>
<th>Control Genotypes</th>
<th>Knockdown Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{UAS - mCherry^{RNAi}}{Y} ) + +</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{UAS - RNAi}{Y} ) + + +</td>
</tr>
<tr>
<td></td>
<td>( \frac{y^1, v^1}{Y} ) attP2 +</td>
<td>( \frac{y^1, v^1}{Y} ) UAS - Dicer2 - +</td>
</tr>
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GD (60000)

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<thead>
<tr>
<th>Courtship Controls</th>
<th>Control Genotypes</th>
<th>Knockdown Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{UAS - mCherry^{RNAi}}{Y} ) + +</td>
<td>( y^1, sc^*, v^1, sev^{21} ) ( \frac{UAS - RNAi}{Y} ) + + +</td>
</tr>
<tr>
<td></td>
<td>( \frac{y^1, v^1}{Y} ) attP2 +</td>
<td>( \frac{y^1, v^1}{Y} ) UAS - Dicer2 - +</td>
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</table>

GD (60000)
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<thead>
<tr>
<th></th>
<th><strong>Control Genotypes</strong></th>
<th><strong>Knockdown Genotypes</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mCherry</td>
<td>$\frac{y^1, v^1, Y; attP40}{Y, attP40} + \frac{v^1, Y; R14H06 - GAL4}{R14H06 - GAL4}$</td>
<td>$\frac{y^1, v^1}{Y; UAS - RNAi} + \frac{Y; R14H06 - GAL4}{R14H06 - GAL4}$</td>
</tr>
<tr>
<td>attP2 (Dicer-2)</td>
<td>$\frac{y^1, v^1}{Y; UAS - Dicer2; R14H06 - GAL4, UAS - mCD8 :: GFP}$</td>
<td>$\frac{y^1, v^1}{Y; UAS - RNAi; R14H06 - GAL4}$</td>
</tr>
<tr>
<td>attP40</td>
<td>$\frac{y^1, v^1}{Y; UAS - mCD8 :: GFP; R14H06 - GAL4}$</td>
<td>$\frac{y^1, v^1}{Y; UAS - RNAi; R14H06 - GAL4}$</td>
</tr>
<tr>
<td>GD (60000)</td>
<td>$\frac{w^{1118}}{Y; UAS - Dicer2; R14H06 - GAL4, UAS - mCD8 :: GFP}$</td>
<td>$\frac{w^{1118}}{Y; UAS - Dicer2; R14H06 - GAL4, UAS - mCD8 :: GFP}$</td>
</tr>
<tr>
<td>KK (60100)</td>
<td>$\frac{y^1, w^{1118}, attP, y^+, w^{3r}}{Y; UAS - Dicer2; R14H06 - GAL4}$</td>
<td>$\frac{y^1, w^{1118}, attP}{Y; UAS - Dicer2; R14H06 - GAL4, UAS - mCD8 :: GFP}$</td>
</tr>
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2.3 Validation of RNAi Knockdown

RNAi efficiency was assessed using a lethality assay where we measured the survival of flies by means of ubiquitous KD of target genes using Act5C-GAL4 driver. This simple phenotypic test allows us to compare ubiquitous knockdown to lethality observed in null mutations. Three biological replicate crosses were made between the heterozygous driver, Act5C-GAL4/CyO and the homozygous UAS-RNAi transgenes at 25°C and 70% humidity with a 12-hour light-dark cycle. Virgin female Act5C-Gal4/CyO were crossed with male UAS-RNAi flies (Table 2). From this crossing scheme, half of the progeny are expected to receive Act5C-GAL4 and UAS-RNAi transgenes while the other half is expected to have the CyO balancer chromosome which contains a dominant marker for curly wings and the UAS-RNAi transgenes. The CyO marker is therefore a visual indicator that there is no Act-Gal4 transgene in that particular fly. The proportion of total flies observed without the CyO marker would therefore indicate survival of the Actin5C-GAL4 driven expression of the RNAi transgene. Therefore, survival percentage was calculated by (% survival = ). Calculations were performed independently for both females and males, in addition to cumulatively. Deviations from expected survival percentage were determined using an unpaired t-test.

In addition to the lethality assay, real time quantitative polymerase chain reaction (RT-qPCR) was performed to determine RNAi knockdown efficiency by looking at the gene expression levels in KD samples (Appendix B). Since RNAi-mediated knockdown can vary considerably between various RNAi lines, the phenotypic effects can also be inconsistent for different RNAi lines that target the same gene. Protocol for qPCR was performed as previously described (Mainland et al., 2017). All experiments were performed on standard media and kept at 25°C and 70% humidity with a 12-hour light-dark cycle. Third instar larvae were collected from crosses made from the RNAi stocks crossed with a UAS-Act-GAL4/CyO-ActGFP driver. Three biological replicates consisting of 10 larvae per biological replicate of each genotype were collected, flash frozen, and stored in -80°C freezer. For each biological replicate, three technical replicates were conducted. Relative expression was normalized to two reference genes, βCOP and eIF2By. One-tailed t-tests were performed to determine if there was a significant reduction in mRNA levels compared to UAS-mCherry-RNAi control. Finally, a literature search was conducted to establish RNAi lines
that have been used in previous studies. These three methods were considered when selecting RNAi lines for courtship conditioning and morphology analysis.

2.4 Memory Assay using Courtship Conditioning

Courtship conditioning was used to test for short- and long-term memory (STM and LTM) deficiency and was performed as previously described (Koemans, Oppitz, et al., 2017). Genetic crosses were made as indicated in (Table 1). F1 male knockdown flies were collected and isolated for four days in individual wells of a 96-well block that contained 500 μL of fly media in each well. In courtship conditioning, a male fly is paired with a previously mated female (PMF) fly who will continually reject the male fly’s courting attempts. Male flies with no learning or memory defect will remember the rejection and demonstrate reduced courting attempts with a different PMF after initial training. Male flies with memory deficiencies do not remember rejection and therefore do not show reduced courting. All male subjects were transferred using gentle aspiration to mitigate any extrinsic stress caused by transferring and knocking out flies using CO₂ (Colinet & Renault, 2012). With the exception of the initial collection of male flies’ post eclosion, CO₂ is not used on subjects in the assay. For STM, the F1 4-day old males are separated into two cohorts, naïve and trained. Male flies in the trained cohort are individually paired with a 4-day old PMF in a new 96-well block with media and trained for one hour. Following training, the male and female flies are separated, and the male fly is gently aspirated into a new well and allowed to rest in isolation for an hour. Once the isolation period is complete, male flies from the trained and naïve cohorts are individually tested and paired with new PMFs. Individual male flies and PMFs are placed in a specially designed mating chamber that contain eighteen 1 cm diameter mating circles, allowing 18 fly pairs to be tested simultaneously. Courtship behaviour was recorded with a digital camera for 10 minutes and 29 seconds. The additional 29 seconds was included to allow the flies to acclimate to their new environment but was not included as part of the testing phase. To test for LTM, the training period is extended to 7-8 hours for the male trained cohort followed by an isolated rest period of 20-24 hours. Standard experiments are conducted on three consecutive days which allowed for a maximum of 54 pairs of flies per genotype and training conditioning to be tested.
2.4.1 Statistical Analysis of Courtship Conditioning Assay

