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# Regulation of Learning and Memory by the Drosophila melanogaster SWI/SNF complex

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# Abstract

The SWI/SNF complex is a highly-conserved ATP-dependent chromatin remodeling complex that is important in the etiology of intellectual disability (ID). I systematically investigated the overall and adult-specific roles of each of the 15 *Drosophila melanogaster* SWI/SNF complex components in memory. Flies with RNAi-mediated knockdown of individual SWI/SNF genes in the mushroom body (MB) were tested for short- and long-term memory impairment using courtship conditioning. Knockdown of several SWI/SNF genes, including *brahma, Bap60, Snr1,* and *e(y)3,* caused loss of memory. Adult-specific knockdown of SWI/SNF genes caused some loss of memory phenotypes, indicating an acute role in adult MB activity. These data provide the first comprehensive neurobiological phenotypic profile of the SWI/SNF complex, demonstrating an essential role for this complex in the regulation of memory. These findings will help reveal the SWI/SNF complex's role in regulating neuronal processes and fly memory, and have implications for understanding SWI/SNF-associated forms of ID.



Keywords: *Drosophila melanogaster*, SWI/SNF complex, BAP complex, PBAP complex, learning and memory, courtship conditioning, chromatin remodeling, epigenetics, intellectual disability.

## Statement of Co-Authorship

Research for this thesis was conducted under supervision of Dr. Jamie M. Kramer. Project was devised by Dr. Kramer, and designed by the author with Dr. Kramer's cooperation. Balancing of some *Drosophila* lines used in this project was completed by Dr. Tara Edwards. All experiments were completed by the author, except for: assistance with fly brain dissection and shared responsibilities of scoring lethality assays completed by Melissa Chubak. Courtship video analysis was completed with assistance from the following work-study students and laboratory volunteers: Nour Abuali, Sumbal Afzaal, Shawn Amadasun, Orli Chapman, Delaney Cosma, Derek Nguyen, Jennifer Desnoyers, Valentina Gnanapragasam, Roxanne Hummel, Cindy Kao, Nerdin Mansour, Morgan Nebsitt, Amanda Noyek, Ryan Park, Ashita Patel, Emma Prescott, Shelby Rice, Jamie Rosen, Carly Sugar, Rangana Talpe Guruge, Nicole Taylor, Virsika Yogeswaran, and Rachel Zevy.

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# List of Abbreviations

| | |
|---|---|
| 12L:12D | 12 hours light, 12 hours dark |
| 20E | 20-hydroxyecdysone |
| AC | adenylyl cyclase |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BAF | BRG1- or HBRM-associated factors |
| BAP | Brahma-associated protein |
| *BCL7-like* | BCL Tumor Suppressor 7-like |
| BRG1 | Brahma-related gene 1 |
| $Ca^{2+}$ | Calcium ion |
| cAMP | Cyclic adenosine monophosphate |
| ChIP | Chromatin immunoprecipitation |
| CI | Courtship Index |
| $CO_2$ | Carbon dioxide |
| CREB | Cyclic adenosine monophosphate response element binding protein |
| CREST | Calcium-responsive transactivator |
| cVA | Cis-Vaccenyl Acetate |
| da | Dendrite arborisation |
| DHR3 | Drosophila hormone receptor 3 |
| DNA | Deoxyribonucleic acid |
| DopEcR | Dopamine/ecdysteroid receptor |
| dsRNA | double-stranded RNA |
| *e(y)3* | enhancer of yellow 3 |

| | |
|---|---|
| EcR | Ecdysone receptor |
| EGFP | Enhanced Green Fluorescent Protein |
| EGFR | Epidermal growth factor receptor |
| EMS | Ethylmethanesulfonate |
| esBAF | embryonic stem cell BAF |
| GAL4 | Galactose/lactose metabolism regulatory protein GAL4 |
| GAL80 | Galactose/lactose metabolism regulatory protein GAL80 |
| $GAL80^{ts}$ | Temperature-sensitive GAL80 |
| GO | Gene ontology |
| HBRM | Human Brahma |
| ID | Intellectual disability |
| LI | Learning Index |
| MAPK | Mitogen-activated protein kinase |
| MB | Mushroom body |
| miR | microRNA |
| Mll | Mixed-linkage leukemia |
| mRNA | Messenger RNA |
| nBAF | neuronal BAF |
| NMDA | N-methyl-D-aspartate |
| npBAF | neuronal progenitor BAF |
| PBAP | Polybromo-associated BAP |
| PBS | Phosphate buffer saline |
| PDE | Phosphodiesterase |
| PFA | Paraformaldehyde |
| pH | Potential of hydrogen |

| | |
|---|---|
| PKA | Protein Kinase A |
| PMF | Previously-mated females |
| qPCR | Quantitative real-time polymerase chain reaction |
| $R^2$ | Coefficient of determination |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| SAYP | Supporter of activation of yellow protein |
| SET | Su(var)3-9, Enhancer-of-zeste and Trithorax |
| SMARCB1 | SWI/SNF related, Matrix associated, Actin dependent regulator of chromatin, subfamily 1 |
| Snr1 | Snf5-related 1 |
| SS18 | Synovial Sarcoma Translocation, Chromosome 18 |
| SWI/SNF | SWItch/Sucrose Non-Fermentable |
| TRiP | Transgenic RNAi Project |
| USP | Ultraspiracle |
| VDRC | Vienna Drosophila Stock Centre |

# 1. Introduction

Inquiry in the field of epigenetics began in 1942, when C.H. Waddington first used the term *epigenesis* to propose that there must be a force *above genetics* that affects phenotypic output apart from the genetic code (Waddington, 2012). Waddington's hypothesis—which was initially hypothesized to explain organismal development—was exceptionally noteworthy given that the concept of genetic heritability was not yet understood. Today, the term *epigenetics* is better defined as: "the study of changes in gene function that are mitotically and/or meiotically heritable, and that do not entail a change in DNA sequence" (Wu and Morris, 2001).

There are many different types of epigenetic modifications that modify and control gene expression (Kouzarides, 2007; Wu and Morris, 2001; Wu et al., 2009). Among these epigenetic processes are covalent modifications of DNA and/or histones by enzymatic activities such as: acetylation, methylation, phosphorylation, and ubiquitination (Kouzarides, 2007). Typically, covalent modifications facilitate the activation or repression of gene expression. For example, covalent modifications by histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) work antagonistically at histone 3 lysine 9 (H3K9) to regulate transcription by either opening or closing chromatin to transcriptional machinery (Barski et al., 2007; Strahl and Allis, 2000). The other major epigenetic process is the non-covalent modification of chromatin structure. This type of modification, called chromatin remodeling, alters nucleosome structure to impact gene expression. Chromatin remodeling complexes use energy derived from ATP hydrolysis to eject, restructure, or move nucleosomes to create open DNA regions for other machinery to regulate gene expression (Wu, 2012). Just as Waddington had initially hypothesized, epigenetic factors play important roles in development and gene regulation (Berger, 2002), and most research has focused on the role of these processes in the context of organismal development (Ho and Crabtree, 2010).

Recent studies have uncovered a role for epigenetic modifications in the brain that have opened up a new research field, called neuroepigenetics, that has grown rapidly in the last 10–25 years (Sweatt, 2013). Neuroepigenetic studies have already provided greater insight into

understanding the molecular changes that regulate nervous system processes, such as memory formation (Gupta et al., 2010; Levenson et al., 2004; Miller and Sweatt, 2007; Miller et al., 2010), neuronal development (Lessard et al., 2007; Yoo et al., 2009), and addiction (Maze et al., 2010; Renthal et al., 2007). The crucial first link between neuronal activity and chromatin remodeling was the discovery of histone modifications in neuronal cells in response to drug-induced chromatin remodeling in hippocampal neurons (Crosio et al., 2003). Since this critical first step, additional impactful research has identified vital epigenetic regulation in the brain. For example, HMT and HAT activity regulate long-term potentiation in the hippocampus, which is critical for long-term memory (Gupta et al., 2010; Levenson et al., 2004; Miller and Sweatt, 2007; Miller et al., 2008). Research on rats identified that trimethylation in the hippocampus at histone 3 at lysine 4 (H3K4) is upregulated in response to fear conditioning, and contextual fear conditioning experiments on mice deficient in the HMT, *Mll*, showed loss of proper long-term memory consolidation (Gupta et al., 2010). Additionally, critical switches in ATP-dependent chromatin remodeling complexes have been shown to regulate neuronal development when neural progenitor cells exiting the cell cycle replace their subunits with subunits necessary for neuronal differentiation (Lessard et al., 2007; Yoo et al., 2009). Finally, epigenetic processes have been shown to impact addictive behaviour, as evidenced by the effect of cocaine treatment on transcriptional repression of target genes by HDAC5 and the HMT, G9a, which regulate cocaine response in mice (Maze et al., 2010; Renthal et al., 2007). Despite the recent advancements in the field of neuroepigenetics, there is still much to be uncovered about the mechanisms by which different epigenetic machinery, including ATP-dependent chromatin remodeling complexes, function in the nervous system.

## 1.1. The SWI/SNF complex

The SWItch/Sucrose Non-Fermentable (SWI/SNF) complex is an ATP-dependent chromatin remodeling complex that was first discovered in yeast (*Saccharomyces cerevisiae)* due to its role in mating type switching and sucrose fermentation (Whitehouse et al., 1999). The SWI/SNF complex is highly conserved in eukaryotes, including flies, rodents, and humans (Son and Crabtree, 2014; Vogel-Ciernia and Wood, 2014) (Table 1). Whole genome analyses in yeast (Sudarsanam et al., 2000), flies (Zraly et al., 2006), and mice (Gresh et al., 2005) reveal an essential functional role for the SWI/SNF complex in gene regulation, as this complex

affects the expression of approximately five percent of all genes in each of these species. The high level of conservation and similar function of the SWI/SNF complex across species is critical because it allows researchers to use model organisms to study this complex's function. In this project, I used the fruit fly (*Drosophila melanogaster*) as a model to study the SWI/SNF complex in the nervous system. Flies are an effective model for conducting genetic screens because of their short generation time and life cycle, ease of handling, and the availability of many published genetic tools and assays. Most significantly, more than 70% of proteins involved in human disease—including members of the SWI/SNF complex—are found in flies (Rubin et al., 2000), making it an exceptional model for studying neuronal function of human disease orthologs (Bilen and Bonini, 2005).

Most studies on the SWI/SNF complex's specific mechanism of chromatin remodeling have been conducted in yeast. The SWI/SNF complex modifies nucleosome structure to either activate or repress gene expression (Smith et al., 2003; Vignali et al., 2000). To regulate gene expression, the SWI/SNF complex is recruited to specific *in vivo* target sites through interactions with DNA-binding transcription factors (Peterson and Workman, 2000). The complex then remodels chromatin through the proposed DNA looping model (Figure 1). Using energy derived from ATP hydrolysis, the SWI/SNF complex breaks histone-DNA interactions to form a micro-DNA loop using a torsional domain. This loop is then forced to travel down the length of the DNA along the nucleosome by a tracking domain within the complex, resulting in nucleosome sliding or ejection (van Holde and Yager, 2003; Whitehouse et al., 1999). Once chromatin remodeling is complete, other covalent modification enzymes, such as HATs, HDACs, and HMTs, work together to maintain the necessary activation or repression of transcription (Narlikar et al., 2002).

**Table 1. Conservation of SWI/SNF complex subunits from yeast to humans.**

| ***SWI/SNF*** *(Saccharomyces cerevisiae)* | **BAP** *(Drosophila melanogaster)* | **BAF** *(Mus musculus)* | **BAF (Human nomenclature)** *(Homo sapiens)* |
|---|---|---|---|
| | Act5C | β-actin | ACTB |
| ARP7 | Bap55 | BAF53a | ACTL6A |
| ARP9 | | BAF53b | ACTL6B |
| SWI1 | osa | BAF250a | ARID1A |
| | | BAF250b | ARID1B |
| | Bap170 | BAF200 | ARID2 |
| | CG9650 | BAF11a | BCL11A |
| | | BAF100b | BCL11B |
| | BCL7-like | BAF40a | BCL7A |
| | | BAF40b | BCL7B |
| | | BAF40c | BCL7C |
| | CG7154 | Brd7 | BRD7 |
| | | Brd9 | BRD9 |
| | d4 | BAF45b | DPF1 |
| | | BAF45c | DPF2 |
| | | BAF45d | DPF3 |
| | polybromo | BAF180 | PBRM1 |
| | e(y)3 (SAYP) | BAF45a | PHF10 |
| SWI2 | brahma | BRM | SMARCA2 |
| | | BRG1 | SMARCA4 |
| SNF5 | Snr1 | BAF47 | SMARCB1 |
| SWI3 | | BAF155 | SMARCC1 |
| | moira | BAF170 | SMARCC2 |
| SWP73 | Bap60 | BAF60a | SMARCD1 |
| | | BAF60b | SMARCD2 |
| | | BAF60c | SMARCD3 |
| | Bap111 | BAF57 | SMARCE1 |
| | CG10555 | BAF55a | SS18 |
| | | BAF55b | CREST/SS18L |

SWI/SNF complex (referred to by the name commonly used in each species) subunits are organized into families and presented alphabetically by their human nomenclature name. Table is adapted from (Son and Crabtree, 2014), and updated using the DIOPT (v6.0.1) ortholog prediction tool (Hu et al., 2011).

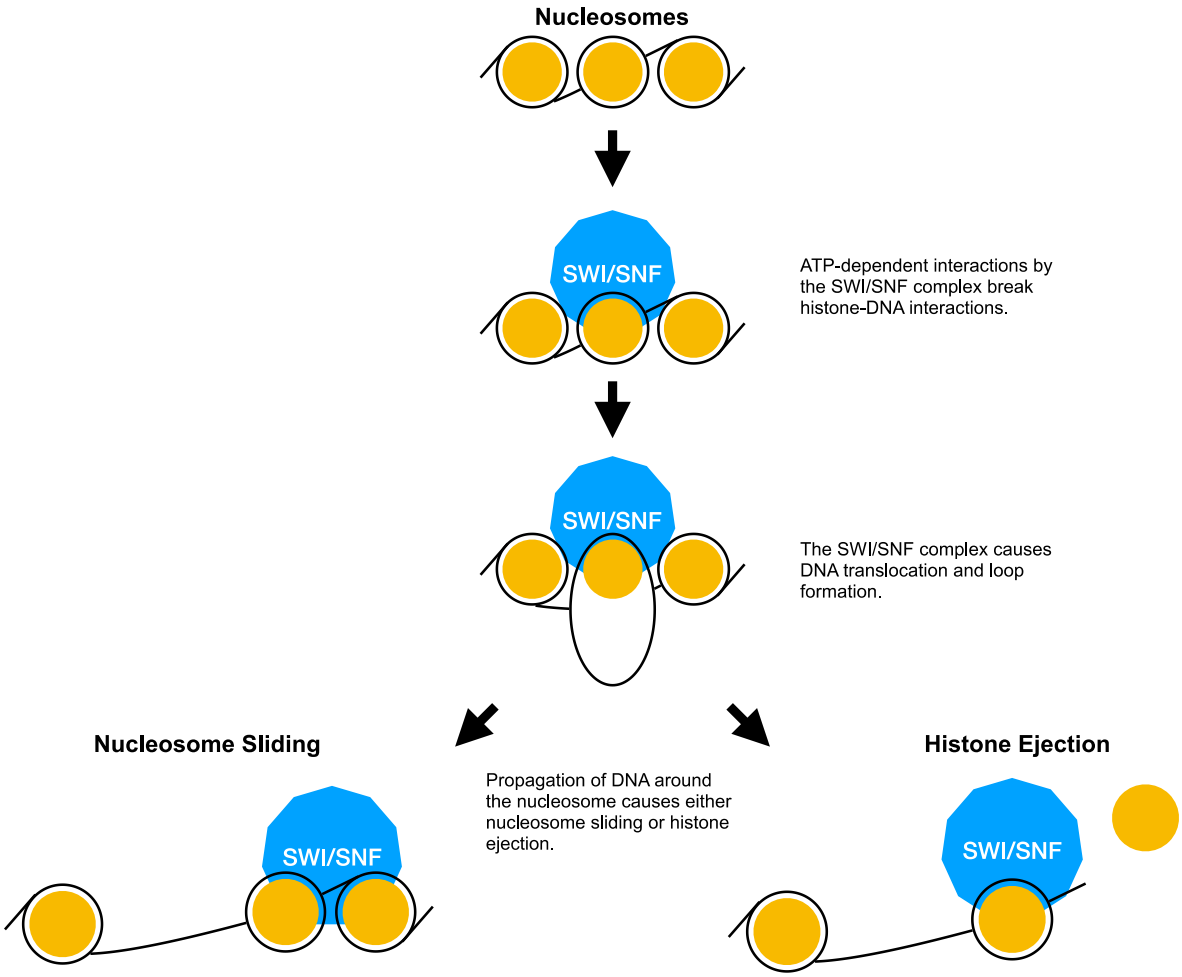



**Figure 1. DNA looping model of chromatin remodeling by the SWI/SNF complex.**

Simplified mechanism of chromatin remodeling by the SWI/SNF complex. The SWI/SNF complex binds and disrupts histone-DNA interactions in an ATP-dependent manner. The SWI/SNF complex creates a DNA loop that slides along the nucleosome, thus changing the position of the DNA with respect to the nucleosome, or causes ejection of an adjacent nucleosome.

### 1.1.1. The Mammalian SWI/SNF complex

The mammalian SWI/SNF complex (Table 1), called the BRG1/HBRM-associated factors (BAF) complex, is more diverse than the *Drosophila* Brahma-associated protein (BAP) complex. The BAF complex is encoded by 29 genes from 15 gene families (Table 1). The BAF complex undergoes combinatorial assembly of its subunits into cell-type specific conformations (Kadoch and Crabtree, 2015). For example, in pluripotent embryonic stem cells, esBAF is found in a unique conformation that is signified by the absence of BAF170 (Ho et al., 2009). When post-mitotic neurons differentiate from neuronal progenitor cells, there is a specific switch in subunit composition from the neuronal progenitor BAF (npBAF) complex to the neuronal BAF (nBAF) complex, whereby npBAF-specific subunits BAF45a and BAF45d, SS18, and BAF53a are specifically replaced by the nBAF-specific subunits BAF45b and BAF45c, CREST, and BAF53b (Lessard et al., 2007; Olave et al., 2002). The combinatorial assembly observed in the BAF complex in neurons implies that this complex regulates gene expression in post-mitotic cells, and thus may regulate important behaviours through acute gene regulation.

The BAF complex has been implicated in developmental processes related to cellular differentiation, cell adhesion, and tumour suppression (Ho and Crabtree, 2010; Ho et al., 2009; Kadoch and Crabtree, 2015; Nguyen et al., 2016). The role of the SWI/SNF complex in cancer biology was first discovered when somatic truncating mutations in the human SWI/SNF gene, SMARCB1, were identified in malignant rhabdoid tumours, and SMARCB1's role in tumour suppression was further explored in murine models (Roberts et al., 2000; Versteege et al., 1998). Subsequent studies have identified mutations in human SWI/SNF complex subunits in several different types of cancers, including pancreatic cancer, lung cancer, colorectal cancer, and leukemia (Kadoch and Crabtree, 2015; Kadoch et al., 2013). More recently, mutations in SWI/SNF genes known to affect cancer have also been identified in patients with intellectual disability (ID), whereby somatic mutations in SWI/SNF genes cause cancer and malignancies, while germline truncating or missense mutations in the same genes are exclusively are related to ID (Santen et al., 2012a).

### 1.1.2. The SWI/SNF complex in intellectual disability

Intellectual disability (ID) is characterized by limitations in intellectual function and adaptive behaviour starting before age 18 (American Psychiatric Association, 2013), and affects 1–3% of the global population (Harris, 2006). There are more than 900 genes that are currently known to be involved in the monogenic forms of ID (Kochinke et al., 2016; Vissers et al., 2016). Dominant *de novo* mutations are the most common cause of ID, and can cause the most severe ID phenotypes (Gilissen et al., 2014; Vissers et al., 2010). Dominant *de novo* copy number variations (CNVs) and single nucleotide variants are estimated to account for approximately 60% of all ID cases, compared to only 2% of ID cases that are caused by rare inherited forms (Gilissen et al., 2014). According to the SysID database, which categorizes ID-associated genes based on phenotypic and functional data (updated: March 9, 2017), there are 291 dominant ID genes. I performed a bioinformatics analysis called Gene Ontology (GO) enrichment analysis (https://geneontology.org) on this collection of 291 primary human ID genes. GO enrichment analyses quantify the representation of annotated GO terms in a subset of inputted genes and compares their prevalence to a random sample of genes. GO terms that are over-represented in a gene set are considered enriched. The results of our GO enrichment analysis revealed that the SWI/SNF complex is the most enriched protein complex amongst all GO cellular components. The nBAF complex (30.06-fold enrichment), npBAF complex (29.23-fold), SWI-SNF complex (28.06-fold), and BAF-type complex (18.30-fold) were four of the top five most enriched GO cellular components terms in the analysis (Figure 2). Other epigenetic- and chromatin remodeling-related terms were also found among the 30 most enriched GO cellular components (Figure 2).

Further substantiating the revealed importance of the SWI/SNF complex in the context of intellectual disability, ID-causing mutations have been identified in 11 of the 29 genes encoding subunits of the human SWI/SNF complex (Dias et al., 2016; Di Donato et al., 2014; Van Houdt et al., 2012; Hoyer et al., 2012; Johnston et al., 2013; Rivière et al., 2012; Santen et al., 2012b, 2013; Tsurusaki et al., 2012; Wieczorek et al., 2013; Wolff et al., 2012) (Figure 3). The SWI/SNF complex's importance in ID is best understood with respect to Coffin-Siris syndrome, which is most commonly caused by mutations in ARID1B (Wieczorek et al., 2013). However, there is some genetic heterogeneity in this syndrome, as forms of Coffin-Siris

syndrome are also caused by mutations in ARID1A, SMARCB1, SMARCA4, and SMARCE1 (Kosho et al., 2014) (Table 1). Patients with mutations in different SWI/SNF genes have different, but overlapping, clinical characteristics, which include: mental retardation, and digital and facial malformations (Santen et al., 2012a). Since disruptions in SWI/SNF complex genes play a critical part in ID, it is important to understand the consequence of obstructing this complex in the nervous system. Since it is difficult to study the functional role of the SWI/SNF complex in humans, members of the SWI/SNF complex have been previously studied in model organisms, including *Drosophila.*

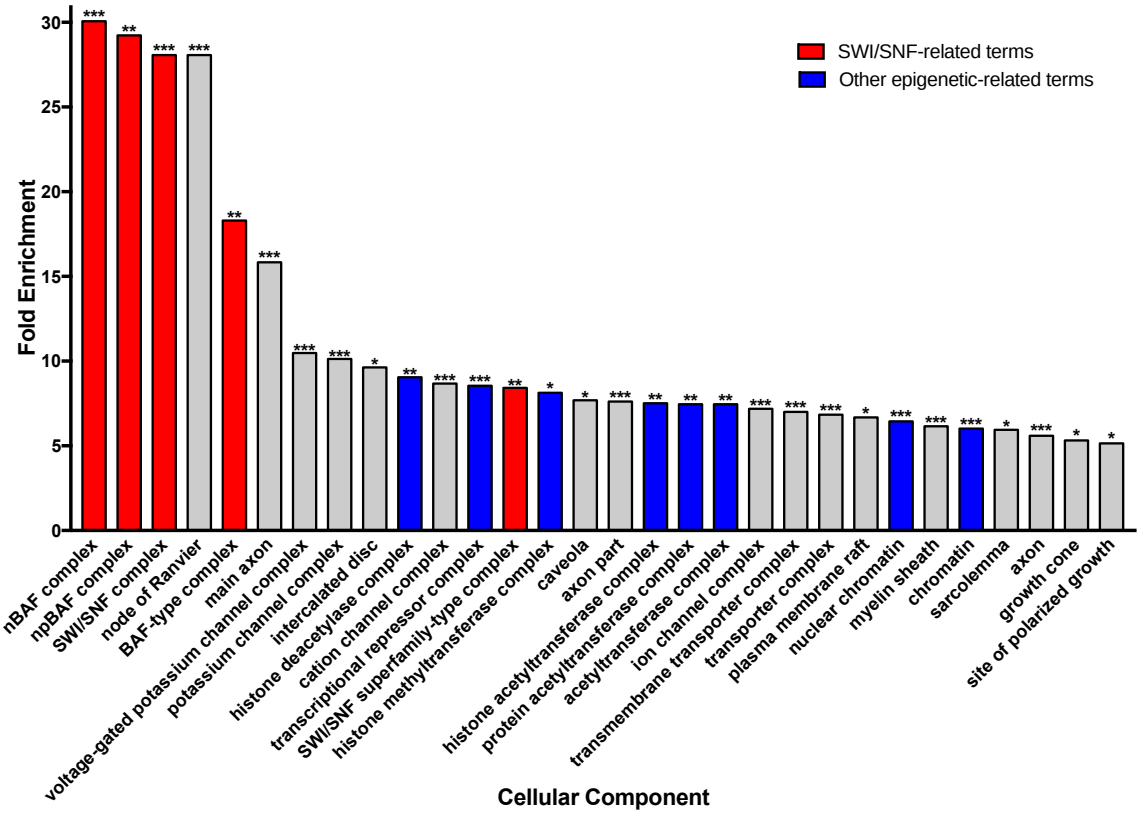



**Figure 2. The SWI/SNF complex is the most enriched cellular component amongst dominant ID genes.**

Gene Ontology enrichment analysis for cellular components of the 291 dominant ID genes. Bar graphs show fold enrichment of the top 30 most-enriched cellular component terms found in the analyses. SWI/SNF-related GO terms are indicated in red, and other epigenetic-related terms are indicated in blue. (Bonferroni correction for multiple comparisons (n=1267), * $P_{adj}$ <0.05, ** $P_{adj}$ <0.01, *** $P_{adj}$ <0.001).

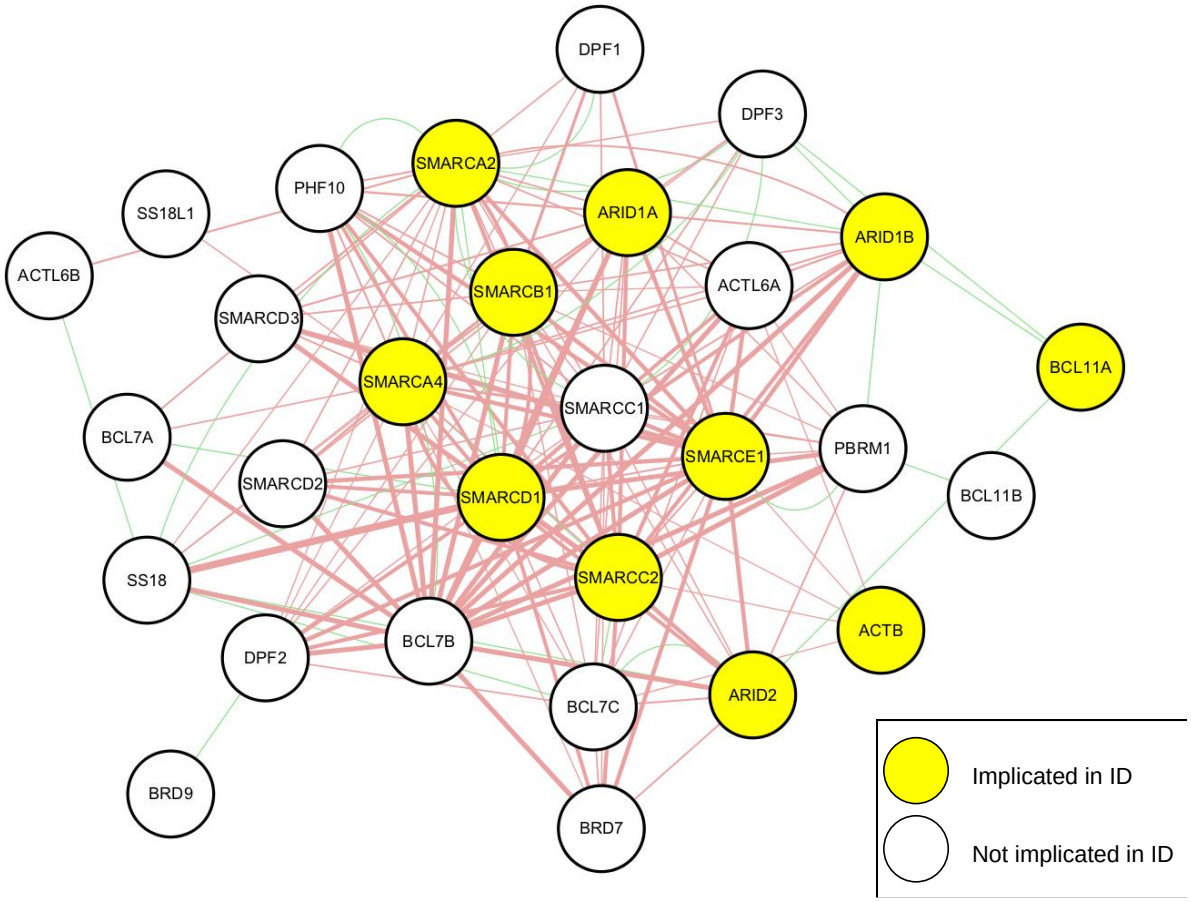



**Figure 3. Eleven of the 29 human SWI/SNF complex subunits are implicated in ID.**

Protein network of human SWI/SNF complex proteins was generated using Cytoscape (Franz et al., 2015), and displays annotated genetic (green) and protein (red) interactions. Subunits highlighted in yellow are implicated in ID.

### 1.1.3. The *Drosophila* SWI/SNF complex

The *Drosophila* SWI/SNF complex (Figure 4), often referred to as the brahma-associated protein (BAP) complex, was first identified through genetic screens for regulators of homeotic gene expression that affect leg development (Kennison and Tamkun, 1988; Tamkun et al., 1992). The BAP complex is highly conserved both structurally and functionally with the mammalian BAF complex (Martens and Winston, 2003). The BAP complex consists of seven core subunits: Actin5C, BAP55, BAP60, BAP111, Brahma, Moira, and Snr1 (Chalkley et al., 2008; Mohrmann et al., 2004). The core subunits are believed to be most important in defining the complex's enzymatic activity and in maintaining the complex's structure (Moshkin et al., 2007). The BAP complex's core ATPase Brahma is an important enzyme in the genome-wide activation of genes transcribed by RNA Polymerase II (Mohrmann and Verrijzer, 2005). Additionally, the BAP complex has been implicated by genome-wide screens as essential in neural stem cell self-renewal (Neumüller et al., 2011), and intestinal stem cell proliferation (Jin et al., 2013). Additional studies have shown that Brahma works antagonistically with Geminin to regulate EGFR-Ras-MAPK signaling, which affects wing development (Herr et al., 2010). Furthermore, Snr1 has been shown to interact with the SET domain of the homeotic regulator Trithorax (TRX) (Marenda et al., 2003), and helps regulate wing, abdomen, and peripheral nervous system development (Zraly et al., 2003).

The BAP complex is found in one of two conformations, the BAP complex or the polybromo-associated BAP (PBAP) complex, that target partially overlapping, but distinct, regions of the genome (Chalkley et al., 2008; Mohrmann et al., 2004). Whole-genome expression profiling revealed that both the BAP and PBAP complexes function distinctly to control transcription, but only the BAP complex, and not the PBAP complex, regulates cell cycle progression through mitosis (Moshkin et al., 2007). The BAP complex is defined by the presence of the subunit Osa, and the PBAP complex is defined by the absence of Osa and presence of Polybromo, BAP170, and SAYP (Chalkley et al., 2008; Mohrmann et al., 2004) (Figure 4). These 'signature' subunits of the BAP and PBAP complexes control the functional specificity of the two complexes, and are not understood to play catalytic roles in chromatin remodeling (Moshkin et al., 2007). Studies have shown that the BAP-specific subunit Osa represses expression of the Wingless-regulated target genes, *nubbin*, *Distal-less,* and

*decapentaplegic* (Collins and Treisman, 2000), and regulates *Drosophila* wing development both by regulating EGFR signaling (Terriente-Félix and de Celis, 2009) and regulating the expression of Apterous-regulated targeted genes (Milán et al., 2004). In contrast, mutations in the PBAP-specific gene *polybromo* result in defective eggshell formation, and the Polybromo protein must be stabilized by BAP170 to perform its function. Additionally, BAP170 plays a role in wing vein patterning (Carrera et al., 2008). *e(y)3* is the most recently identified member of the PBAP complex, and is required to incorporate both Polybromo and BAP170 into the PBAP complex (Chalkley et al., 2008). The *e(y)3* protein, SAYP, has also been shown to interact with Brahma to regulate transcription by forming a nucleosome barrier ahead of a paused RNA Polymerase II (Vorobyeva et al., 2012).

In addition to the core, BAP-specific, and PBAP-specific genes, four additional genes (*BCL7-like*, *CG7154*, *CG9650*, *CG10555*) are predicted orthologs of known mammalian SWI/SNF complex components based on the Drosophila RNAi Screening Center's (DRSC) Integrative Ortholog Prediction Tool (DIOPT v6.0.1) (Hu et al., 2011). Although not confirmed members of the *Drosophila* BAP complex, the four predicted orthologs were also studied in this project.