Quantification of courtship behaviour required manual observation and scoring. These behaviours include male orientation towards the female, male following the female, male wing “tapping”, male “licking” of female genitalia and attempted copulation (Koemans, Oppitz, et al., 2017). A courtship index (CI) was calculated for each male-female pair by determining the time the male spent courting the female over a 10-minute period. The CI is the proportion of time spent displaying courtship behaviour during a 10-minute testing period. Once the CI is obtained for both naïve and trained cohorts, a memory index (MI) can be calculated based on the following formula: MI = ((CI_{naïve} - CI_{trained}) / CI_{naïve}). Statistically, memory deficiency can be identified in one of two ways. First, a comparison within a genotype comparing the CI between both naïve and trained cohorts to determine if there is a reduction in mean courtship activity. Second, a comparison between genotypes comparing the MI of the KD genotype and its respective control. Statistical analysis of each genotype’s CI was compared using a one-tailed Mann-Whitney test on GraphPad Prism v.7.03. A significant reduction (P > 0.05) between the two cohorts’ CIs is an indication that the genotype has retained the memory of the training event and subsequently reduced their courting behaviour (Figure 4A). Alternatively, when analyzing the MI between genotypes, a randomization test (random sampling with replacement, 10 000 replicates) was performed using a custom bootstrapping R script (Koemans, Oppitz, et al., 2017) to compare MIs of knockdown to control flies (Figure 4B). A significant reduction in MI (P<0.05) between the control and KD genotypes indicates some level of memory deficiency was caused by the knockdown. Box and whisker graphs were made using GraphPad Prism v.7.03 with whiskers showing values in the 10-90th percentiles. It is important to note that a significant reduction in CI can appear in some cases indicating memory retention in knockdown flies, a memory phenotype may still be present when compared to their respective genetic controls. These two tests are independent and as such, are both sufficient to define memory defect in this study.

2.5 Brain Dissections and Confocal Microscopy

Male and female adult fly brains were assessed for gross MB morphology by examining individual brains with R14H06-directed GFP expression. Visualization of the MB was made possible through standard genetic techniques to combine R14H06-GAL4 driver with UAS-
mCD8::GFP to allow for MB specific visualization. Crosses for knockdown experiments utilizing short hairpin RNAi constructs were made using males from the driver line with the genotype w1118; P{UAS-mCD8::GFP.L}LL5, P{UAS-mCD8::GFP.L}2/CyO; P{GMR14H06-GAL4}attP2)/TM6 crossed with homozygous virgin females with the UAS-RNAi transgene. Similarly to crosses made for courtship conditioning, RNAi constructs that utilized long hairpin RNA molecules required co-expression of Dicer-2 to achieve optimal knockdown. Therefore, knockdown experiments using long hairpin RNAi used males with the genotype w1118; P{UAS-Dcr-2.D}2/CyO; P{GMR14H06-GAL4}attP2), P{UAS-mCD8::GFP.L}LL6/TM6. Prior to eclosion of F1 progeny, parents were removed and newly eclosed adult flies were removed and aged for five days like flies used in courtship experiments. The brains of both male and female adult flies were dissected in 1X phosphate buffered saline (PBS) (pH 7.2) and then fixed with 4% paraformaldehyde (PFA) for 40 minutes at room temperature. Brains were then mounted in Vectashield (Vector Laboratories) and imaged using a Zeiss LSM800 confocal laser scanning microscope with Airyscan. Confocal z-slices were analyzed using ZEN and processed using ImageJ software (Fiji) (Schindelin et al., 2012). Images were scanned using 25X zoom to capture the MB within the Drosophila brain. Intervals of each frame should equal to/or less than the thickness of the section thickness, z-stacks varied in size depending on the size of brain as well as mounting procedure.

Scoring and classification of MB phenotypes were based on previously established phenotypes identified in the lab by M. Chubak (Chubak et al., 2018). While there is a high degree of natural variation in the size of the Drosophila MB, confocal stacks were qualitatively assessed for gross morphological defects. Previously, four distinct MB phenotypes were observed following knockdown of SWI/SNF subunits, including missing α and β lobes, crossing of β-lobe fibers over the midline, extra dorsal projections and stunted γ-lobes. Knockdown brains were qualitatively compared to their respective genetic background controls.
Chapter 3

3 RESULTS

3.1 Analysis of Memory in Controls Flies

RNAi stocks from different libraries have different genetic backgrounds that the RNAi transgene gets inserted into (Dietzl et al., 2007; Ni et al., 2008, 2011; Perkins et al., 2015). The controls used for courtship conditioning have the same genetic background but without the RNAi construct insertion. The exception to this is the mCherry-RNAi control which contains an RNAi construct targeting the mCherry fluorescent protein that has no effect on endogenous Drosophila genes (Ni et al., 2011; Perkins et al., 2015).

Short- and long-term memory courtship analysis was performed on the five genetic controls used (Figure 4A-B). Each of the five control genotypes, mCherry, attP2, attP40, GD and KK, demonstrated significant reduction of CI between naïve and trained flies in both short- and long-term memory. This indicates that the training session was effective and that the trained cohort was able to reduce their courting attempts during the testing phase. The MI for each of the control groups ranged from $\text{MI}_{kk}= 0.2899$ to $\text{MI}_{\text{attP40}} = 0.1455$ for STM and $\text{MI}_{\text{mCherry}} = 0.2323$ to $\text{MI}_{\text{attP40}} = 0.0831$. While a reduction in courtship can be observed indicating learning occurred, the MIs are slightly lower than reported MIs found in the (Chubak et al., 2018; Keleman et al., 2012). No apparent morphological defects were observed in control MB images (Figure 4C). GFP expression is strongest in $\gamma$ lobes and weaker $\alpha/\beta$ lobes. Proper development of the MB is required for proper learning and courtship memory to occur. Therefore, we analyzed gross MB morphology to determine if KD of KDMs caused any observable structural defects.
Figure 4. Analysis of genetic controls used in courtship conditioning and MB gross morphology.

(A) Boxplots show distribution of Courtship Indices (CI) for naïve (N) and trained (T) control male flies tested in short-term memory and long-term memory. Male flies are generated through crossing of control UAS-RNAi flies to R14H06-GAL4 driver flies. Mean CI represented by (+). Mann-Whitney Test used to compare CI naïve to CI trained flies within each genotype. Total flies tested listed in the (n=) row. (B) Grey bars representing Memory Index (MI) calculated from CIs. The control genotype is listed below each bar. (C) Confocal projections analyzing gross morphology of genetic controls used in courtship conditioning. UAS-mCD8::GFP was used for visualization of MB. Natural variation in MB sizes but no apparent defects observed to general α, β or γ lobe structure. Qualitative observation was used to determine if MB had structural deformities.
3.2 MB-Specific KD of H3K4 Demethylases

3.2.1 Knockdown of Su(var)3-3 in the MB Impairs Short- and Long-Term Memory

To assess the role of LSD1 in neurons we studied the Drosophila ortholog, su(var)3-3. Homozygous mutations of this gene result in sex dependent lethality in male flies (Stefano et al., 2008). Therefore, lethality assay using the ubiquitous driver Act-Gal4 was only considered for males. The results show lethality in 2/3 lines used and reduced viability in the other line (v106147) (Table 3). However, no further experimentation was conducted on BL33726 despite showing reduced mRNA expression due to difficulty collecting flies for experimentation. Through a literature review, publications using BL32853 and BL36867 found positive phenotypes as a result of knockdown of these RNAi lines (Jafari & Alenius, 2020 (preprint); Lee & Spradling, 2014). With these factors in consideration, BL32853 and BL36837 were selected and used in courtship conditioning and MB morphology analysis.

Knockdown of su(var)3-3 in 1/2 lines resulted in reduced courtship (p = 0.0013) after 1 hour of training (Figure 5A). This is an indication that learning occurred in the RNAi line BL36867. However, when compared to the control, both RNAi lines display a downward trend of reduced memory in comparison to the control, mCherry (Figure 5B). In terms of LTM, after 7-8 hours of training both RNAi lines showed no significant reduction in courtship signifying that the flies did not learn. However, when compared to the control there is no significant difference between the knockdown and control. This could be explained by the low number of tested flies since the power of this statistical analysis decreases in cohorts with fewer flies. Despite that, there is still a downward trend in memory retention in knockdown flies suggesting that su(var)3-3 is required in both STM and LTM. Here we observed that a stronger reduction in MI was associated with the more potent RNAi line. In addition, low courtship indices were also observed in the more potent line, BL32853, in both naïve and trained flies. No major morphological defects were observed in both RNAi lines suggesting that the cause of memory loss is in the knockdown of su(var)3-3 is not due to structural deformities to the MB (Figure 5C).
Figure 5. *Su(var)*3-3 is required in the MB for Short- and Long-Term Memory

(A) Boxplots show distribution of Courtship Indices (CI) for naïve (N) and trained (T) male flies in STM and LTM. Mean CI represented by (+). Mann-Whitney Test used to compare CIm naïve to CIm trained flies within each genotype. Number of flies tested listed in the (n=) row. (B) Bar graph representing Memory Index (MI) calculated from CIs. Grey bars represent control and purple bars represent *Su(var)*3-3 KD (C) Confocal projections analyzing gross morphology of the MB where KD occurred. *UAS-mCD8::GFP* was used for visualization of MB. No morphological defect was observed following KD. Scale bars: 50 µm.
3.2.2 Knockdown of \textit{KDM2} in the MB Impairs Short- and Long-Term Memory

The second KDM that was analyzed is \textit{KDM2}. The effect of \textit{KDM2} in \textit{Drosophila} viability is controversial (Lagarou et al., 2008; L. Li et al., 2010; Zheng et al., 2014). However, the most recent study looking at this gene has reported that \textit{KDM2} is not required for fly viability (Liu et al., 2016). Here we observed that ubiquitous knockdown of \textit{KDM2} using Act-Gal4 did not affect the survival of the flies (Table 3). A literature review found knockdown of BL33699, BL31360 and v31402 resulted in reduced mRNA expression (Kavi & Birchler, 2009; Liu et al., 2016). With those factors in consideration, BL31360 and v31402 were selected since they are from different libraries and consistent phenotypes observed can control for off-target effects and differences in genetic background. BL31360 was selected over BL33699 since a greater reduction in mRNA expression was observed in the qRT-PCR analysis performed by Liu et al.

One way a learning defect can be determined is by looking within genotype between the naïve and trained cohorts. In the case for both STM and LTM, no significant difference was observed between the two cohorts which suggests that there is a learning deficiency in \textit{KDM2} KD flies. However, the randomization test found mixed results. This could be due to the variability of the data set which can occur in behavioural assays. As a caveat of the data set, the low MI observed in the STM test for 60000 and 36303dcr in LTM could explain that despite a downward trend of memory retention in the KD flies, when compared to the control, no significance was observed. Finally, no major morphological defects were observed suggesting that KD of \textit{KDM2} does not affect the overall structure of the MB.
Figure 6. KDM2 is required in the MB for Short- and Long-Term Memory

(A) Boxplot distribution of CIs for each condition, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mean CI represented by (+). Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) Bar graphs showing MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent control while purple bars represent KDM2 KD. (C) Confocal images of KDM2 knockdown in the MB show no visible structural defects. Scale bars: 50 µm
3.2.3 Knockdown of *Lid* in the MB Impairs Short- and Long-Term Memory

One of the more well studied KDMs is *Lid*. Mutations in its human ortholog, specifically *KDM5A*, *KDM5B* and *KDM5C*, are found in patients with ID. This implicates a potential role for *KDM5* in the regulation of transcription in development or activity in neuronal tissues (Vallianatos & Iwase, 2015). Here we use the *Drosophila* ortholog as a model to better understand the link between mutations in the *KDM5* family proteins and cognitive defects. Null mutations in *Lid* result in semi-lethality which means that less than 50% of the mutant progeny survive (Shalaby et al., 2017). Lethality assay using *Act-GAL4* observed semi-lethality in 2/4 lines tested, specifically BL28944 (19.75 ± 2.41 p = 0.0343) and v103830 (17.65 ± 3.89, p = 0.001) (Table 3). Through a literature review, we found that the RNAi line, v103830 was able to recapitulate phenotypes observed in knockout *Lid* flies (Pinzón et al., 2017). A second study observed significant reduction in mRNA expression using BL28944 and v103830 (Liu et al., 2016). BL28944 and v103830 was selected and used in courtship conditioning and MB morphology analysis.

Short-term memory analysis of the two selected RNAi lines found that there was no significant reduction between naïve and trained flies indicating that flies did not learn (Figure 7A). The randomization test between genotypes found a downward trend in KD flies. In LTM, courtship activity was not significantly reduced in BL28944 but was reduced in v103830 (Figure 7B). A downward trend in the memory index was observed in both lines. Furthermore, there were no observable MB morphological defects observed following knockdown of *Lid* in post-mitotic MB neurons (Figure 7C).
Figure 7. *Lid* is required in the MB for Short- and Long-Term Memory

(A) Boxplot distribution of CIs for each condition, (N) for naïve and (T) for trained. Total number of flies represented below the x-axis on the n = row. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) Bar graph represents MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent appropriate controls to the KD shown in purple. (C) Confocal images of *Lid* knockdown in the MB. Scale bars: 50 µm
3.2.4 Knockdown of *NO66* in the MB Impairs Short and Long-Term Memory

The final H3K4 demethylase that we studied is *NO66* (CG2982). Null mutation of *NO66* does not affect fly viability (Shalaby et al., 2017). As such, no lethality was expected when ubiquitously knockdown occurred using the Act-GAL4 driver. Here we observe that knockdown of BL33596 and v107819 do not affect fly viability (Table 3). Due to difficulty maintaining BL33596 no further research was conducted on the line. However, a literature review found positive phenotypes that recapitulated phenotypes observed in null mutants using v107819 (Pinzón et al., 2017). Only one RNAi line was therefore tested since no other viable stocks were available on BDSC or VDRC.