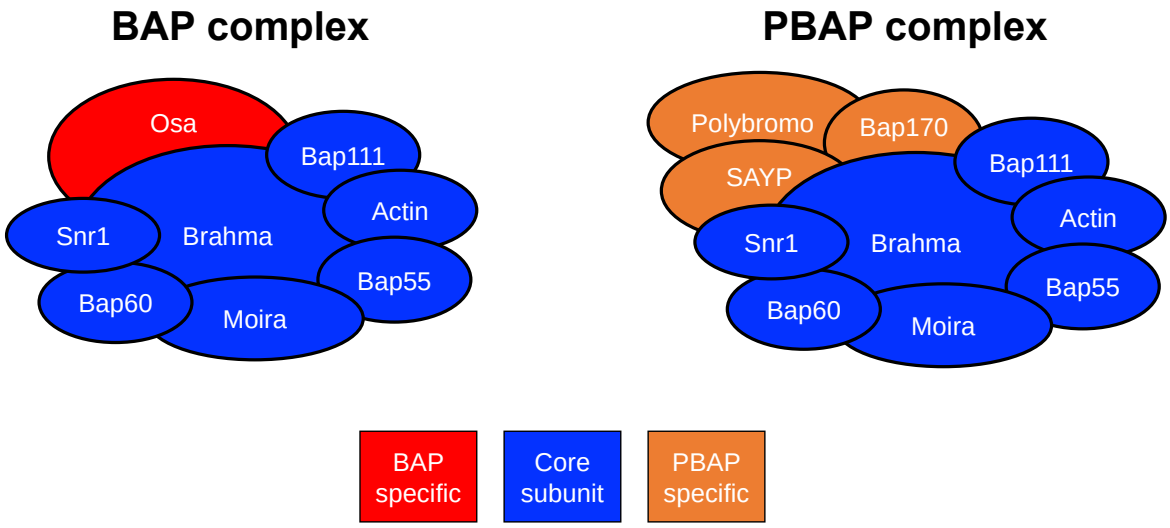



**Figure 4. The *Drosophila* SWI/SNF complex is found in two conformations.**

Visual representation of the *Drosophila* BAP and PBAP complexes. The seven core subunits are indicated in blue. The BAP-specific subunit Osa is indicated in red. In the PBAP complex, Osa is replaced by the three PBAP-specific subunits, Polybromo, Bap170, and SAYP (encoded by *e(y)3*), indicated in orange. Image is a cartoon representation aimed to compare complex composition and orientation of molecules do not indicate direct binding.

### 1.1.4. The SWI/SNF complex in the nervous system

Despite the SWI/SNF complex's known importance in neurodevelopmental disorders, its role in the nervous system has not been studied extensively. Some studies have indicated that the SWI/SNF complex plays a key role in both neuronal development and cognitive function. In murine models, it has been shown that the neuron-specific subunit BAF53b is critical in neuronal gene expression required for dendritic arborisation, branching, and synapse formation (Staahl and Crabtree, 2013; Wu et al., 2007). In mice, the absence of BAF53b had no effect on the interactions between the other nBAF complex subunits, but these mice still die two days after their birth (Wu et al., 2007). Neuronal cultures collected from BAF53b knockout mice have severe defects in synapse formation, activity-dependent dendritic outgrowth, and axonal myelination (Wu et al., 2007). Attempted rescue of these phenotypes by overexpression of the npBAF-specific subunit BAF53a proved unsuccessful; however, substitution of the critical subdomain 2 region in BAF53a for the subdomain 2 region found in BAF53b successfully rescued dendritic outgrowth and deficits in gene expression (Wu et al., 2007). BAF53b has also been shown to be necessary in cognitive behaviour, as both heterozygous null mice (*Baf53b*$^{+/-}$) and transgenic mice with a deletion of only the BAF53b hydrophobic domain (*Camk2a-BAF53*Δ*HD*) displayed severe defects in object location memory (Vogel-Ciernia et al., 2013). Reintroduction of BAF53b into the adult hippocampus was able to restore memory deficits, indicating an adult-specific role for BAF53b in cognitive function (Vogel-Ciernia et al., 2013). Additional research has shown that subdomain 2-deficient mice (*BAF53b*Δ*SB2*) were deficient in long-term potentiation, memory, and phosphorylation of synaptic cofillin (Vogel Ciernia et al., 2017). Synaptic cofillin is critical in proper actin cytoskeleton remodeling at the dendritic spine, indicating an important link between memory phenotypes and biological function of BAF53b in neurons (Vogel Ciernia et al., 2017). Together, these findings indicate that individual SWI/SNF subunits—and not necessarily the entire complex—have critical, and distinct, functions in post-mitotic neurons.

A genetic screen in *Drosophila* revealed that knockdown of *Bap55*, *Bap60*, and *brahma* in class I dendrite arborisation (da) neurons caused dendrite misrouting, and knockdown of *Snr1* resulted in primary branch extension and reduced lateral branching (Parrish et al., 2006). Additionally, knockdown of *Bap55* also resulted in reduced dendrite arborisation (Parrish et

al., 2006). An additional screen in dendrites of olfactory projection neurons indicated that *Bap55* is also required for dendrite targeting (Tea and Luo, 2011). Despite these previous studies in flies, there are no functional studies that describe the consequences of altering SWI/SNF gene expression in the context of nervous system processes. In this project, I studied the role of each *Drosophila* SWI/SNF complex gene in the context of cognition and memory.

## 1.2. Learning and memory in *Drosophila melanogaster*

An important functional output of the nervous system is the capacity for learning and memory. *Drosophila* are an excellent model for studying learning and memory because the molecular mechanisms underlying memory in flies are similar to those in other species, including mammals (Dunning and During, 2003; Frank and Greenberg, 1994; Margulies et al., 2005). Humans with ID often have impaired memory, making this intellectual ability a good behaviour in which to study the importance of ID genes in the brain (American Psychiatric Association, 2013). The capability for *Drosophila* memory was first discovered in an olfactory conditioning experiment (Quinn et al., 1974). In this paradigm, flies were tasked to discriminate between two odours: one coupled to an electric foot-shock, and a different odour presented without any shock (Quinn et al., 1974). Using the established olfactory conditioning paradigm, the first memory mutant in any species was identified in *Drosophila*. An ethylmethanesulfonate (EMS) mutagenesis screen identified *dunce* as a mutant deficient in learning (Dudai et al., 1976). Drosophila *dunce* mutants were incapable of shock avoidance in olfactory learning experiments, but otherwise displayed regular behaviour, including the ability to sense both the odorants and electric shock (Dudai et al., 1976). In a separate EMS mutagenesis screen, a second learning and memory gene, *rutabaga*, was also identified (Livingstone et al., 1984). Both *dunce* and *rutabaga* encode enzymes involved in the cyclic AMP (cAMP) second messenger system, which is an important pathway in the *Drosophila* nervous system, and in the molecular mechanism of learning and memory conserved across species (Davis et al., 1995).

### 1.2.1. The mushroom body

The mushroom body (MB) is the learning and memory centre of the *Drosophila* brain (McGuire et al., 2001). The MB consists of symmetrically paired neuropil structures that sense olfactory cues from the antennal lobe through mushroom body projection neurons (Lee et al., 1999). MB neurons are derived from Kenyon cells, which project dendrites into the calyx and axons into the central brain, where they form the α, β, α', β', and γ lobes (Aso et al., 2009; Lee et al., 1999). The adult MB is formed from the division of four distinct neuroblast cells that divide continuously throughout development. These neuroblasts differentiate into γ neurons during late embryonic, and early larval stages of development, followed by α' and β' neuron development in the larval stage, and into α and β neuron development during the pupal stage (Lee et al., 1999).

The mushroom body is a critical brain structure in both *Drosophila melanogaster* olfactory memory and courtship conditioning (Davis, 1993, 2011; Heisenberg, 1998; McBride et al., 1999). Mushroom body ablation impairs the ability for both short- and long-term courtship conditioning memory, an important (McBride et al., 1999). Many genes involved in olfactory learning and memory have enriched expression in the MB, particularly those encoding components of the cAMP signaling pathway (McGuire et al., 2001), including *dunce* in the MB neuropil (Nighorn et al., 1991) and *rutabaga* (Han et al., 1992). Studies have shown that loss of memory phenotypes caused by *rutabaga* mutation can be rescued by GAL4-UAS-mediated expression of functional *rutabaga* in the developing MB (Zars et al., 2000), or by temperature-sensitive GAL80 ($GAL80^{ts}$) mediated, adult-specific expression in the MB (McGuire et al., 2001). These studies indicate that the MB is the memory centre of the *Drosophila* brain, and that manipulation using transgenic techniques can alter memory. In this study, I use MB-cell-type-specific transgenic techniques to isolate the specific effects of the *Drosophila* SWI/SNF complex in the context of memory.

### 1.2.2. Molecular mechanism of memory

Memories are formed, stored, and retrieved in the brain through the understanding and processing of external sensory cues at the molecular level. Memory formation in neurons

occurs through changes in neurons that require signaling through the cAMP pathway. The cAMP pathway is sometimes called the "learning pathway" because it is conserved across species in the processes of learning and memory (Dunning and During, 2003; Frank and Greenberg, 1994). The role of the cAMP pathway in the nervous system was first identified in the gill-withdrawal reflex in the sea slug, *Aplysia* (Brunelli et al., 1976).

In *Drosophila*, the fast-acting mechanisms that culminate in short-term memory require cAMP signaling in MB γ neurons (Blum et al., 2009; Zars et al., 2000), and persist for only 1–3 hours (Davis, 2011; Margulies et al., 2005). Glutamate binding through several receptor channels such as NMDA-type and AMPA-type receptors leads to an influx of calcium ion ($Ca^{2+}$) into the neuron (Davis, 2011). In response to high levels of intracellular $Ca^{2+}$, calmodulin activates Rutabaga, an adenylyl cyclase (AC) which converts ATP into cAMP (Levin et al., 1992; Livingstone et al., 1984). The cAMP second messenger then activates Protein Kinase A (PKA), which phosphorylates downstream protein targets that affect biological activities at the post-synaptic membrane. In contrast, *dunce* encodes cAMP phosphodiesterase (PDE), which works antagonistically to Rutabaga and degrades cAMP, thus decreasing its intracellular levels (Dudai et al., 1976). Despite their antagonistic roles, mutations in both *rutabaga* and *dunce* have similar effects on both short-term memory (Dudai et al., 1976; Livingstone et al., 1984) and synaptic plasticity at both the excitatory and inhibitory synapse (Lee and O'Dowd, 2000), indicating that the proper maintenance of cAMP homeostasis is more important than the absolute levels of cAMP in neurons.

Much of what is known about the molecular mechanisms underlying long-term memory are due to parallel studies in *Drosophila* and *Aplysia*. Long-term memories in *Drosophila* can last for more than 24 hours (Davis, 2011), and require MB α/β neurons cAMP signaling independent from—but parallel to—short-term memory signaling in the MB γ neurons (Blum et al., 2009). In comparison to the formation of short-term memory, long-term memory formation requires consistent conditioning stimuli that cause more persistent and longer-lasting PKA activation. Active PKA that is translocated to the nucleus phosphorylates the cAMP response element binding protein (CREB) (Yin and Tully, 1996; Yin et al., 1994). Nuclear CREB works as a transcription factor that regulates cAMP signaling-dependent gene expression (Yin and Tully, 1996; Yu et al., 2006). Because of the evident importance of CREB

in the formation of long-term memory, but not short-term memory, it is widely understood that short-term memory formation is protein synthesis-independent, while long-term memory is protein synthesis-dependent (Dunning and During, 2003; Frank and Greenberg, 1994; Yin and Tully, 1996) (Figure 5).

As mentioned above (1.2), the role of the cAMP pathway in memory is conserved between *Drosophila* and mammals. Studies have shown that mice with targeted knockout of CREB are deficient in long-term potentiation and display loss of memory phenotypes (Bourtchuladze et al., 1994). Furthermore, several known ID genes have been shown to affect the cAMP pathway, including the CREB binding protein (CREBBP) gene, which is mutated in patients with a rare form of ID called Rubinstein-Taybi syndrome (Rubinstein and Taybi, 1963). The meaningful similarities between memory consolidation pathways in mammals and flies reinforce the use of *Drosophila* as an effective model for studying the molecular mechanisms underlying learning and memory.

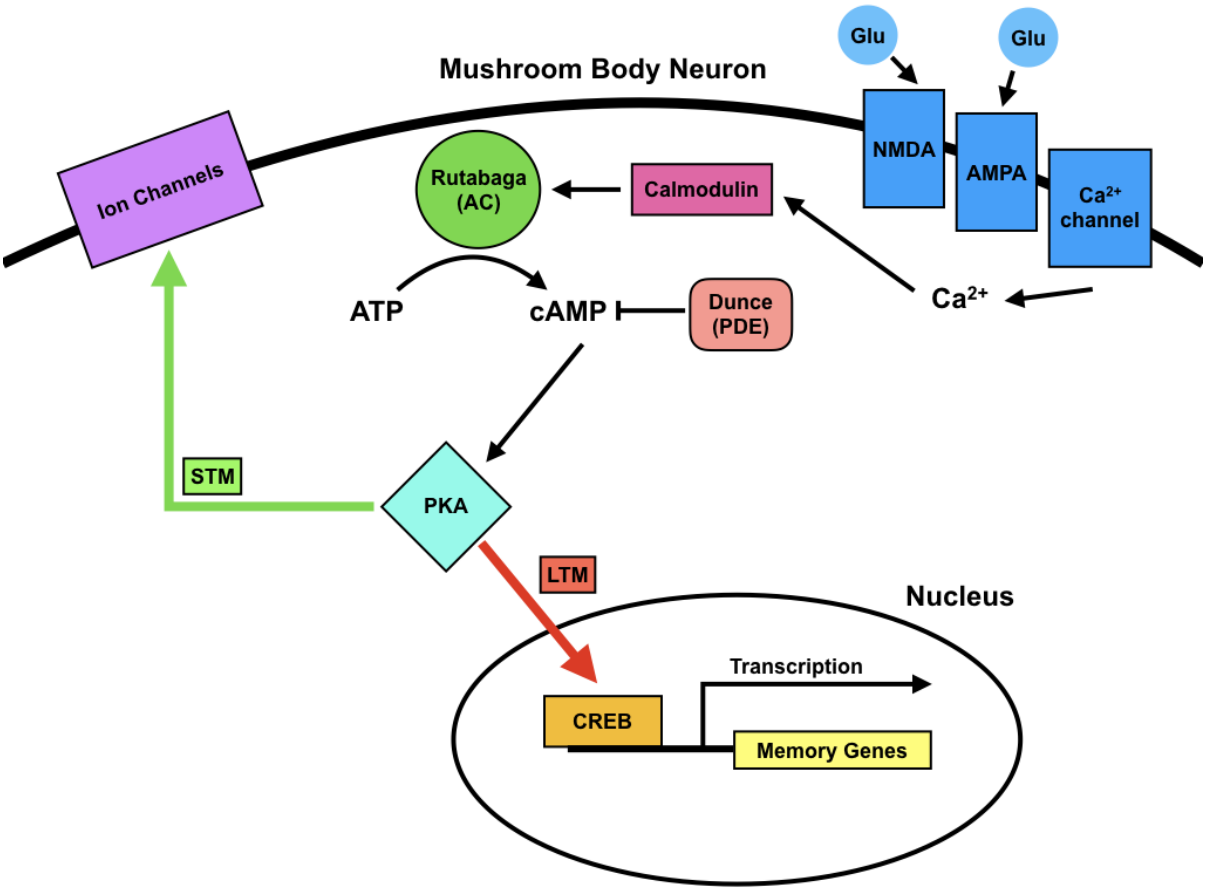



**Figure 5. Molecular mechanisms of *Drosophila* short- and long-term memory.**

Simplified diagram of the mechanisms of short- and long-term memory with specific focus on the role of the cAMP pathway. Activation of Rutabaga adenylyl cyclase leads to increased cAMP in the MB. High levels of cAMP phosphorylate downstream targets of PKA, resulting in short-term memory signaling. Persistent activation of PKA results in signaling to the nucleus, resulting in long-term memory via CREB-dependent transcriptional activity. Figure adapted from: (Bolduc and Tully, 2009).

### 1.2.3. Courtship conditioning as a paradigm for studying memory

Following the initial olfactory conditioning experiments that revealed the capacity for *Drosophila* memory, many other experimental paradigms have been used to study memory in *Drosophila*, including: classical (Pavlovian) conditioning (Tully and Quinn, 1985), appetitive learning (Schroll et al., 2006), visual place learning and spatial memory (Ofstad et al., 2011), and courtship conditioning (Siegel and Hall, 1979). Of these paradigms, courtship conditioning is the most ecologically relevant paradigm because training is less restricted by the confines of the laboratory, as courtship is a natural *Drosophila* behaviour (Gailey et al., 1982; Kamyshev et al., 1999; Koemans et al., 2017; Siegel and Hall, 1979). In the courtship conditioning assay, we try to limit extrinsic stresses that may affect *Drosophila* behaviour by transferring flies between wells and chambers by gentle aspiration, and not anesthetizing them with carbon dioxide ($CO_2$) throughout the assay, except during initial collection of male subjects, and during the initial pairing of training previously-mated females (PMFs) with un-anesthetized males (Ejima and Griffith, 2011).

*Drosophila* courtship is defined by a set of established, and easily recognizable, movements and behaviours (Bastock and Manning, 1955). During a courtship attempt, a male fly orients himself towards a female fly and chases her, taps her with his forelimb, performs an audible courtship song by vibrating his wing, licks her genitalia, and finally attempts to copulate with her by curling his abdomen towards her from behind (Greenspan and Ferveur, 2000; Hall, 1994; Sokolowski, 2001). However, PMFs reject male flies' courtship attempts. When male flies are exposed to PMFs, they suppress their subsequent courtship efforts, through a form of associative learning (Ejima et al., 2005; Siegel and Hall, 1979). Suppression of courtship behaviour occurs due to a response to the association of the conditioned stimulus of courtship rejection with an unconditioned stimulus, the pheromone cis-Vaccenyl Acetate (cVA). During copulation, male flies transfer cVA to female flies through their ejaculate, which reduces the female's receptivity to future mating attempts (Ejima et al., 2007). Therefore, cVA is present on PMFs but is absent on virgin females, providing an indicator of receptivity to male flies trying to mate. The behavioural response to cVA by male flies is mediated through dopamine neurons that signal through the MB γ lobe (Keleman et al., 2012). In the courtship conditioning assay, male flies are subjected to training with PMFs, and their capacity for courtship

suppression in subsequent testing with a different PMF is compared to the courtship behaviour of naïve, untrained male flies. The courtship conditioning assay can be used to analyze both short- and long-term memory, with experimental design differing only by adjusting training and separation times.