Following training in both STM and LTM, male flies that have a *NO66* knockdown did not show a significant reduction in courtship suggesting that learning did not occur (Figure 8A). From the CI, the MI was calculated for each genotype and compared to determine if there was a difference between genotypes. A significant decrease was observed in STM (p = 0.0002) but not in LTM (p = 0.293) (Figure 8B). Subsequently, we aimed to determine whether these effects were caused by MB development defects and analyzed gross MB morphology. Confocal imaging of individual knockdown brains found no visible morphological defects (Figure 8C).
Figure 8. *NO66* is required in the MB for Short- and Long-Term Memory

(A) Boxplot distribution of CIs for each cohort, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) Bar graphs represent MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent corresponding control to the purple bars that symbolize *NO66* KD (C) Confocal images of *NO66* knockdown in the MB. Scale bars: 50 µm
3.3 MB specific KD of H3K9 Demethylases

Only 2/3 of the H3K9 demethylases were studied since the lethality assay used on the available TRiP line for *KDM4A* (BL34629) was completely lethal (Table 3). Null mutations of individual H3K9 demethylases do not affect viability, therefore this line was excluded since there were off-target effects that were affecting fly viability (Shalaby et al., 2017).

3.3.1 *KDM4B* is not required in the MB for Short- and Long-Term Memory

As previously mentioned, *KDM4B* is a H3K9 demethylase that does not affect fly viability (Shalaby et al., 2017). Lethality assay found no effect on fly survival following ubiquitous knockdown of *KDM4B* in both lines tested (Table 3). A literature review found positive and consistent phenotypes in knockdown of both BL35676 and BL57721 (Jafari and Alenius, 2020 (preprint)).

No significant loss of short-term memory was observed following knockdown of *KDM4B*. This is shown by the significant reduction in courtship between naïve and trained flies and no significant difference between the MI of knockdown and control genotypes (Figure 9A-B). However, knockdown of BL57721 had no significant decrease between naïve and trained flies in the LTM test. This could be due to the relatively low numbers of flies tested (Figure 9A). Since no memory phenotype was observed, MB morphology was not analyzed.
Figure 9. *KDM4B* is not required in the MB for Short- and Long-Term Memory.

(A) Boxplot distribution of CIs for each cohort, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent appropriate control to the KD which is shown in orange.
3.3.2 Knockdown of *JHDM2* in the MB Impairs Short- and Long-Term Memory

To investigate the role of *JHDM2* and MB courtship memory we began by testing the viability of ubiquitous knockdown of *JHDM2* and compared it with known literature. Our results are consistent with published work (Table 3) (Shalaby et al., 2017). Both RNAi lines have been used in previous publications and have found positive phenotypes (Pinzón et al., 2017; Park et al., 2019). Experimentation on BL3295 was challenging due to inadequate number of flies collected from the line therefore only BL58264 was used.

In both STM and LTM courtship assays no reduction in courting activity was observed between naïve and trained flies in the knockdown cohorts. This suggests that learning did not occur in KD flies (Figure 10A). When compared to the respective control, mCherry, there is a downward trend where KD flies have a lower MI than the control in both STM (p = 0.0502) and LTM (p = 0.014) (Figure 10B). Following courtship conditioning, MB morphology was analyzed, and no major morphological defects were observed (Figure 10C).
Figure 10. *JHDM2* is required in the MB for Short- and Long-Term Memory.

(A) Boxplot distribution of CIs for each cohort, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) Bar graphs depict MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent control that are compared to *JHDM2* KD shown in yellow. (C) Confocal images of *JHDM2* knockdown in the MB. Scale bars: 50 μm
3.4 MB specific KD of H3K27 Demethylase

Null mutants of the following H3K27 demethylases, *Jarid2* and *UTX*, are known to affect fly viability and indeed cause complete lethality (Shalaby et al., 2017). However, following ubiquitous knockdown of *Jarid2*, both RNAi lines that were available did not affect fly viability and thus this gene was not further studied (Table 3).

3.4.1 Knockdown of *UTX* in the MB Impairs Short- and Long-Term Memory

The final KDM we investigated in this screen is *UTX*. Null mutations of this gene are known to cause complete lethality in mutants. Therefore, any deviations using the ubiquitous driver *Act-GAL4* were eliminated during our RNAi selection process. There was complete or almost complete reduction in survival seen in 3/4 *UTX* RNAi lines tested (Table 3). It should be noted that the transgene for the RNAi line v37446 is inserted in chromosome 1, the sex chromosome. Since male *UAS-RNAi* flies were crossed to female *Act5C-Gal4/CyO* flies, none of the F1 male flies had our gene of interest and were therefore excluded in the lethality assay. A literature review found consistent and positive phenotypes following knockdown of BL34076 (Gervais et al., 2019) and v37664 (Katz et al., 2014). Therefore, v37664 was chosen based on complete lethality and BL34076 was chosen over v37663 since they are from a different library than v37664. Consistent phenotypes observed from different genetic libraries can control for any potential off-target effects.

Following courtship conditioning experimentation, both BL34076 and v37664 resulted in no significant change between naïve and trained flies in both short- and long-term memory assays (Figure 11A). A downward trend in the MIs can be observed in both knockdowns, however, only BL34076 is significantly different from its respective control, mCherry (Figure 11B). While no significant change in MI was observed in v37664, this could be attributed to the low MIs of the controls and the variability of the data set (Figure 11B). Following courtship conditioning, MB morphology was analyzed and similarly to other KDM KDs, no visible defects to the MB was observed (Figure 11C).
Figure 11. *UTX* is required in the MB for Short- and Long-Term Memory.

(A) Boxplot distribution of CIs for each cohort, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) Bar graphs show MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent corresponding controls used for each *UTX* KD. (C) Confocal images of *UTX* knockdown in the MB. Scale bars: 50 µm
3.5 Knockdown of trr in the MB Impairs Short- and Long-Term Memory

Previous studies in the lab found a role for trr in short-term memory, however LTM was not tested (Koemans, Kleefstra, et al., 2017). The Trithorax related complex is a COMPASS like complex that mediates H3K4 methylation as well as H3K27 demethylation through UTX. In addition, UTX displayed memory deficiency in both short- and long-term memory after MB-specific knockdown. Therefore, we decided to test whether or not this effect extended to the COMPASS complex. Previous studies in our lab has validated significant knockdown with 2/3 lines trr lines used, specifically BL36916 and BL29563 (Mainland et al., 2017). We decided to continue experimentation on v110276 since null mutations of trr affect fly viability and knockdown from all three lines caused complete lethality (Table 3).

A significant loss of both STM and LTM was observed following knockdown of trr in the MB. Three out of three RNAi lines used resulted in no significant reduction in courtship in both short- and long-term memory tests (Figure 12A). When compared to their respective controls, a significant reduction in MI was observed in three out of three RNAi lines used (Figure 12B). Finally, no gross morphological defects were observed upon knockdown of trr (Figure 12C).
Figure 12. *trr* is required in the MB for Short- and Long-Term Memory.

(A) Boxplot distribution of CIs for each cohort, (N) for naïve and (T) for trained. Total number of flies represented on the n = row below the x-axis. Mann-Whitney test for statistical difference between each cohort. Exact P-values indicated above boxplots. (B) MI derived from CIs. Randomization test used to compare between KD and controls. Exact P-values above. Grey bars represent appropriate controls to the blue bars that signify *trr* KD. (C) Confocal images of *trr* knockdown in the MB. Scale bars: 50 µm
Table 3. Lethality assay RNAi efficiency of KDM RNAi stocks.
% survival ± SE for all RNAi lines tested. Significant changes in survival between Act-GAL4/UAS-RNAi and CyO/ UAS-RNAi were determined with an unpaired t-test. Flies were reared at 25°C at 70% humidity with a 12-hour light-dark cycle.

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3.6 Summary of Objective 1: Relative Memory Index of KDMs and \textit{trr}

The first objective of this project was to screen KDMs and test whether or not they play a role in \textit{Drosophila} learning and memory. The results are summarized as a relative MI to its respective control (Figure 13). We observed that knockdown of 6/7 KDMs resulted in memory loss either by no reduction in CI between trained and naïve flies or a reduction in MI compared to its genetic control. These results suggest that H3K4 demethylases is required in the MB for short- and long-term memory. Furthermore, knockdown of only one H3K9 demethylase was found to affect \textit{Drosophila} memory. With that being said, a much stronger phenotype was observed following knockdown of the H3K4 methyltransferase, \textit{trr} (Figure 14). Utilizing both methods, within genotype comparison and between genotype comparison, to determine if a memory defect is present, we found that \textit{trr} is required in the MB for both short- and long-term memory.
Figure 13. Relative Memory Index for Short- and Long-Term Courtship Memory Following Knockdown of KDMs.

Bar graphs represent relative MI to respective controls of each RNAi used in courtship conditioning (M_knockdown/M_control) for both short- (A) and long-term (B) memory. Pounds (#) present no significant reduction between naïve and trained male flies within a genotype due to training (Mann-Whitney test, p > 0.05). Asterisks (*) are an indication of significant reduction in MI compared to their respective controls (randomization test, p < 0.05).
Figure 14. Relative Memory Index of trr on Short- and Long-Term Courtship Memory.

Relative MI represented in bar graph to respective controls of each RNAi used in courtship conditioning ($\text{MI}_{\text{knockdown}} / \text{MI}_{\text{control}}$) for both short- (A) and long-term (B) memory. Pounds (#) represents no significant reduction between naïve and trained male flies within a genotype due to training (Mann-Whitney test, $p > 0.05$). Asterisks (*) are an indication of significant reduction in MI compared to their respective controls (randomization test, $p < 0.05$)
Chapter 4

4 DISCUSSION

In this study, I screened several KDMs to determine if they had a role in *Drosophila* development or function of MB neurons. The results of this project found that 6/7 demethylases tested caused a loss of short- and long-term courtship memory (Figure 13). A strong memory loss was observed in the MB-specific knockdown of *UTX* and *trr* (Figure 13-14). Both of these genes are found in the COMPASS complex which has established roles in memory. Additionally, MB-specific knockdown of these genes did not cause any notable qualitative phenotypes providing evidence that the cause of the memory phenotypes observed in courtship conditioning are not due to structural defects in the mushroom body.

4.1 H3K4 Methylation and Demethylation Plays a Critical Role in *Drosophila* Memory

This study demonstrated that MB-specific knockdown of H3K4 demethylase, *Su(var)3*-3, *KDM2*, *lid* and *NO66*, caused a loss of short- and long-term courtship memory. Previous studies have shown that 4/6 human H3K4 demethylases are associated with impaired cognitive function (Collins et al., 2019). With that being said, it remains unclear as to what role H3K4 demethylases play in cognitive function. However, many studies have established that H3K4 methylation is an important regulatory element of memory formation. Memory experimentation with rats have demonstrated that there was a significant increase in H3K4me3 observed following fear conditioning when compared to naïve animal cohorts (Gupta et al., 2010). Histone methylation is a dynamic process and loss of methyltransferase or demethylase activity can result in a decrease or increase in overall H3K4 methylation levels. Therefore, as an important regulatory mechanism of chromatin plasticity, it is often hypothesized to be a critical player in memory formation (Kerimoglu et al., 2013).

One of the more well studied H3K4 demethylases is *lid* and its human ortholog, *KDM5C*. In addition to removing H3K4me3, *lid*, also has two other domains that recognize the methylation status of the lysine residue. The C-terminal PHD motif binds to H3K4me2/3 while the N-terminal PHD recognizes when the lysine residue is unmethylated (Lloret-Illinares et al., 2008). *Lid* has been
shown to affect transcription in a demethylase-dependent manner and thus can activate or repress gene transcription (Zamurrad et al., 2018). At promoter regions, lid can affect transcription by demethylating H3K4me3 which is a hallmark of transcriptionally active genes (Johansson et al., 2014). In humans, KDM5A, KDM5B and KDM5C are found in patients with ID. This implicates a role for KDM5 in the development or activity of neuronal tissues (Vallianatos & Iwase, 2015). In line with results found in this study, they found a learning and memory defect in lid mutants without affecting the MB neuronal morphology. RNA sequencing in this study found mild changes to gene expression observed in mutant flies. This suggests that lid acts by fine tuning expression of multiple genes within memory pathways (Zamurrad et al., 2018).

Another interesting H3K4 KDM is Su(var)3-3 and its human ortholog, LSD1. Initial studies of Su(var)3-3 mutations found it is involved in the suppression of heterochromatic gene silencing and removal of histone marks by Su(var)3-3 is a prerequisite for subsequent heterochromatin formation by H3K9 methylation (Rudolph et al., 2007). Another study found that there is interplay between two of the H3K4 demethylases, Su(var)3-3 and lid. The study found that while mutations in lid cause an increase in H3K4 methylation levels, it also suppresses Su(var)3-3 mutant phenotypes (Stefano et al., 2008). Finally, Su(var)3-3 and its downstream targets are involved in a wide variety of biological function including embryonic development (Rudolph et al., 2007) and neurogenesis (J. Wang et al., 2015).