## 1.3. Rationale and Objectives

Although the highly-conserved SWI/SNF complex has been shown to play various important roles in neuronal processes across multiple species—including contributing to ID in humans—there is still more known about the SWI/SNF complex's role and function in cancer biology than in neurons. Even though 11 out of 29 human SWI/SNF genes have been implicated in the etiology of ID, only the contributions of BAF53b in acute nervous system processes in mice has been studied extensively. It is important to identify whether other members of the SWI/SNF complex influence proper nervous system function to better understand the mechanisms by which the SWI/SNF complex mediates gene regulation in post-mitotic neuronal development and function.

Because of the high level of conservation of both the SWI/SNF complex and the molecular mechanisms of memory in *Drosophila* with respect to humans, I used flies as a model system to investigate the role of each of the 15 *Drosophila* SWI/SNF subunits in the formation of both short- and long-term memory. I hypothesized that members of the SWI/SNF complex influence both short- and long-term memory formation through gene regulation that is important in MB-neuron development, and/or the post-developmental processes during the formation of memory in adult flies.

In this research project, I aimed to:

1. Systematically analyze whether knockdown of individual *Drosophila* SWI/SNF subunits in the MB impairs the ability for normal short- and long-term memory using courtship conditioning.

2. Determine if the *Drosophila* SWI/SNF complex has an adult-specific role in the MB for the regulation short- and long-term courtship memory.

This project is the first to provide a broad-scale analysis of the role of each *Drosophila* SWI/SNF complex subunit in learning and memory. It is also the first study in any organism to conduct a screen for the adult-specific role for the SWI/SNF complex in the regulation of memory formation, as opposed to studying the effects of gene manipulation beginning during development. Since it is unknown whether loss of memory phenotypes in SWI/SNF-knockdown flies are caused by shortcomings in neuronal development (such as altered cell morphology and identity) or adult-specific brain processes (such as the dynamic transcriptional regulation required for long-term memory), this study provides the foundation on which to build further investigation into the genetic regulatory mechanisms underlying identified memory phenotypes.

# 2. Materials & Methods

## 2.1. Fly stocks and husbandry

All *Drosophila melanogaster* stocks were maintained at room temperature on standard cornmeal-yeast media. All stocks were obtained from either the Bloomington Drosophila Stock Center (Bloomington, IN, USA), or the Vienna Drosophila Resource Center (VDRC) (Vienna, Austria). Inducible RNAi stocks targeted against each SWI/SNF gene, and their respective appropriate controls, were obtained from Bloomington's Transgenic RNAi Project (TRiP) collections, and VDRC's GD and KK libraries (See Appendix A for a list of stocks used in this thesis and their descriptions). TRiP RNAi stocks are created by transgenic insertion of RNAi hairpins into either the attP40 (second chromosomal) or attP2 (third chromosomal) genomic landing site using either VALIUM10 or VALIUM20 vectors (Ni et al., 2008, 2011; Perkins et al., 2015). GD library RNAi stocks are created by random *P*-element insertion using the pMF3 transformation vector (Dietzl et al., 2007). KK library RNAi stocks are created by targeted phiC31 integration at the VIE260b landing site on the second chromosome (Dietzl et al., 2007). The genetic background stocks into which transgenic RNAi constructs are inserted were used as controls (Appendix A). These stocks have the same genetic composition as the RNAi stocks in each respective library, but have no P-element or transformation vector insertion (Dietzl et al., 2007; Perkins et al., 2015). The exception to this is TRiP VALIUM20 insertions on the third chromosome. Instead of the attP2 genetic background stock, a hairpin stock targeting mCherry (mCherry-RNAi) was used as a control due to the presence of scutoid [*sc**], which is found on the X chromosome of VALIUM20 RNAi stocks, but not in the attP2 genetic background stock (Appendix A), making it the most similar genetic background control (Ni et al., 2008; Perkins et al., 2015). In all experiments, flies from different RNAi stocks were crossed to flies from the same GAL4 driver stock. Therefore, knockdown flies each had the same genetic background as their appropriate control. All genotypes for control genotypes and sample genotypes for SWI/SNF knockdowns are recorded in

Table 2.

**Table 2. List of controls and sample SWI/SNF knockdown genotypes used in both MB-specific knockdowns, and adult-specific knockdowns.**

*UAS-RNAi* represents a generic SWI/SNF RNAi stock of that genotype. Full genotypes of each SWI/SNF RNAi stock and its appropriate control genotype are listed in Appendix A.

| Control Name | Control Genotype | Knockdown Genotype |
|---|---|---|
| attP40 | $\frac{y^1 v^1}{Y};\frac{attP40}{+};\frac{+}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y};\frac{UAS\text{-}RNAi}{+};\frac{+}{R14H06\text{-}GAL4}$ |
| mCherry-RNAi | $\frac{y^1 sc^* v^1}{Y};\frac{+}{+};\frac{UAS\text{-}mCherry\text{-}RNAi}{R14H06\text{-}GAL4}$ | $\frac{y^1 sc^* v^1}{Y};\frac{+}{+};\frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| attP2 | $\frac{y^1 v^1}{Y};\frac{+}{+};\frac{attP2}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y};\frac{+}{+};\frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| attP2 (Dicer2) | $\frac{y^1 v^1}{Y};\frac{+}{UAS\text{-}Dicer2};\frac{attP2}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y};\frac{+}{UAS\text{-}Dicer2};\frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| GD | $\frac{w^{1118}}{Y};\frac{+}{UAS\text{-}Dicer2};\frac{+}{R14H06\text{-}GAL4}$ | $\frac{w^{1118}}{Y};\frac{UAS\text{-}RNAi}{UAS\text{-}Dicer2};\frac{+}{R14H06\text{-}GAL4}$ or $\frac{w^{1118}}{Y};\frac{+}{UAS\text{-}Dicer2};\frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| KK | $\frac{y^1 w^{1118}}{Y};\frac{attP,y^+ w^{3'}}{UAS\text{-}Dicer2};\frac{+}{R14H06\text{-}GAL4}$ | $\frac{y^1 w^{1118}}{Y};\frac{UAS\text{-}RNAi}{UAS\text{-}Dicer2};\frac{+}{R14H06\text{-}GAL4}$ |
| **Control Name** | **Adult-Specific Control Genotype** | **Adult-Specific Knockdown Genotype** |
| attP40 | $\frac{y^1 v^1}{Y};\frac{attP40}{tubP\text{-}GAL80^{ts}};\frac{+}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y};\frac{UAS\text{-}RNAi}{tubP\text{-}GAL80^{ts}};\frac{+}{R14H06\text{-}GAL4}$ |

| | | |
|---|---|---|
| mCherry-RNAi | $\frac{y^1 sc^* v^1}{Y}; \frac{+}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}mCherry\text{-}RNAi}{R14H06\text{-}GAL4}$ | $\frac{y^1 sc^* v^1}{Y}; \frac{+}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| attP2 | $\frac{y^1 v^1}{Y}; \frac{+}{tubP\text{-}GAL80^{ts}}; \frac{attP2}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y}; \frac{+}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| attP2 (Dicer2) | $\frac{y^1 v^1}{Y}; \frac{UAS\text{-}Dicer2}{tubP\text{-}GAL80^{ts}}; \frac{attP2}{R14H06\text{-}GAL4}$ | $\frac{y^1 v^1}{Y}; \frac{UAS\text{-}Dicer2}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| GD | $\frac{w^{1118}}{Y}; \frac{UAS\text{-}Dicer2}{tubP\text{-}GAL80^{ts}}; \frac{+}{R14H06\text{-}GAL4}$ | $\frac{w^{1118}}{Y}; \frac{UAS\text{-}RNAi}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}Dicer2}{R14H06\text{-}GAL4}$ or $\frac{w^{1118}}{Y}; \frac{UAS\text{-}Dicer2}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}RNAi}{R14H06\text{-}GAL4}$ |
| KK | $\frac{y^1 w^{1118}}{Y}; \frac{attP,y^+ w^{3'}}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}Dicer2}{R14H06\text{-}GAL4}$ | $\frac{y^1 w^{1118}}{Y}; \frac{UAS\text{-}RNAi}{tubP\text{-}GAL80^{ts}}; \frac{UAS\text{-}Dicer2}{R14H06\text{-}GAL4}$ |

## 2.2. Mushroom body-specific knockdown of SWI/SNF genes using the GAL4-UAS system

Knockdown of SWI/SNF genes in the mushroom body (MB) was conducted using the GAL4-UAS system combined with transgenic RNA interference (RNAi) technology. RNAi is an effective method of knocking down gene expression used in experimental biology that works by targeting the degradation of specific mRNA sequences, thus neutralizing the translation of specific proteins. GAL4 is a yeast transcription factor that activates expression of genes under control of an Upstream Activating Sequence (UAS) enhancer (Brand and Perrimon, 1993). The GAL4-UAS system allows for tissue-specific manipulation of gene expression, which is required in this study because null mutations in many *Drosophila* SWI/SNF genes are known to be embryonic lethal. Mushroom body-specificity is achieved using the R14H06-GAL4 'driver' construct, which is highly and specifically expressed in the MB (Jenett et al., 2012).

Male R14H06-GAL4 (BL48667) 'driver' flies were crossed with female 'responder' flies expressing UAS-RNAi sequences specific to a *Drosophila* SWI/SNF mRNA transcript and to several control genotypes (Appendix A). Crosses were incubated at 25ºC, 70% relative humidity, and 12L:12D light cycle. In progeny of these crosses, GAL4 induces expression of double-stranded hairpin RNAs (dsRNA) that are processed into silencing RNAs (siRNAs) that direct sequence-specific degradation of the target mRNA, culminating in knockdown of individual SWI/SNF gene products (Brand and Perrimon, 1993). $F_1$ males from these crosses were collected at eclosion and used for courtship conditioning experiments (Figure 6).

Transgenic RNAi sequences in flies can either be transcribed into short- or long-hairpin transcripts. Dicer-2 is an endogenous regulatory protein that aids in effectively processing RNA hairpins into single-stranded RNA that target specific mRNA sequences for degradation (Pham et al., 2004). Endogenous Dicer-2 is sufficient for effective processing of RNAi in flies with short-hairpin transgenes obtained from TRiP's VALIUM20 collection (Groth et al., 2004; Ni et al., 2008, 2011; Perkins et al., 2015). However, RNAi-mediated knockdown using flies from long-hairpin RNAi libraries (TRiP's VALIUM10 collections and VDRC's GD and KK

libraries) is more effective when additional GAL4-mediated Dicer-2 protein is co-expressed (Dietzl et al., 2007; Lee et al., 2004). For MB-specific knockdown of RNAi stocks (and controls) from these libraries, *UAS-Dicer2* is co-expressed (Appendix A).

MB-specific knockdown of SWI/SNF genes was conducted using at least two different RNAi stocks targeting the same gene but with different target sequences and preferentially from different transgenic libraries, when possible, to control for off-target effects and differences in genetic background. Therefore, experiments were completed on flies with 31 unique RNAi-mediated knockdowns, targeting 15 SWI/SNF genes.

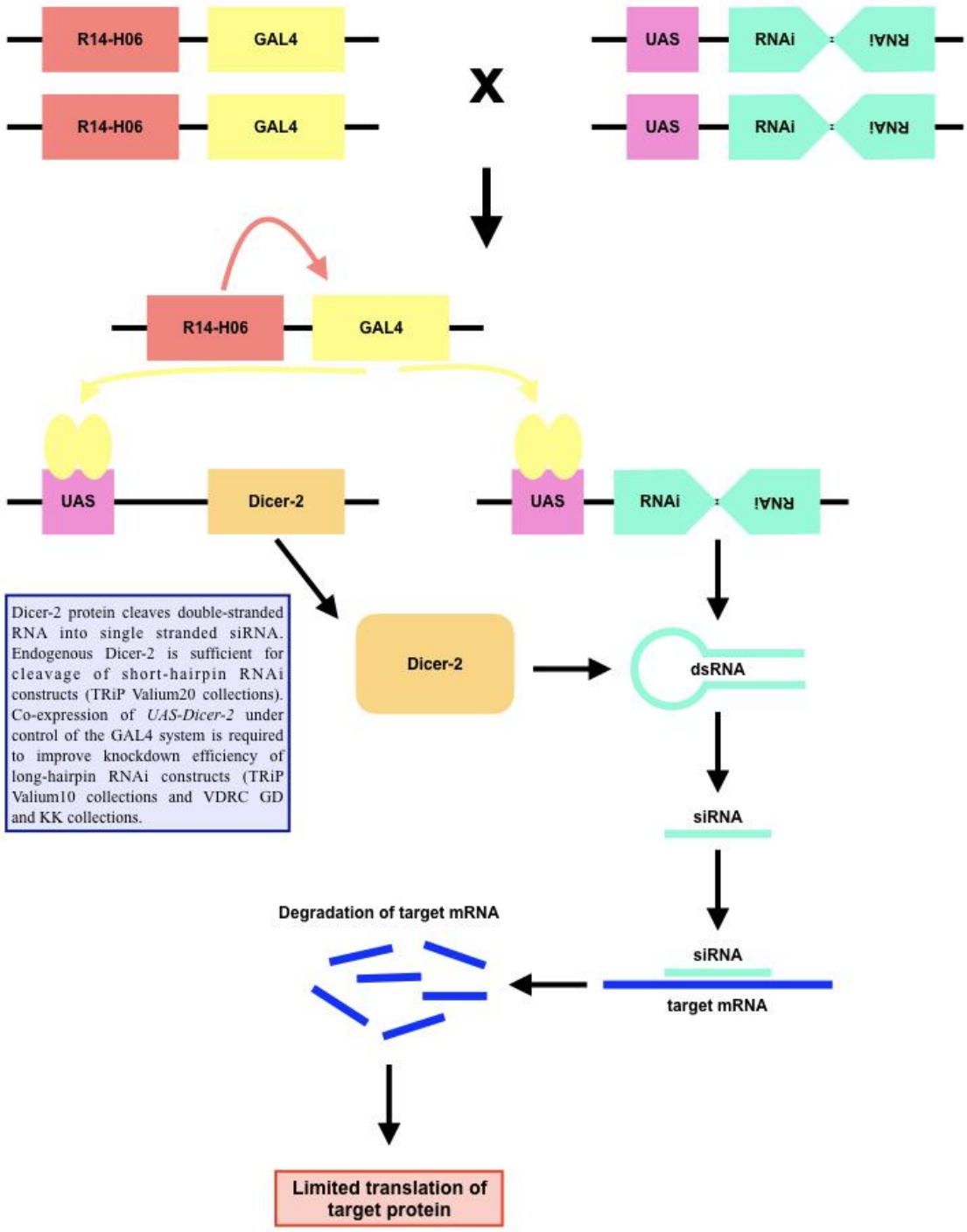



**Figure 6. The GAL4-UAS system allows for MB-specific knockdown of SWI/SNF gene expression.**

R14H06-GAL4 drives MB-specific expression of RNAi constructs under control of an UAS. Long-hairpin RNAi constructs require Dicer-2 protein co-expression for effective knockdown of gene expression.

### 2.2.1. Lethality assay for testing RNAi efficiency

To measure lethality caused by ubiquitous RNAi knockdown, female flies heterozygous for the ubiquitous expression driver, Actin5C-GAL4 (BL25374), were crossed with male UAS-RNAi stocks in three biological replicates at 25ºC, with 70% relative humidity, and a 12L:12D cycle. Due to heterozygosity of the Actin5C-GAL4 driver, which is balanced over the CyO chromosome, 50% of all progeny are expected to have active GAL4-UAS expression, while 50% of flies are expected to have the curly wing marker on the CyO balancer chromosome (and no GAL4-UAS activity). $F_1$ progeny were scored for the presence of the curly wing marker. The proportion of total flies observed without the curly wing marker indicated survival with Actin5C-GAL4-driven expression of the RNAi construct. Survival percentage was calculated by $\frac{n_{\text{progeny with straight wings}}}{n_{\text{progeny with curly wings}}}$, and was calculated both independently for male and female flies, and cumulatively. Deviation from expected population frequencies was analyzed using a $\chi^2$ test.

## 2.3. Adult-specific knockdown of SWI/SNF genes using the temperature-sensitive GAL80 system

The temperature-sensitive GAL80 ($GAL80^{ts}$) system, also called the Temporal and Regional Gene Expression Targeting (TARGET) system, was used to perform adult-specific knockdown of SWI/SNF genes in the mushroom body (McGuire et al., 2004). GAL80 is a transcription factor that binds to GAL4 to repress transcription (del Valle Rodríguez et al., 2011). By combining a ubiquitously-expressed $GAL80^{ts}$ construct (tubP-$GAL80^{ts}$) with a MB-specific GAL4-UAS driver (R14H06-GAL4), GAL4-mediated transgene expression can be temporally regulated (McGuire et al., 2003, 2004; del Valle Rodríguez et al., 2011). At 18ºC, active $GAL80^{ts}$ represses GAL4-mediated transcription. $GAL80^{ts}$ is inactivated at 29ºC, thus permitting GAL4-mediated transcriptional activation.

Male flies containing ubiquitously-expressed $GAL80^{ts}$ and the MB-specific R14H06-GAL4 driver (genotype: tubP-$GAL80^{ts}$;R14H06-GAL4) were crossed to female responder flies containing UAS-RNAi constructs and to several control genotypes. Crosses were incubated at 18ºC, with 70% relative humidity, and a 12L:12D light cycle, inhibiting RNAi-

mediated knockdown during *Drosophila* development. For courtship conditioning experiments, $F_1$ males were collected at eclosion and transferred to 29ºC, 70% relative humidity, and 12L:12D light cycle conditions that allow for adult-specific GAL4-UAS-mediated SWI/SNF knockdown in the MB for five days prior to testing (Figure 7).

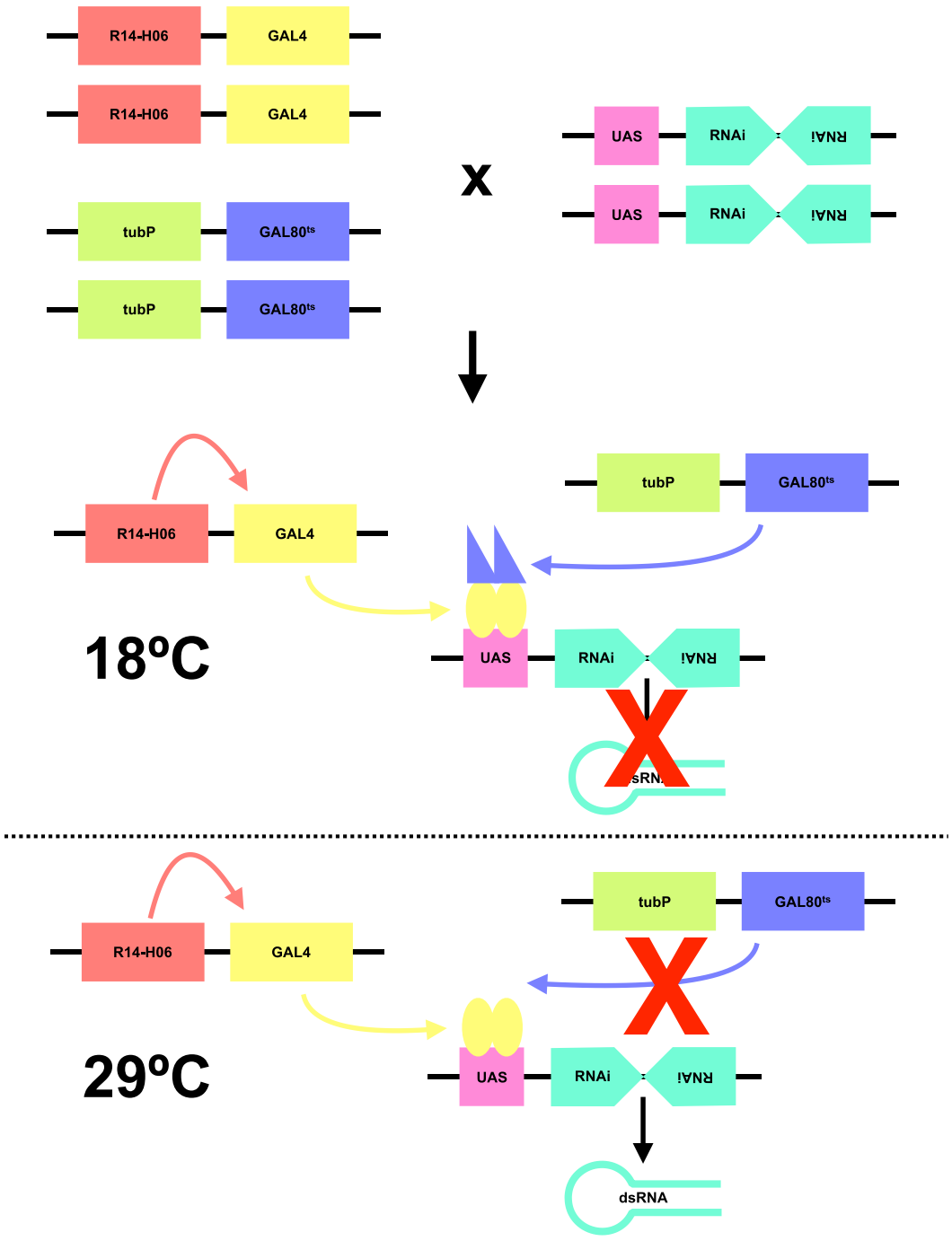



**Figure 7. The temperature-sensitive GAL80 (GAL80$^{ts}$) allows for adult-specific knockdown of SWI/SNF gene expression in the MB.**

When flies are raised at 18ºC, *tubP-GAL80$^{ts}$* inhibits regular R14H06-GAL4-UAS-mediated knockdown of SWI/SNF genes in the MB during embryonic and larval development. At eclosion, adult flies are transferred to 29ºC where *tubP-GAL80$^{ts}$* activity is inhibited, allowing for unobstructed R14H06-GAL4-UAS-mediated knockdown in the MB in an adult-specific manner.

### 2.3.1. Validation of GAL80$^{ts}$-mediated adult-specific GAL4-UAS activity

To validate the effectiveness of the GAL80$^{ts}$ system in *Drosophila* mushroom bodies, fluorescence microscopy was used to observe the expression/repression of GAL4-induced expression of GFP in the MB under the control of GAL80$^{ts}$ in response to changing temperatures. Male GAL80$^{ts}$;R14H06-GAL4 flies were crossed with female UAS-EGFP responder flies (BL6658). Crosses were reared at either 18ºC or 29ºC, with 70% relative humidity, and a 12L:12D light cycle. Male third instar larvae and adults were collected for brain dissection. *Drosophila* brains were dissected in 1x phosphate buffer saline (PBS, pH 7.2), and fixed with 4% paraformaldehyde (PFA) for 45 minutes at room temperature. Brains were then mounted in Vectashield (Vector Laboratories) on microscope slides. Whole brains were imaged by compound fluorescence microscopy (Zeiss Axioimager Z1). Images were processed using FIJI software (https://fiji.sc).

## 2.4. Courtship conditioning assay

Courtship conditioning was performed as described previously (Koemans et al., 2017). Male knockdown flies were collected at eclosion and raised in isolation for five days in individual wells of a 96 2mm-well flat-bottom block (Qiagen) filled with 0.5 ml of *Drosophila* media. Collected males were randomly assigned to either untrained (naïve) or trained cohorts. Flies in trained cohorts are paired with a five-day-old PMF in a clean well of a flat-bottom block filled with media within 30 minutes of the incubator lights turning on. Training lasts one hour for short-term memory experiments, and seven hours for long-term memory experiments. During training, PMFs reject the male subject's courtship attempts. Following training, male subjects are separated from the PMF used in training, and placed in isolation in a separate well. Isolation lasts one hour for short-term memory experiments, and 24 hours for long-term memory experiments. Flies in the naïve cohort remain isolated through the training and isolation periods. Following isolation, both naïve and trained males are individually paired with a new PMF in specialized courtship chambers capable of holding up to 18 distinct pairs of flies. The courtship chambers are placed under a video camera and courtship behaviour is filmed for 10 minutes.

Standard experiments are conducted on three consecutive days, allowing assays to be completed on up to 54 pairs of flies per genotype and training condition. For genotypes in which cohorts of at least 30 flies per condition were unachievable during initial testing, experiments were repeated to attempt to increase overall cohort size. If cohorts of 30 flies per condition were unachievable after re-testing a given genotype, experimentation was ceased.

### 2.4.1. Quantification and analysis of *Drosophila* courtship behaviour

To quantify courtship behaviour, observers sufficiently trained to identify *Drosophila* courtship behaviours analyzed videos collected in courtship conditioning experiments. Videos were specifically assigned to different scorers to ensure that the same scorer was scoring both the naïve and trained flies of the same genotype from the same experiment, and that no scorer was scoring all videos of a given genotype. Although videos were purposefully designated, scorers were naïve to the overall nature and purpose of the study, and to the genotypes of the flies in their assigned videos. All scorers received approximately the same number of videos of control genotypes. In addition to trained scorers, Actual Track software (Actual Analytics, Ltd.) was used to score experiments (Koolen et al., 2012). Scorers calculated and recorded a courtship index (CI) for each fly pair, defined as the proportion of time during the 10-minute video in which the male displays courtship behaviours towards the PMF (Keleman et al., 2012; McBride et al., 1999). A learning index (LI)—the reduction in mean courtship activity of trained males compared to naïve ($LI = \frac{\overline{CI}_{naive} - \overline{CI}_{trained}}{\overline{CI}_{naive}}$) (Keleman et al., 2012)—is calculated to describe the capacity for memory of each genotype.

To confirm consistency between courtship scoring completed by different trained observers, each of 16 scorers (14 work-study students and volunteers, Actual Track software, and myself) were assigned the same two videos, which consisted of 23 total fly pairs, and scored them for courtship. Scorers were blind to each other's results during scoring. Comparisons between scorers were completed by calculating a coefficient of determination ($R^2$) of Pearson's correlation to determine the correlation between CIs collected between each pair of trained scorers.

### 2.4.2. Statistical analysis of courtship memory

Analysis of baseline courtship behaviour in SWI/SNF knockdown flies was conducted by comparing combined $CI_{naive}$ from both short- and long-term memory experiments of knockdown flies to the combined $CI_{naive}$ of the appropriate control genotype using a one-way ANOVA with selected multiple comparisons (Bonferroni multiple comparisons test).

Statistically, loss of memory can be identified using two complimentary methods, one which compares courtship activity within genotypes, and another that compares courtship memory between genotypes and control groups. Reduction of courtship behaviour between naïve ($CI_{naive}$) and trained ($CI_{trained}$) flies of the same genotype was compared using a one-tailed Mann-Whitney test. No significant reduction in CI due to training ($P>0.05$) indicates a loss of memory. Alternatively, a randomization test (random sampling with replacement, 10,000 replicates) (Kamyshev et al., 1999) using a custom R script (R Core Team, 2013) is used to compare LIs of knockdown genotypes to control genotype flies. Loss of memory in knockdowns is indicated by a significant reduction in LI ($P<0.05$) compared to control flies. In some cases, the randomization test indicates borderline reduction in LI ($0.05<P<0.10$), while the Mann-Whitney test indicates no reduction in CI due to training ($P<0.05$). Conversely, a significant reduction in LI ($P<0.05$) compared to control genotypes is observed in some cases, even when there is a clear reduction in CI due to training ($P<0.05$), demonstrating that some memory is still observed in knockdown flies but it is significantly weaker than memory observed in genetic controls. Exact P-values for all tests are shown in Appendix B.

# 3. Results

## 3.1. Validation of scoring method of courtship conditioning

Courtship behaviour for more than 10,000 individual fly pairs was analyzed in this study. Therefore, it was important to ensure that there is consistency between different scorers in the quantification of courtship behaviour. I compared differences in courtship scoring between all 15 trained observers and the Actual Track software using a coefficient of determination ($R^2$) of Pearson's correlation. Overall, a high correlation was observed between trained individual observers and Actual Track software (Figure 8A). All but three individuals produced a mean $R^2 \geq 0.699$ (Figure 8B). The three scorers that produced $R^2 < 0.699$ ($R^2_{S1} = 0.427$, $R^2_{S2} = 0.625$, $R^2_{S14} = 0.572$) were excluded from the analyses (Figure 8B). These data illustrate the potential variability that can arise from individual differences in manual scoring of behaviour, and indicate that with effective training and inspection of scorers, variability can be limited.

Importantly, the Actual Track software produced the fourth highest mean $R^2$ amongst all scorers for the two test videos ($R^2_{AT} = 0.821$) (Figure 8B). On a larger scale, comparison of courtship scores of 204 total trials over 14 videos scored by both Actual Track and myself also resulted in a high coefficient of determination ($R^2 = 0.775$, $P<1x10^{-4}$) (Figure 8C). These results indicate that the Actual Track software is an accurate method of measuring courtship behaviour in *Drosophila*, and is preferred due to increased consistency, absence of bias, and time saved by automated scoring. Due to technical difficulties with Actual Track in the early stages of my project, a combination of automated and manually-scored data was included in this thesis.

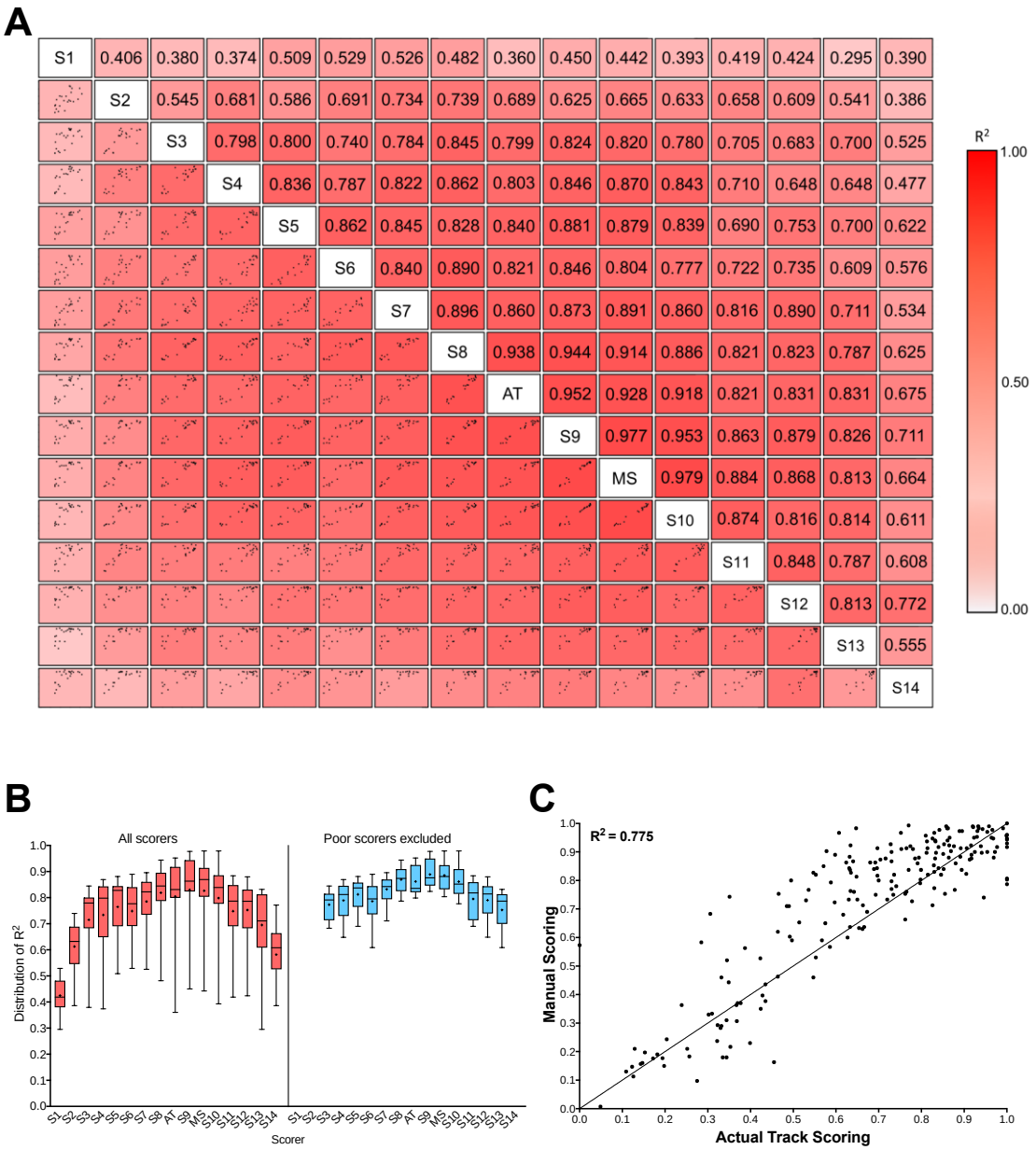



**Figure 8. Validation of courtship scoring method.**

A) Heat map reflects the correlation of courtship scores between pairs of trained observers. Correlation plots comparing scorers is plotted below the diagonal axis, and the corresponding $R^2$ values between each pair of scorers is plotted above the diagonal axis. Box color saturation reflects $R^2$. Scorers with higher median correlations are represented towards the centre of the matrix. Scores compiled by Actual Track software are indicated by AT, scores compiled by the author are indicated by MS, and scores compiled by each of the 14 scorers trained by the author are indicated numerically. B) Boxplots represent the distribution of $R^2$ for each scorer compared to other scorers before (red) and after (blue) excluding the three poor scorers (S1, S2, S14). Mean $R^2$ for each scorer is represented by (+). C) Scatter plot shows correlation over 204 trials between the author (Manual Scoring) and Actual Track Scoring ($R^2$ = 0.775, P <1x $10^{-4}$).