Finally, a study on several JmjC genes including lid, KDM2, NO66 found that these genes function to regulate sleep and circadian rhythm (Shalaby et al., 2018). Specifically, lid displayed high levels of arrhythmicity, KDM2 showed a subtle shortening of the circadian period length and NO66 mutants exhibited reduced sleep and increased activity phenotype (Shalaby et al., 2018). Therefore, KDMs may function as regulators of behaviour rather than play a role in development since null mutations do not affect viability. In addition, no major morphological defect was observed in the MB in this study which is another indication that these genes are not essential in development. While the contribution of histone methylation and demethylation is appreciated in the formation of memory, the mechanism behind these enzymes remain unclear.
4.2 The H3K9 Demethylases

4.2.1 \textit{KDM4A} and \textit{KDM4B} are Biologically Redundant

While this study did not find any conclusive evidence that H3K9 demethylases play a role in \textit{Drosophila} courtship memory we cannot be certain that these genes do not play a role in regulating behaviour. Previous studies have identified that loss of one KDM4 family member is not sufficient to affect histone methylation (Wilson & Krieg, 2019). Flies homozygous for loss-of-function mutations in either \textit{KDM4A} or \textit{KDM4B} are viable, fertile and have normal gross morphology (Tsurumi et al., 2013). However, when a \textit{KDM4A} and \textit{KDM4B} double mutant was created, the mutants were not viable and were lethal at the larval stage. The lethality was rescued following \textit{Act-Gal4} driven expression of \textit{KDM4A}. In addition, \textit{KDM4A} and \textit{KDM4B} double mutants had significantly smaller and more condensed nuclei in their brains at the second instar stage. This is an indication of chromatin compaction which is consistent with loss of H3K9 demethylation. This led researchers to believe that proper H3K9 demethylation requires at least one function copy of either \textit{KDM4A} or \textit{KDM4B} (Tsurumi et al., 2013). This suggests that \textit{KDM4A} and \textit{KDM4B} are biologically redundant and could possibly explain why there was no loss of memory observed following knockdown of \textit{KDM4B} ((Katoh & Katoh, 2004; Lloret-linares et al., 2008). Another possibility to explain why there was no loss of memory could be due to insufficient knockdown observed (Figure 9C).

Previous studies have identified interactions between \textit{KDM4A}, a gene that we did not look at, and the ecdysone signaling pathway. The study shows that \textit{Drosophila KDM4A} and \textit{KDM4B} are essential for mediating ecdysteroid hormone signaling during larval development (Tsurumi et al., 2013). Ecdysone is a steroidal hormone that controls the molting of insects and arthropods. The ecdysone signaling pathway is critical in various developmental events in flies like molting and metamorphosis (Truman & Riddiford, 2002). In addition, there is evidence to suggest that ecdysone plays a critical role in regulation of \textit{Drosophila behaviour}, including courtship memory (Ishimoto et al., 2009). It still remains unclear how \textit{KDM4A} affects the ecdysone signaling pathway, whether it is a direct downstream target of the ecdysone receptor or a secondary effect through regulation of other crucial transcription factors (Tsurumi et al., 2013). Therefore, while the results of this study did not find any conclusive evidence to suggest that H3K9 demethylases play a role in memory it could be due to biological redundancy in the genes. It should be noted
that reports of KDM4A mutants have shown to display abnormal courtship behaviour which could affect future memory studies using courtship conditioning as a memory assay (Tsurumi et al., 2013).

4.2.2 JHDM2 Regulates Behaviour in the Nervous System

The JmjC domain-containing histone demethylase 2, JHDM2, is homologous to the mammalian KDM3 demethylase. Knockdown of JHDM2 was found to play a role in both short- and long-term memory but had no effect on MB morphology (Figure 10). This enzyme has the ability to demethylate H3K9me1 and H3K9me2 (Yamane et al., 2006). This differs from KDM4A and KDM4B which demethylates H3K9me2 and H3K9me3 (Hyun et al., 2017). Furthermore, overexpression or depletion of JHDM2 has demonstrated to have activity against H3K9 methylation. Indeed, JHDM2 associates with H3K9M nucleosomes and overexpression in Drosophila resulted in not only a loss of H3K9 methylation but also heterochromatic silencing defects (Herz et al., 2014). In the study looking at sleep and circadian rhythm, knockout KDM3 mutants exhibited high levels of arrhythmicity. This is an indication that JHDM2 may play a role in regulating behaviour (Shalaby et al., 2018). Another study found that knockout of KDM3 enhances ethanol sensitivity in Drosophila which is another indication that this gene has an effect on the nervous system in regulating behavioural responses ((Pinzón et al., 2017)

4.3 UTX and trr Plays a Critical Role in Drosophila memory

In this study, MB-specific knockdown of UTX caused loss of both short- and long-term memory (Figure 11). While mutations in the human ortholog of UTX, KDM6A, is a recognized ID gene known to cause Kabuki Syndrome (Van Laarhoven et al., 2015), the mechanism remains unclear. Interestingly, UTX is the only KDM that is part of a complex. In Drosophila, the COMPASS, “Complex of Proteins Associated with Set1” complex is responsible for mono-, di- and trimethylation of H3K4. Initially identified in yeast, Drosophila express three Set1 homologs, dSet1, Trithorax (trx) and Trithorax-related (trr). UTX is an additional component associated with trr to help direct the enzyme’s specificity for certain genomic regions (Collins et al., 2019). In line with previous research conducted on the effects of trr on Drosophila memory (Koemans, Kleefstra, et al., 2017), our results from this study found a strong memory loss following MB-
specific knockdown of *trr* (Figure 12). These defects may be a result of changes in cell type specific transcriptional profile of the MB or perhaps memory-dependent transcriptional activation (Koemans et al., 2017).

It is still unclear as to why *UTX* is the only demethylase found in a methyltransferase complex. However, we understand that active enhancers are typically marked with H3K4me1 and H3K27 acetylation, allowing them to be distinguished from inactive enhancers (Creyghton et al., 2010). In *Drosophila*, H3K27ac is catalyzed by a CREB-binding protein (CBP)-related enzyme. Since lysine residues cannot be modified by both methylation and acetylation simultaneously, it has been suggested that the histone demethylase, *UTX*, can facilitate in CBP-mediated H3K27 acetylation through the ability to remove methyl groups from H3K27 (Tie et al., 2012). Therefore, the physical association between *UTX* and *trr* supports a model where removal of a repressive mark and the simultaneous deposition of an active mark can lead to activation of a target gene (Agger et al., 2007).