## 3.2. Validation of SWI/SNF RNAi stocks by lethality assay

Although the GAL4-UAS system is usually used to control gene expression in specific tissues, ubiquitous GAL4-UAS expression can be used to mimic effects caused by mutations. Ubiquitous GAL4-mediated expression of *Drosophila* SWI/SNF RNAi constructs were used to test knockdown effectiveness for each RNAi stock selected for courtship conditioning experiments. Since null mutations in most *Drosophila* SWI/SNF complex genes are known to be embryonic lethal, it was expected that ubiquitous RNAi-mediated knockdown of SWI/SNF genes would result in lethality. In this experiment, if ubiquitous GAL4-mediated knockdown of an individual SWI/SNF gene resulted in lethality, it is assumed that the RNAi line would also be effective to cause tissue-specific knockdown of that gene in the MB. In contrast, if an expectedly lethal ubiquitous knockdown did not cause lethality, it was concluded that the RNAi stock was insufficient for SWI/SNF gene knockdown in the MB, and was not included in courtship conditioning experiments.

Ubiquitous knockdown of 22/31 SWI/SNF RNAi stocks selected for this study using the Actin-GAL4 driver caused complete lethality (% survival $\leq$ 5.00, $P<1x10^{-4}$) (Table 3). No reduction in survival compared to expected population frequencies was observed in five SWI/SNF RNAi stocks (Table 3). Knockdown of *polybromo* (BL32840) revealed no reduction in survival (102% survival ± 12.2, P=0.482). Null mutations in *polybromo* are known to be non-lethal, and thus lethality in *polybromo* knockdowns was not predicted (Mohrmann et al., 2004). Each of the other four RNAi lines in which no significant reduction in survival was observed were either RNAi targeting genes that were expected to be lethal (*Bap55* - BL31708: 88.3% survival ± 23.3 SE, P=0.884; and *brahma* - BL34520: 77.8% survival ± 15.6 SE, P=0.137) or targeting genes where the effect of null mutations of lethality is unknown (*BCL7-like* - BL35714: 96.9% survival ± 13.2 SE, P=0.983; and *CG10555* - BL50606: 98.0% survival ± 37.4 SE, P=0.998). For each of these latter four genes, lethality was observed by Actin5C-GAL4-mediated knockdown in additional RNAi lines (Table 3). Except for the *polybromo* RNAi line, each of the RNAi lines that did not induce lethality were excluded from the study.

Ubiquitous knockdown of four of the 31 SWI/SNF RNAi lines caused a partial reduction in survival compared to expected population frequencies, but did not induce complete lethality

(Table 3). This was observed for Act5C-GAL4-mediated knockdowns of *Snr1* (v12644: 53.8% survival ± 18.8 SE, P=0.010), *BCL7-like* (v20410: 43.6% survival ± 5.78 SE, $P<1x10^{-4}$), *Act5C* (BL42651: 31.4% survival ± 36.2 SE, $P=5.8x10^{-3}$), and *Bap60* (BL33954: 17.8% survival ± 12.4 SE, $P<1x10^{-4}$). These RNAi stocks were included in courtship studies, and in the cases of *Snr1* and *Bap60* RNAi, caused weaker memory phenotypes than other RNAi lines targeting the same genes, which induced complete lethality (see Figure 10). The observed partial lethality suggests that knockdown using these RNAi stocks have a significant, but incomplete effect on gene expression.

**Table 3. Survival of Actin-GAL4-mediated knockdown of SWI/SNF RNAi stocks used in courtship conditioning assays.**

| Knockdown (stock no.) | Total Survival (% ± SE) | $n_{total}$ | Male Survival (% ± SE) | $n_{male}$ | Female Survival (% ± SE) | $n_{female}$ | $\chi^2$ | P |
|---|---|---|---|---|---|---|---|---|
| polybromo (32840) | 102 ± 12.2 | 198 | 81.5 ± 16.5 | 98 | 127 ± 7.79 | 100 | 2.46 | 0.482 |
| CG10555 (50606) | 98.0 ± 37.4 | 196 | 100 ± 66.3 | 88 | 96.4 ± 23.4 | 108 | 0.0370 | 0.998 |
| BCL7-like (35714) | 96.9 ± 13.2 | 128 | 90.3 ± 26.1 | 59 | 103 ± 34.5 | 69 | 0.167 | 0.983 |
| Bap55 (31708) | 83.3 ± 23.3 | 77 | 85.2 ± 27.0 | 50 | 80.0 ± 29.3 | 27 | 0.653 | 0.884 |
| mCherry (35785) | 80.2 ± 8.40 | 245 | 73.1 ± 18.4 | 116 | 87.0 ± 6.50 | 129 | 3.42 | 0.331 |
| brahma (34520) | 77.8 ± 15.6 | 208 | 61.7 ± 23.0 | 97 | 94.7 ± 21.3 | 111 | 5.54 | 0.137 |
| Snr1 (v12644) | 53.8 ± 18.8 | 120 | 45.2 ± 12.8 | 45 | 59.6 ± 22.3 | 75 | 11.2 | 0.010 |
| BCL7-like (v20410) | 43.6 ± 5.78 | 168 | 37.7 ± 14.7 | 73 | 48.4 ± 5.01 | 95 | 26.4 | $< 1.0 \times 10^{-4}$ |
| Act5C (42651) | 31.4 ± 36.2 | 46 | 31.2 ± 30.9 | 21 | 31.6 ± 41.5 | 25 | 12.5 | $5.8 \times 10^{-3}$ |
| Bap60 (33954) | 17.8 ± 12.4 | 185 | 23.1 ± 15.8 | 96 | 12.7 ± 8.04 | 89 | 91.0 | $< 1.0 \times 10^{-4}$ |
| Bap55 (v24703) | 5.00 ± 4.40 | 147 | 1.61 ± 1.85 | 63 | 7.69 ± 7.80 | 84 | 121 | $< 1.0 \times 10^{-4}$ |
| CG9650 (v104402) | 4.80 ± 3.30 | 175 | 10.9 ± 8.60 | 71 | 1.00 ± 0.900 | 104 | 146 | $< 1.0 \times 10^{-4}$ |
| Snr1 (32372) | 4.04 ± 3.92 | 103 | 7.84 ± 9.52 | 55 | 0 | 48 | 88.2 | $< 1.0 \times 10^{-4}$ |
| Act5C (v101438) | 3.74 ± 1.75 | 46 | 4.17 ± 1.88 | 21 | 3.39 ± 1.70 | 25 | 191 | $< 1.0 \times 10^{-4}$ |
| Bap111 (35242) | 3.55 ± 0.970 | 146 | 0 | 71 | 7.14 ± 2.61 | 75 | 127 | $< 1.0 \times 10^{-4}$ |
| osa (38285) | 2.47 ± 1.55 | 83 | 2.78 ± 3.03 | 37 | 2.22 ± 1.75 | 46 | 75.2 | $< 1.0 \times 10^{-4}$ |
| osa (v7810) | 2.13 ± 0.980 | 144 | 1.49 ± 2.08 | 68 | 2.70 ± 1.80 | 76 | 132 | $< 1.0 \times 10^{-4}$ |
| Bap170 (26308) | 1.70 ± 3.70 | 120 | 1.56 ± 4.76 | 65 | 1.85 ± 3.03 | 55 | 112 | $< 1.0 \times 10^{-4}$ |
| Bap111 (26218) | 1.41 ± 1.96 | 72 | 0 | 41 | 3.33 ± 6.67 | 31 | 68.1 | $< 1.0 \times 10^{-4}$ |
| CG7154 (v37670) | 1.23 ± 1.15 | 82 | 2.63 ± 2.56 | 39 | 0 | 43 | 78.1 | $< 1.0 \times 10^{-4}$ |
| moira (v110712) | 1.20 ± 1.33 | 168 | 2.63 ± 4.17 | 78 | 0 | 90 | 160 | $< 1.0 \times 10^{-4}$ |
| brahma (v37720) | 0.962 ± 0.790 | 105 | 2.04 ± 1.96 | 50 | 0 | 55 | 101 | $< 1.0 \times 10^{-4}$ |
| CG7154 (v107992) | 0.909 ± 0.850 | 111 | 1.82 ± 1.67 | 56 | 0 | 55 | 107 | $< 1.0 \times 10^{-4}$ |
| moira (v6969) | 0.826 ± 0.930 | 122 | 0 | 58 | 1.59 ± 1.75 | 64 | 118 | $< 1.0 \times 10^{-4}$ |
| Bap170 (v34582) | 0.719 ± 1.15 | 140 | 0 | 61 | 1.28 ± 1.96 | 79 | 136 | $< 1.0 \times 10^{-4}$ |
| CG10555 (v105802) | 0 | 96 | 0 | 42 | 0 | 54 | 96.0 | $< 1.0 \times 10^{-4}$ |
| CG9650 (40852) | 0 | 112 | 0 | 43 | 0 | 69 | 112 | $< 1.0 \times 10^{-4}$ |

| **Knockdown (stock no.)** | **Total Survival (% ± SE)** | **$n_{total}$** | **Male Survival (% ± SE)** | **$n_{male}$** | **Female Survival (% ± SE)** | **$n_{female}$** | **$\chi^2$** | **P** |
|---|---|---|---|---|---|---|---|---|
| CG9650 (v23170) | 0 | 49 | 0 | 24 | 0 | 25 | 22.0 | $< 1.0 \times 10^{-4}$ |
| brahma (31712) | 0 | 61 | 0 | 27 | 0 | 34 | 42.0 | $< 1.0 \times 10^{-4}$ |
| Bap60 (32503) | 0 | 122 | 0 | 65 | 0 | 57 | 122 | $< 1.0 \times 10^{-4}$ |
| e(y)3 (32346) | 0 | 30 | 0 | 13 | 0 | 17 | 30.0 | $< 1.0 \times 10^{-4}$ |
| e(y)3 (v105946) | 0 | 120 | 0 | 46 | 0 | 74 | 120 | $< 1.0 \times 10^{-4}$ |

## 3.3. Objective 1: Analysis of knockdown of SWI/SNF complex genes on conditioned courtship memory

### 3.3.1. Analysis of conditioned courtship memory in control genotypes

Knockdown of SWI/SNF complex genes in the MB was conducted using RNAi stocks from different RNAi collections (Dietzl et al., 2007; Ni et al., 2008, 2011; Perkins et al., 2015) that have been produced and made available for use by the scientific community (2.1, Appendix A). RNAi stocks from different collections have different genetic backgrounds into which the RNAi transgene is inserted. These genetic backgrounds, with no RNAi construct inserted, were used as controls for courtship conditioning experiments (Appendix A). The exception to this is the mCherry-RNAi control, which has the same genetic background as RNAi stocks from the VALIUM20 TRiP collection inserted into the attP2 landing site, but contains an RNAi construct targeting the mCherry fluorophore that has no effect on endogenous *Drosophila* genes (Ni et al., 2011; Perkins et al., 2015). mCherry-RNAi has a more similar genetic background to RNAi stocks than the attP2 genetic background (See: Section 2.1, Appendix A), and expression of this non-targeting RNAi controls for the effect of active GAL4-UAS expression, and for the production and processing of dsRNA.

Analyses of short- and long-term courtship memory on the six control genotypes crossed to the R14H06-GAL4 driver (See: Section 1.1, Appendix A) were conducted to validate the efficacy of the courtship conditioning protocol and to examine any effects that may arise due to differences in genetic background. In crosses of R14H06-GAL4 flies to the GD, KK, and attP2 (Dicer) controls, *Dicer-2* is co-expressed (See: Section 1.1, Appendix A). Each of the six control genotypes demonstrated significant reduction of CI relative to naïve flies in both short- and long-term memory (Figure 9A). The LIs for each of the control groups ranged from $LI_{attP2}$=0.335 to $LI_{KK}$=0.468 for short-term memory experiments and from $LI_{GD}$=0.206 to $LI_{mCherry\text{-}RNAi}$=0.318 for long-term memory experiments (Figure 9B). These LIs are similar to those published in other studies (Keleman et al., 2012; Zografos et al., 2016). As such, the courtship conditioning protocol—as used in this study—is effective for eliciting both short- and long-term memory in tested *Drosophila* control strains.

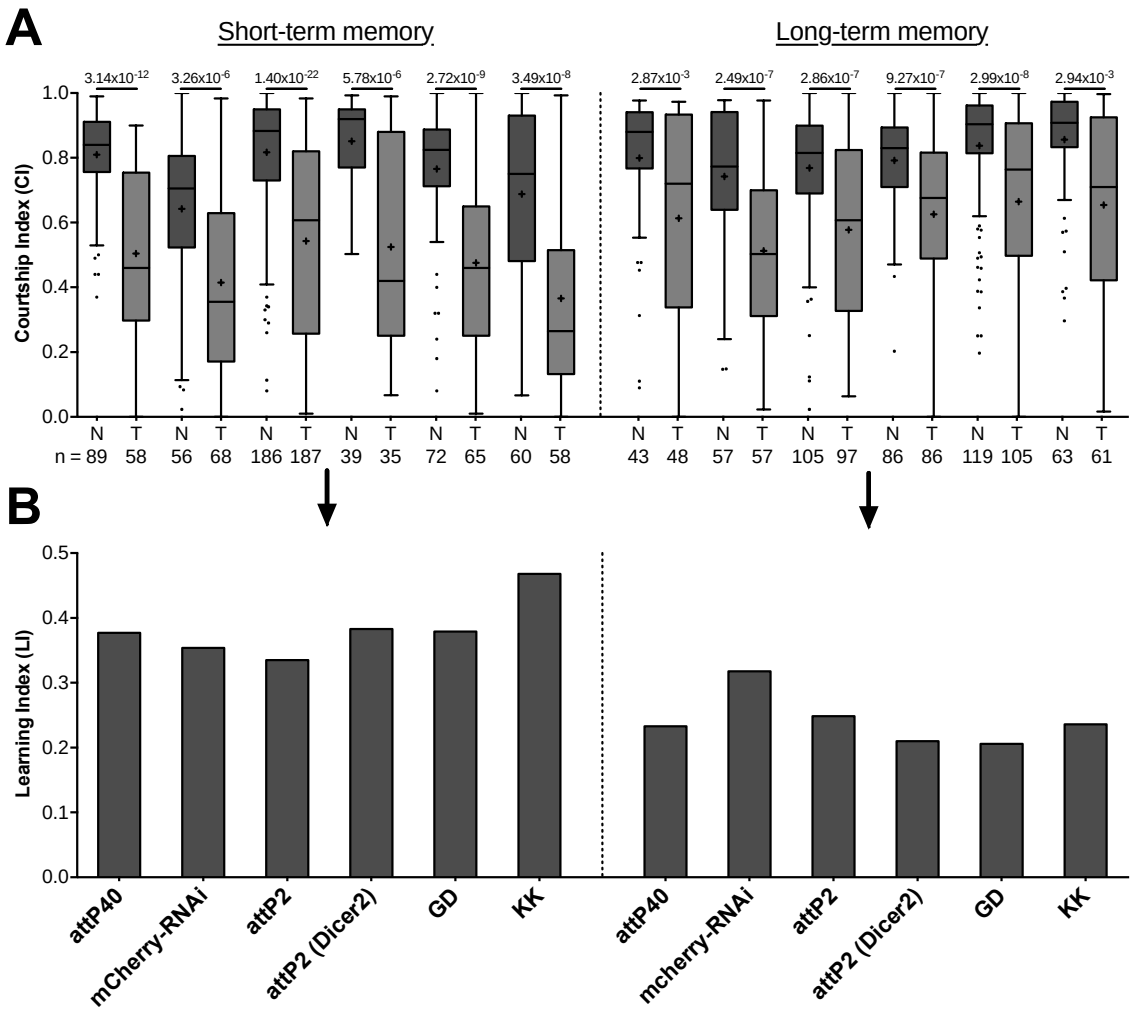



**Figure 9. Analysis of courtship in control genotypes using the R14H06-GAL4 driver.**

Courtship indices (A) and learning indices (B) of different control flies crossed to R14H06-GAL4 driver flies used in courtship conditioning assays. A) Boxplots represent distribution of CI of naïve (N) and trained (T) male flies. Mean CI is represented by (+). Total flies tested for each genotype and condition are listed in the (n=) row. One-tailed Mann-Whitney test was used to compare $CI_{trained}$ to $CI_{naive}$. B) Bars represent LIs calculated from CIs.

### 3.3.2. MB-specific knockdown of SWI/SNF impairs short-term memory

To determine if MB-specific knockdown of SWI/SNF subunits caused loss of courtship memory, LIs of knockdown flies were compared to the appropriate control. Since six different control groups were used, a relative LI ($LI_{knockdown}/LI_{control}$) was calculated to normalize LIs across different genotypes by comparing each knockdown to the appropriate control group. Courtship conditioning experiments revealed that knockdown of 11/15 SWI/SNF genes caused loss of short-term memory (Figure 10A).

Significant loss of short-term courtship memory was observed in MB-specific knockdowns using at least one RNAi stock for six out of the seven core SWI/SNF complex genes. For the core subunits, *Bap60*, *Snr1*, and *brahma*, loss of short-term memory was observed in knockdowns using two independent RNAi lines. For *Bap55*, only one RNAi line was tested, showing loss of memory. Knockdown of both *Bap111* and *moira* caused inconsistent phenotypes where loss of memory is observed in one out of two RNAi lines tested. Difference between the two moira-RNAi lines are not obvious and may be due to sample size or variability in the samples (Appendix B-12). No loss of memory was observed in knockdowns of *Act5C* (Figure 10A). In both knockdowns of *Bap60*, and *Snr1*, one RNAi line caused a stronger reduction in LI than the other (Figure 10A). In both cases, stronger reduction in LI was associated with the more potent RNAi line, as indicated by lethality results upon ubiquitous knockdown with Actin5C-GAL4 (Bap60-RNAi (BL32503): 0% survival, (BL33954): 17% survival; Snr1-RNAi (BL32372): 4% survival, (BL12644): 19% survival) (Table 3). These results suggest that the core SWI/SNF subunits are important in the MB for normal short-term memory.