Several studies have identified a role in H3K4 methylation in learning and memory. In fact, dysregulation of H3K4 methylation is associated with a variety of neurodevelopmental disorders including ID, autism spectrum disorder, schizophrenia spectrum, substance-related and additive disorders (Collins et al., 2019). For further characterization of the mechanistic role of *Drosophila trr* and *UTX*, future studies should look at the genes that are up- and down-regulated in response to knockdown using RNA-sequencing (Koemans et al., 2017).

### 4.4 Limitations

RNAi is a commonly used tool to effectively study gene function. Despite pre-screening of lines used; RNAi genetic studies are limited due to their potential off-target effects of siRNAs as well as insufficient target gene knockdown. The assay also does not provide any information about the overall expression level of the protein following knockdown. Ideally, at least 2 RNAi lines were used and sourced from different transgenic libraries to prevent false positives from occurring. To better quantify the effectiveness of RNAi knockdown, future studies should strive to measure protein levels using Western blotting. While mRNA expression of RNAi lines was analyzed, it is important to remember that mRNA levels do not always equate to protein levels (Fortelny et al., 2017; Wilhelm et al., 2014). Furthermore, inconsistencies in mRNA expression was observed in
some cases when compared with the literature (Liu et al., 2016). While knockdown was observed in other studies no knockdown was observed in this study. This could be due to several reasons including differences in many conditions like sub-optimal primers despite validation, different tissues used (whole larvae vs. wing imaginal discs), and even different stages of Drosophila (larvae vs. adult) used could affect mRNA expression levels. Overall, inconsistencies between phenotypes observed in RNAi lines were not a major limitation as results observed in most cases were consistent.

For two of the KDM genes, NO66 and JHDM2, experiments were only conducted using one RNAi because knockdowns using other available stocks were incapable of eclosing sufficient number of F1 males for courtship conditioning experiments. Therefore, it is difficult to make any strong conclusions about the results from these two genes. Future studies should aim to validate phenotypes by using a second RNAi line.

Another limitation in this study is the variability observed in the data set. The randomization test used to calculate the MI between controls and knockdowns has been shown to be effective in detecting memory loss (Kamyshev et al., 1999), the power of this statistical analysis decreases with increased variability in CI as well as low numbers of flies.

Finally, while gross morphology was analyzed for defects in MB structure. We cannot say for sure that knockdown of KDMs did not affect MB neuronal circuitry since the analysis is relatively crude. In particular, fine details that can affect memory cannot be seen despite overall normal structure.

4.5 Future Research

Although the *Drosophila* nervous system is less complex than the mammalian nervous system, the molecular mechanisms behind memory formation is highly conserved between species (Frank and Greenberg, 1994). While the specific mechanisms in which KDMs operate to regulate memory in the MB remains unknown, future studies can help identify specific transcription targets as a result of KDM knockdowns. Transcriptome studies like RNAseq can help identify genes that are upregulated or downregulated following knockdown using a protocol we have already established in the lab to isolate MB nuclei (Jones et al., 2018). The viability of KDM null mutants suggest that these genes play more of a “fine tuning” role in biological processes rather than controlling essential gene expression machinery since many of the genes are non-lethal (Shalaby et al., 2017).
With that in mind, future research can also validate the results from this study through the usage of available mutants on viable lines or MB-specific CRISPR knockout to validate the phenotypes observed in the knockdown experiments. If the memory phenotypes observed in these knockout experiments are consistent with the memory defects observed using RNAi knockdown then it is likely that the results we found in this study are accurate and not due to any off-target effects.

4.6 Conclusions

In summary, this study provides an initial screen of KDMs and their role in *Drosophila* short- and long-term courtship memory. While there is still much to be discovered about the role of KDMs in the nervous system this research provides a foundation for future investigation. Loss of memory was observed in 6 out of 7 KDMs tested. KDMs are broadly required in MB neurons for short- and long-term memory formation. These genes likely affect memory through regulation of MB neuron function rather than play a role in the development of MB structure. Finally, KDMs may be required for fine tuning behavioural processes including memory formation. As a result, these findings provide a foundation for understanding KDM mutations in cognitive function, specifically ID, and may lead to mechanistic studies to understand how KDMs regulate memory. While the role for demethylation in memory formation is not as well established as that of methylation, the importance of the regulatory complexity of erasing chromatin marks in neurons should not be overlooked (Collins et al., 2019).
5 REFERENCE


genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila.

_Nature._


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http://dx.doi.org/10.1038/ncomms13471

http://dx.doi.org/10.1016/j.cub.2012.08.048


Intrinsic neurons of Drosophila mushroom bodies express short neuropeptide F: Relations to extrinsic neurons expressing different neurotransmitters. *Journal of Comparative Neurology.*


Koemans, T. S., Kleeistra, T., Chubak, M. C., Stone, M. H., Reijnders, M. R. F., de Munnik, S.,...


Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V.,


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Yamane, K., Toumazou, C., Tsukada, Y. ichi, Erdjument-Bromage, H., Tempst, P., Wong, J., &
6 APPENDICES

Appendix A: List of all fly stocks used in this project
All Drosophila stocks were obtained from either Bloomington Drosophila Stock Center (BDSC) or Vienna Drosophila Resource Center (VDRC)