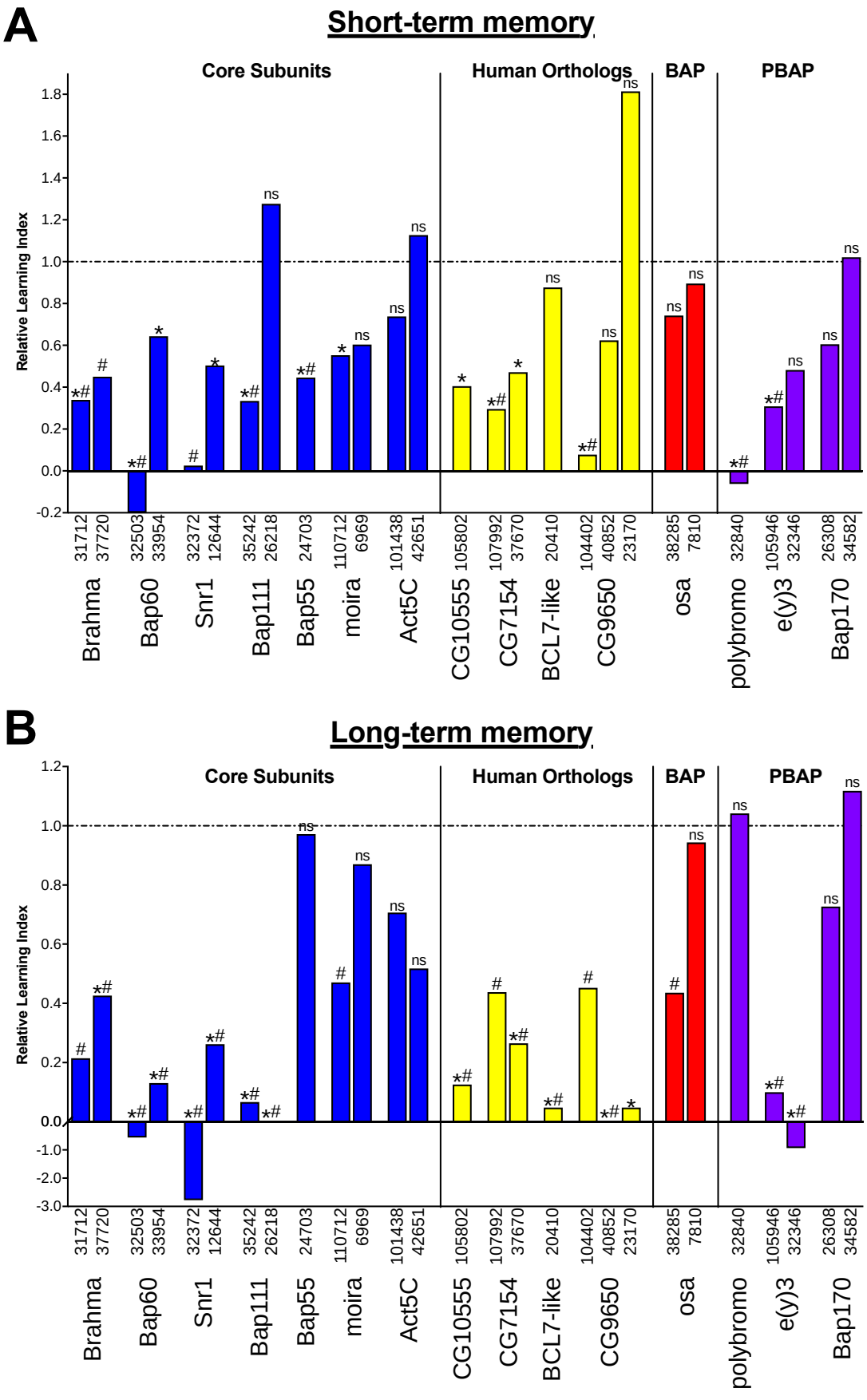
Significant loss of short-term courtship memory was also observed in MB-specific knockdowns of three of the four *Drosophila* orthologs of known human SWI/SNF complex genes. Consistent loss of short-term memory was observed in knockdown of *CG7154* in two independent RNAi lines. Loss of short-term memory was also observed in knockdown of *CG10555*, but was only tested in one RNAi line. Knockdown of *CG9650* caused loss of memory in only one of three RNAi lines. No loss of memory was observed in knockdown of BCL7-like, which was tested in one RNAi line (Figure 10A). These findings suggest that the

*Drosophila* orthologs of known SWI/SNF genes are important in the MB for normal short-term memory.

Loss of short-term memory was observed in MB-specific knockdowns of two of the three PBAP-specific genes. Loss of short-term memory was observed in knockdown of *polybromo*, which was only tested in one RNAi line, and in one of the two *e(y)3* RNAi lines tested. No significant memory phenotypes were observed in MB-specific knockdowns of *Bap170* (Figure 10A). In contrast, no loss of short-term memory was observed in MB-specific knockdowns of the BAP-specific gene, *osa* (Figure 10A). These findings suggest that the PBAP complex, and not the BAP complex, plays an important role in the regulation of short-term courtship memory.

**Figure 10. Effect of knockdown of SWI/SNF complex genes on short- and long-term courtship memory.**

Bar plots show relative LI to respective appropriate control group ($LI_{knockdown}/LI_{control}$) for SWI/SNF gene knockdowns for both short- (A) and long-term (B) memory. Asterisks (*) indicate significant reduction in relative LI compared to appropriate control group (randomization test, $P<0.05$). Pounds (#) represent no significant reduction between trained flies and naïve flies within the same genotype due to training (One-tailed Mann-Whitney test, $P>0.05$).


A
Short-term memory
Core Subunits
Human Orthologs
BAP
PBAP
Relative Learning Index
Brahma
Bap60
Snr1
Bap111
Bap55
moira
Act5C
CG10555
CG7154
BCL7-like
CG9650
osa
polybromo
e(y)3
Bap170
B
Long-term memory
Core Subunits
Human Orthologs
BAP
PBAP
Relative Learning Index
Brahma
Bap60
Snr1
Bap111
Bap55
moira
Act5C
CG10555
CG7154
BCL7-like
CG9650
osa
polybromo
e(y)3
Bap170

### 3.3.3. MB-specific knockdown of SWI/SNF genes impairs long-term conditioned courtship memory

Significant loss of long-term memory is observed in MB-specific knockdowns of 11/15 *Drosophila* SWI/SNF genes (Figure 10B). Significant loss of long-term courtship memory was observed in MB-specific knockdowns using at least one RNAi stock for five out of the seven core SWI/SNF complex genes. For the core subunits, *Bap60, Snr1, brahma,* and *Bap111,* loss of long-term memory was observed in knockdowns using two independent RNAi lines. Knockdown of *moira* caused inconsistent phenotypes in which loss of long-term memory is only observed in one out of the two RNAi lines tested. No loss of long-term memory is observed in knockdowns of *Bap55* or *Act5C* (Figure 10B). These results suggest that the core SWI/SNF subunits are important in the MB for normal long-term memory maintenance.

Loss of long-term memory phenotypes was observed in each of the four *Drosophila* orthologs of known human SWI/SNF complex genes. Consistent loss of long-term memory was observed in knockdown of both *CG7154* and *CG9650* in all RNAi lines tested. Loss of long-term memory was also observed in knockdowns of *CG10555* and *BCL7-like,* but was only tested in one RNAi line each (Figure 10B). These findings suggest that the *Drosophila* orthologs of known mammalian SWI/SNF genes are important in the MB for normal long-term memory maintenance.

Consistent loss of long-term memory was observed in MB-specific knockdowns of the PBAP-specific gene, *e(y)3,* using two independent RNAi lines. No loss of long-term memory was observed in knockdowns of *polybromo* and *Bap170* (Figure 10B). Conversely, inconsistent phenotypes were observed in knockdowns of the BAP-specific gene, *osa.* In only one of the two RNAi lines (BL38285), no significant reduction in CI due to training was observed, and no reduction in LI is observed in either RNAi line by the randomization test (Figure 10B). These findings suggest that *e(y)3* is an important PBAP gene in the regulation of long-term memory, and provide weak evidence towards a role for the BAP complex in normal long-term memory control.

### 3.3.4. Analysis of baseline courtship behaviour in MB-specific knockdowns of SWI/SNF genes

To properly describe memory using the courtship conditioning assay, it is important that the naïve cohort of a given genotype court PMFs often and consistently. Low or variable naïve courtship indices ($CI_{naive}$) impair the ability to determine discernible differences between flies that court less often in general, and a reduction in courtship behaviour due to training. To determine if MB-specific knockdown of SWI/SNF complex genes impairs baseline courtship behaviour in *Drosophila*, the $CI_{naive}$ of each SWI/SNF knockdown used in this study was compared to the appropriate control group. Significant reduction in $CI_{naive}$ compared to control was observed in 11 of 27 genotypes tested in this study (Figure 11A–F). Knockdown of *Bap111* was the only gene that showed significant reduction in $CI_{naive}$ in both RNAi constructs used in this study (Figure 11B, D). Nine of the 11 genotypes with an observed reduction in baseline courtship behaviour were from MB-specific knockdowns using stocks from the TRiP collection (Figure 11A, B, D). Significant reduction of baseline courtship was observed in two KK lines (Figure 11E), and was not observed in GD lines (Figure 11F). These results suggest that there is a differential effect on *Drosophila* courtship behaviour between knockdowns mediated by different RNAi constructs. Reduction of baseline courtship behaviour is not indicative of the capacity for observable courtship memory, as some of the genotypes with observed reduction in $CI_{naive}$ still showed reduction in $CI_{trained}$ in courtship conditioning assays (see Figure 10, Appendix B). Additionally, the 95% confidence interval of the mean $CI_{naive}$ was calculated to describe the variability of baseline courtship behaviour for each genotype. Overall, absolute size of the 95% confidence intervals for both SWI/SNF knockdowns and controls were very narrow, ranging from 0.044 to 0.157 (Figure 11G). In general, 95% confidence intervals of $CI_{naive}$ were smaller for control genotypes compared to knockdowns, as each control genotype was found within the 15 smallest intervals (Figure 11G).

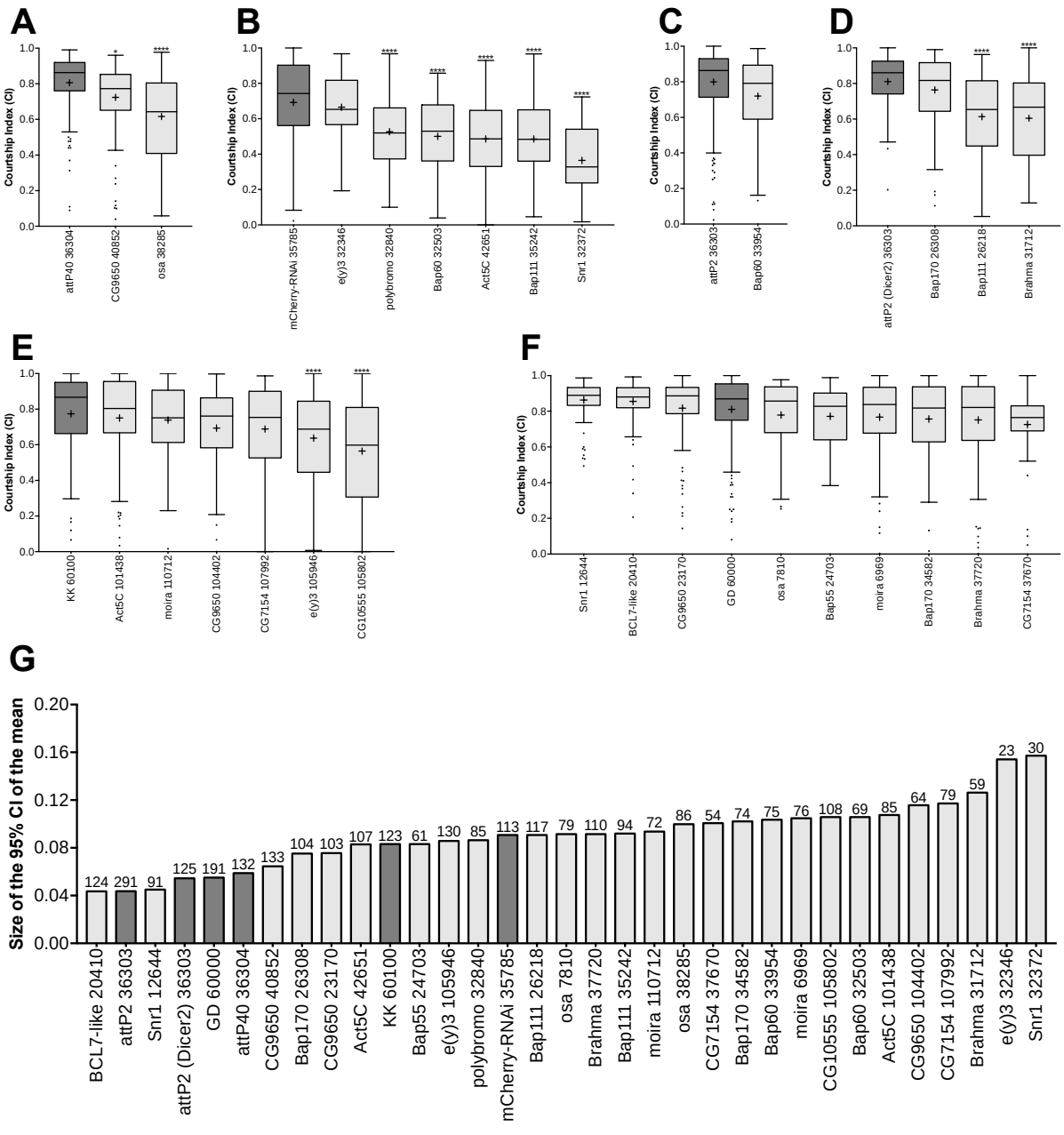



**Figure 11. Analysis of baseline courtship behaviour in SWI/SNF knockdowns.**

A-F) Boxplots display the distribution of naïve courtship indices for SWI/SNF knockdown (light grey boxes) and control (dark grey boxes) flies. Mean courtship index is indicated by (+). Boxplots are sorted on different axes based on their appropriate control. Differences between means were calculated using a one-way ANOVA, with selective comparison of knockdowns to their appropriate control (Bonferroni correction for multiple comparisons (n=26), * $P_{adj}$ <0.05, **** $P_{adj}$ <1x10$^{-4}$). G) Bar graphs display the absolute size of the 95% confidence interval (CI) of the mean $CI_{naive}$. Total number of naïve flies tested is indicated as data labels.

## 3.4. Objective 2: Analysis of adult-specific knockdown of SWI/SNF genes on conditioned courtship memory

### 3.4.1. The GAL80$^{ts}$ system allows for adult-specific gene regulation in the mushroom body

The R14H06-GAL4 driver used to knockdown SWI/SNF gene expression drives GAL4 expression in post-mitotic mushroom body neurons that originate during embryonic development (Lee et al., 1999). Thus, short- and long-term memory phenotypes that are observed using the R14H06-GAL4 driver (in Objective 1) may be the result of dysregulation of SWI/SNF function during developmental processes or in acute SWI/SNF complex function in the adult mushroom body. The studies done in Objective 2 aimed to use the temperature-sensitive GAL80 (GAL80$^{ts}$) system to isolate the adult-specific contributions of SWI/SNF complex genes on the acute processes that lead to short- and long-term memory formation in *Drosophila* by permitting regular *Drosophila* development until adulthood before initiating adult-specific knockdown of SWI/SNF genes in the developed mushroom body. I tested the efficiency of this system using UAS-GFP. GFP expression under control of the GAL80$^{ts}$ system in the mushroom body of male *Drosophila* (genotype: GAL80$^{ts}$;R14H06-GAL4/UAS-EGFP) larval and adult brains was observed under 18ºC and 29ºC conditions. No GFP expression was observed in the mushroom body in neither larval (Figure 12A) nor adult (Figure 12B) flies raised at 18ºC, validating that GAL80$^{ts}$ effectively inhibits GAL4-mediated gene regulation under these conditions. In contrast, GFP expression was observed in both larval (Figure 12D) and adult (Figure 12E) MBs in flies raised at 29ºC. GFP expression was also observed in MBs of adult flies raised until eclosion at 18ºC (when GAL4-mediated gene regulation is inhibited) that were collected and transferred to 29ºC conditions for five days following eclosion. EGFP expression was observed in MBs of these flies (Figure 12C). These results confirm that at 29°C, GAL80$^{ts}$ is inactivated, thus allowing for temperature-sensitive induction of GAL4-mediated transgene expression.