<table>
<thead>
<tr>
<th>Control and Genetic Toolkit</th>
<th>Stock Name</th>
<th>Stock No.</th>
<th>Source</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCherry</td>
<td>35785</td>
<td>BDSC</td>
<td>y¹ sc¹ v¹ sev²¹; P{y¹+t7.7 v¹+t1.8=VALIUM20-mCherry}attP2</td>
<td>Short hpRNA targeting mCherry. TRiP library genetic background for attP2 landing site, controls for sc¹</td>
<td></td>
</tr>
<tr>
<td>attP2</td>
<td>36303</td>
<td>BDSC</td>
<td>y¹ v¹; P{y¹+t7.7=CaryP}attP2</td>
<td>Background control for VALIUM 1 and 10 TRiP RNAi collection</td>
<td></td>
</tr>
<tr>
<td>attP40</td>
<td>36304</td>
<td>BDSC</td>
<td>y¹ v¹; P{y¹+t7.7=CaryP}attP40</td>
<td>Background control for attP40 site TRiP RNAi collection</td>
<td></td>
</tr>
<tr>
<td>GD</td>
<td>60000</td>
<td>VDRC</td>
<td>w¹¹¹¹</td>
<td>GD library genetic background control</td>
<td></td>
</tr>
<tr>
<td>KK</td>
<td>60100</td>
<td>VDRC</td>
<td>y¹ w¹¹¹¹; P{attP, y¹ w¹}</td>
<td>KK library genetic background control</td>
<td></td>
</tr>
<tr>
<td>Act5C-GAL4</td>
<td>25374</td>
<td>BDSC</td>
<td>y¹ w¹; P{Act5C-GAL4-w¹}El/CyO</td>
<td>Expresses GAL4 ubiquitously under the control of Act5C (FBgn0000042) promoter</td>
<td></td>
</tr>
<tr>
<td>R14H06-GAL4</td>
<td>48667</td>
<td>BDSC</td>
<td>w¹¹¹¹; P{y¹+t7.7 w¹+mC=GMR14H06-GAL4}attP2</td>
<td>Expresses GAL4 under the control of a rutabaga (FBgn0003301) enhancer</td>
<td></td>
</tr>
<tr>
<td>UAS-Dicer2</td>
<td>24650</td>
<td>BDSC</td>
<td>w¹¹¹¹; P{w¹+mC=UAS-Dcr-2.D}²</td>
<td>Expresses Dicer-2 under UAS control</td>
<td></td>
</tr>
<tr>
<td>UAS-mCD8::GFP</td>
<td>5137</td>
<td>BDSC</td>
<td>y¹ w¹; P{w¹+mC=UAS-mCD8::GFP.L}LL5, P{UAS-mCD8::GFP.L}²</td>
<td>Expresses GFP under UAS control. Used to build fly lines that co-express Dicer-2 and R14H06-GAL4</td>
<td></td>
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<table>
<thead>
<tr>
<th>Inducible RNAi Stocks</th>
<th>Gene Name</th>
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<th>Source</th>
<th>Genotype</th>
<th>Control</th>
<th>Description</th>
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<tr>
<td>Su(var)3-3</td>
<td>32852</td>
<td>BDSC</td>
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<td>mCherry</td>
<td>UAS-RNAi against Su(var)3-3</td>
<td></td>
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<tr>
<td>Su(var)3-3</td>
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<td>BDSC</td>
<td>y¹ sc¹ v¹ sev²¹; P{y¹+t7.7 v¹+t1.8=TRiP.GL01006}attP40</td>
<td>mCherry</td>
<td>UAS-RNAi against Su(var)3-3</td>
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<td>Gene</td>
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<td>Source</td>
<td>RNAi Construct</td>
<td>Gal4</td>
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<td>Su(var)3-3</td>
<td>106147</td>
<td>VDRC</td>
<td>$w^{118}; P{KK102965}VIE-260B$</td>
<td>KK</td>
<td>Long hpRNA UAS-RNAi against Su(var)3-3</td>
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<td>Lid</td>
<td>28944</td>
<td>BDSC</td>
<td>$y^f v^1; P{y^{+t7.7}; v^{+t1.8}=\text{TRiP.HM05155}}attP2$</td>
<td>attP2</td>
<td>Long hpRNA UAS-RNAi against Lid</td>
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<tr>
<td>Lid</td>
<td>103830</td>
<td>VDRC</td>
<td>$P{KK102745}VIE-260B$</td>
<td>KK</td>
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<td>42203</td>
<td>VDRC</td>
<td>$w^{118}; P{GD14113}v42203$</td>
<td>GD</td>
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<td>GD</td>
<td>Long hpRNA UAS-RNAi against Lid</td>
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<td>Kdm2</td>
<td>31360</td>
<td>BDSC</td>
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<td>Long hpRNA UAS-RNAi against Kdm2</td>
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<td>Kdm2</td>
<td>33699</td>
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<td>$y^f sc^* v^1; P{y^{+t7.7}; v^{+t1.8}=\text{TRiP.HMS00574}}attP2$</td>
<td>attP2</td>
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<td>$w^{118}; P{GD7173}v31402$</td>
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<td>NO66 (CG2982)</td>
<td>33596</td>
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<td>UAS-RNAi against KDM4A</td>
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<td>JHDM2</td>
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<td>mCherry</td>
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<td>mCherry</td>
<td>UAS-RNAi against UTX</td>
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<td>37663</td>
<td>VDRC</td>
<td>$w^{118}; P{GD4409}v37663/TM3$</td>
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<td>Gene</td>
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<td>Repository</td>
<td>Strain</td>
<td>RNAi Expression</td>
<td>Notes</td>
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<td>Jarid2</td>
<td>32891</td>
<td>BDSC</td>
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<td>UAS-RNAi against Jarid2</td>
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<td>Jarid2</td>
<td>26184</td>
<td>BDSC</td>
<td>y^{1} v^{1} ; P{y^{+7.7} v^{+1.8}=TRiP.JF02081} attP2</td>
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<td>trr</td>
<td>29563</td>
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<td>y^{1} v^{1} ; P{y^{+7.7} v^{+1.8}=TRiP.JF03242} attP2</td>
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<td>Long hpRNA UAS-RNAi against trr</td>
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Appendix B: qPCR results for lines used in this study

As part of validating the RNAi lines used in this study, RT-qPCR was performed on the following loss-of-function experiments to determine if mRNA expression is reduced following knockdown. For each biological replicate, 10 whole third instar larvae were collected from each cross. For each experiment 3 biological replicates were collected. The primers used were ordered commercially and validated for efficiency using a cDNA dilution series (efficiency = 10^{-1/slope}). The reaction was carried out in a Bio-Rad CFX384 Real-Time System under the following cycling conditions: 2 min at 95°C, then 40 cycles at 95°C for 5s and 65°C for 30s. For each biological replicate, three RT-qPCR technical replicates were conducted. The relative expression was then normalized to two reference genes, eIF2By and βCOP. The results of this experiment found several lines had overexpression or no reduction of mRNA levels compared to the UAS-mCherry-RNAi control. While this can be an indication that the RNAi lines used in this study have potential off-target effects it doesn’t mean the lines do not work. mRNA levels do not always equate to protein levels. A common method of detecting protein levels is a Western blotting, however this method is not effective for tissue-specific RNAi knockdown. In this case, immunohistochemistry would be the ideal form to determine if the knockdown was successful. It is peculiar to see that following knockdown of several of the KDMs we see overexpression (Appendix B: C,D,E,F,I,K,L). This could be due to a technical problem in the experiment like the presence of primer dimers, or potential overcompensation of the gene following knockdown or stalling of RNA. It should be noted that courtship and qPCR experiments were done simultaneously due to time constraint.
7 CURRICULUM VITAE

Name: Crystal Keung

Post-Secondary Education and Degrees:
- Western University, London, Ontario, Canada
  - 2018-2020 M.Sc. University of Toronto
  - 2014-2018 B.Sc.

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  - 2018-2019

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Keung C, Kramer JM (2019, May) The Role of KDMs in Learning and Memory in the Mushroom Body of *Drosophila melanogaster*. Poster presentation at the 39th Southern Ontario Neuroscience Association (SONA), Windsor, ON, Canada
  - Best poster presentation (Master’s category)

Keung C, Kramer JM (2019, June) The Role of KDMs and *trr* in the Mushroom Body of *Drosophila melanogaster*. Poster presentation at the 15th Canadian Drosophila Research Conference (CANFLY), Toronto, ON, Canada

Awards:
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  - Western Graduate Research Scholarship
  - Children’s Health Research Institute
    - Epigenetics Trainee Fellowship ($10,000)