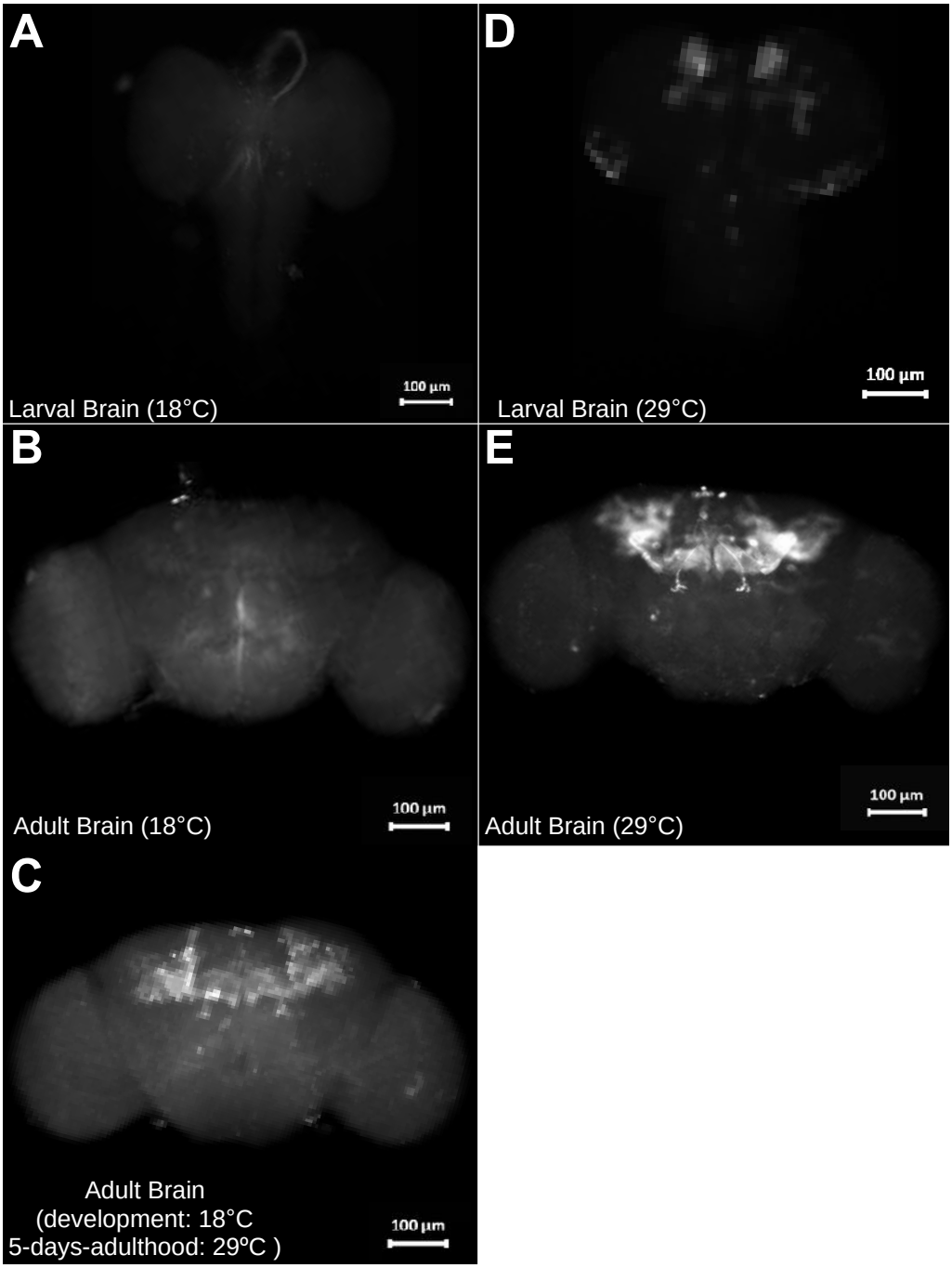



**Figure 12. The GAL80$^{ts}$ system allows for adult-specific expression in the MB.**

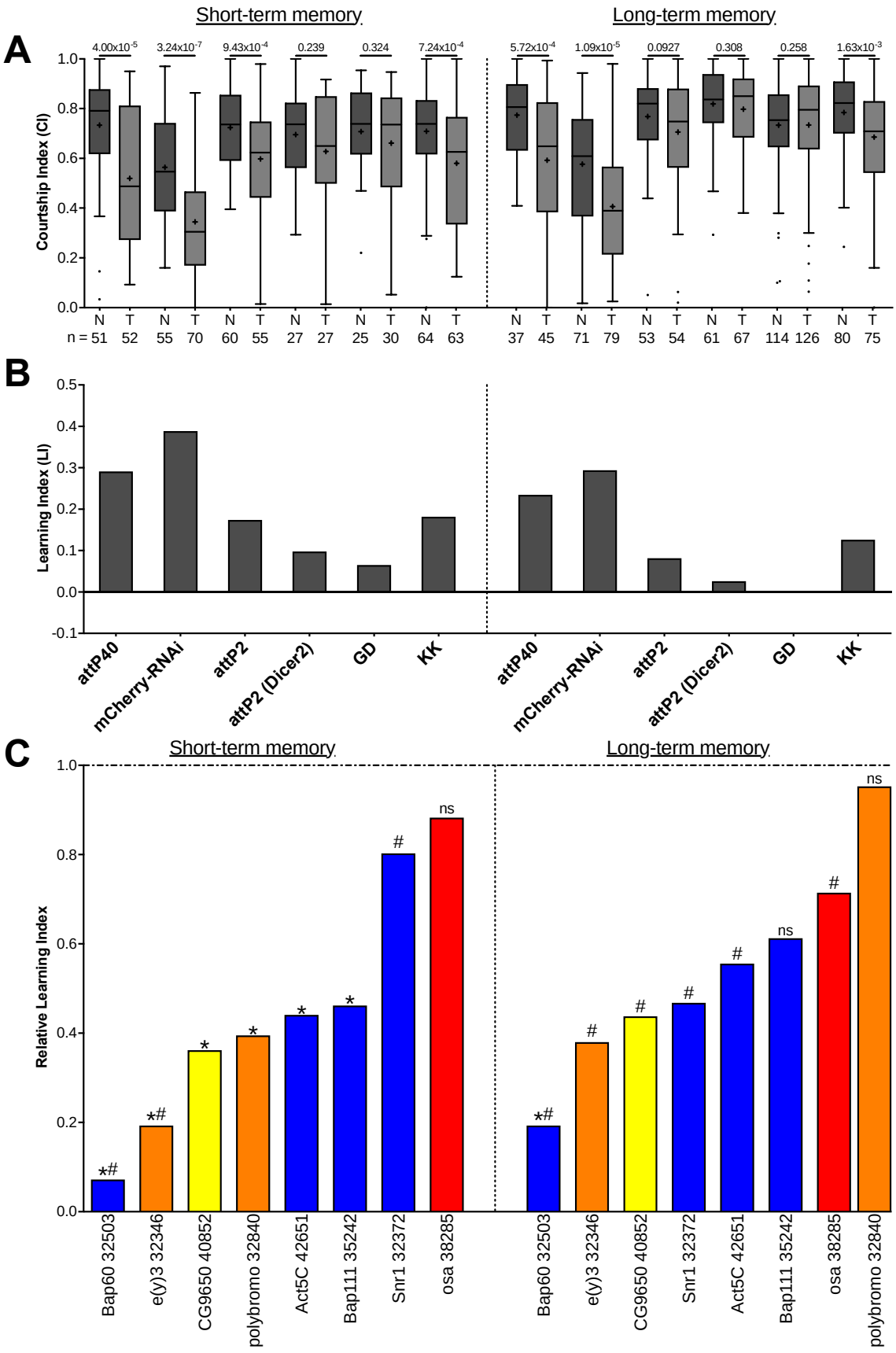
The GAL80$^{ts}$ system was validated by observing EGFP expression in the MB by fluorescence microscopy. No EGFP expression is observed in neither larval (A) nor adult (B) MBs of male flies raised in 18ºC conditions. EGFP expression is observed in both larval (D) and adult (E) MBs of male flies raised at 29ºC. EGFP expression is observed in adult MBs of flies raised until eclosion in 18ºC conditions and transferred to 29º conditions at eclosion for five days.

### 3.4.2. Analysis of conditioned courtship memory in genetic background controls using the GAL80$^{ts}$;R14H06-GAL4 driver

Analyses of short- and long-term courtship memory were conducted on control genotypes to determine the efficacy of the courtship conditioning assay protocol in flies with adult-specific activation of the GAL4-UAS system when flies are raised at 18°C until eclosion, and then transferred to 29°C for five days as adults (See: Section 2.3). Regular capacity for short- and long-term memory was observed in mCherry-RNAi control files and attP40 control flies crossed to the GAL80$^{ts}$;R14H06-GAL4 driver (Figure 13A, B). Significant reduction was observed in CIs of trained flies compared to naïve flies in the attP2 control group for short-term memory experiments, and in the KK background control group for both short- and long-term memory experiments (Figure 13A, B). However, the resulting LI was very low compared to what would be expected based on my own data from Objective 1 (Figure 9A, B) and other published reports (Keleman et al., 2012; Zografos et al., 2016). No significant reduction in courtship behaviour was observed in trained flies relative to naïve flies in both short- and long-term memory experiments in the GD background control group, the attP2 control group with Dicer2 co-expression, and the attP2 control group in long-term memory experiments (Figure 13A, B). These findings indicate that only the attP40 and mCherry-RNAi control genotypes effectively suppress courtship behaviours in response to training under these experimental conditions. As a result, only results of knockdowns using RNAi stocks for which attP40 and mCherry-RNAi are the appropriate controls were included in adult-specific studies (Appendix A).

**Figure 13. Effects of adult-specific knockdown of SWI/SNF complex genes on short- and long-term courtship memory.**

Courtship indices (A) and learning indices (B) of control flies used in courtship conditioning assays. Boxplots represent distribution of CI of naïve (N) and trained (T) male flies. Total flies tested for each genotype and condition are listed in the (n=) row. One-tailed Mann-Whitney test was used to compare $CI_{trained}$ to $CI_{naive.}$ Bar plots (C) show relative LI to respective appropriate control group ($LI_{knockdown}/LI_{control}$) for SWI/SNF gene knockdowns for both short- and long-term memory. Asterisks (*) indicate significant reduction in relative LI compared to appropriate control group (randomization test, $P<0.05$). Pounds (#) represent no significant reduction between trained flies and naïve flies within the same genotype due to training (One-tailed Mann-Whitney test, $P>0.05$).


A
Short-term memory
Long-term memory
4.00x10-5
3.24x10-7
9.43x10-4
0.239
0.324
7.24x10-4
5.72x10-4
1.09x10-5
0.0927
0.308
0.258
1.63x10-3
Courtship Index (CI)
n = 51 52 55 70 60 55 27 27 25 30 64 63 37 45 71 79 53 54 61 67 114 126 80 75
B
Learning Index (LI)
attP40
mCherry-RNAi
attP2
attP2 (Dicer2)
GD
KK
C
Short-term memory
Long-term memory
Relative Learning Index
ns
Bap60 32503
e(y)3 32346
CG9650 40852
polybromo 32840
Act5C 42651
Bap111 35242
Snr1 32372
osa 38285

### 3.4.3. Adult-specific knockdown of SWI/SNF genes in the MB impairs short-term conditioned courtship memory

Because only the attP40 and mCherry-RNAi control genotypes effectively suppress courtship behaviours in response to training under these experimental conditions, only RNAi lines that correspond to these controls were analyzed (Appendix A). As a result, only one RNAi line was tested for eight of the 15 SWI/SNF genes. Loss of short-term memory was observed in adult-specific knockdowns of seven of the eight SWI/SNF genes tested. Significant loss of short-term memory was observed in knockdowns of the core SWI/SNF genes, *Bap60*, *Act5C*, *Bap111*, and *Snr1*; the *Drosophila* ortholog of the mammalian SWI/SNF gene, *CG9650*; and the PBAP-specific genes, *e(y)3* and *polybromo* (Figure 13C). In contrast, no loss of short-term memory was observed in flies with adult-specific knockdown of the BAP-specific gene, *osa* (Figure 13C). These results suggest that some components of the SWI/SNF complex may play an adult-specific role in the acute regulation of normal short-term memory.

### 3.4.4. Adult-specific knockdown of SWI/SNF genes in the MB impairs long-term conditioned courtship memory

Loss of long-term memory was observed in adult-specific knockdowns of six of the eight SWI/SNF genes tested. Significant loss of long-term memory was observed in knockdowns of the core SWI/SNF genes, *Bap60*, *Snr1*, and *Act5C*; the *Drosophila* ortholog of the mammalian SWI/SNF gene, *CG9650*; the PBAP-specific gene, *e(y)3*; and the BAP-specific gene, *osa* (Figure 13C). In contrast, no memory phenotypes were observed in knockdowns of *Bap111* and *polybromo* (Figure 13C). These results provide evidence towards a role for the SWI/SNF complex in the acute regulation of normal long-term memory in the adult *Drosophila* brain.

# 4. Discussion

In this study, I demonstrated that members of the SWI/SNF complex are necessary for the regulation of short- and long-term courtship memory in *Drosophila*. MB-specific knockdown caused loss of courtship memory in 11/15 genes in short-term memory experiments, and 11/15 genes in long-term memory experiments (Figure 10). More specifically, strong loss of memory phenotypes observed in MB-specific knockdowns of *brahma, Bap60, Snr1,* and *e(y)3* suggest a role for core SWI/SNF subunits and the PBAP complex in the regulation of *Drosophila* courtship memory. Additionally, adult-specific knockdown of SWI/SNF genes in the MB caused decreased capacity for short- and long-term memory, providing evidence for an adult-specific role for the SWI/SNF complex in the acute regulation of memory.

## 4.1. Bap60 plays a critical role in *Drosophila* memory

Knockdown of *Bap60* caused the strongest, and most consistent, loss of memory phenotypes of all SWI/SNF genes analyzed in this study. Significant loss of both short- and long-term memory was observed in flies with both developmental and adult-specific knockdown of *Bap60* in the MB. *Bap60* is a conserved and essential member of the core *Drosophila* SWI/SNF complex (Figure 4). *Bap60* has not been shown to have a direct role in SWI/SNF-mediated chromatin remodeling. Instead, it interacts with various transcription factors to direct the site-specific recruitment of the Brahma ATPase to various promoters, resulting in transcriptional activation or repression (Möller et al., 2005). Unbiased screens identified a role for *Bap60* in the *Drosophila* nervous system. RNAi-mediated knockdown of *Bap60* in class I dendrite arborisation (da) neurons caused dendrite misrouting, indicating that *Bap60* plays an important role in the regulation of proper nervous system development (Parrish et al., 2006). *Bap60* has also been shown to have a role in the regulation of nervous system-regulated behaviours, including regulation of circadian rhythms. Knockdown of *Bap60* (and other *Drosophila* core SWI/SNF genes) in *tim*-expressing clock neurons extends the circadian period length by one-to-two hours (Kwok et al., 2015). Furthermore, experiments conducted in our laboratory have revealed a role for *Bap60* in mushroom body development. Mushroom

body-specific knockdown of *Bap60* using the R14H06-GAL4 driver caused defects in MB γ neuron remodeling, extra dorsal projections, and β lobe crossing (Chubak, personal communication, 2017). These previous findings combined with consistent loss of memory phenotypes observed in my experiments suggest that *Bap60* plays a critical role in the regulation of *Drosophila* nervous system processes, including courtship memory. However, most studies on *Bap60* have focused on its contribution within the greater context of the *SWI/SNF* complex. Additional studies in *Drosophila* should specifically target this gene to determine its direct effect on neuronal plasticity in the mushroom body to determine its greater role in regulating transcriptional processes in the fly brain.

## 4.2. Regulation of the ecdysone signaling pathway by the PBAP complex may influence memory

In this study, MB-specific knockdown of the SWI/SNF complex core genes, *brahma* and *Snr1*, and the PBAP-specific gene, *e(y)3*, caused loss of short- and long-term courtship memory phenotypes. Previous studies have identified interactions between these SWI/SNF complex genes and the ecdysone signaling pathway in transcriptional regulation during development (Vorobyeva et al., 2011; Zraly et al., 2006). Transcriptome microarray analyses showed that mutations in the core SWI/SNF genes, *brahma* and *Snr1*, resulted in dysregulation of the late expressed ecdysone inducible genes (*Eig*), and that the Brahma ATPase directly associates with promoters of these genes *in vivo* (Zraly et al., 2006). Initially, it was believed that the *Drosophila* PBAP complex did not interact with the ecdysone signaling pathway (Carrera et al., 2008). However, this finding was refuted based on the identification of the *e(y)3*-encoded protein, SAYP, as a PBAP signature subunit (Chalkley et al., 2008). Flies with mutations in *e(y)3* have a bent-leg phenotype that is similar to the phenotype observed in flies with mutations in the ecdysone signaling pathway (Chalkley et al., 2008). Direct interactions between *e(y)3* and the ecdysone signaling pathway was confirmed when it was observed that the DHR3 nuclear receptor—a component of the ecdysone-induced transcriptional cascade—interacts with SAYP to activate gene expression during embryonic and pupal development (Vorobyeva et al., 2011). The direct relationship between SAYP and ecdysone was validated by ChIP analyses following ecdysone treatment, which revealed that DHR3 specifically binds

to the promoters to help regulate the transcription of SAYP-regulated genes (Vorobyeva et al., 2011).

Ecdysone and its homologs—known as ecdysteroids—are among the most important steroid hormones in *Drosophila.* The ecdysone signaling pathway is critical in various developmental events in flies, including regulation of larval molting, metamorphosis (Truman and Riddiford, 2002), and tissue growth (Colombani et al., 2005). There is also evidence of a role for the ecdysone signaling pathway in the regulation of mushroom body development (Boulanger et al., 2011; Kraft et al., 1998; Lai et al., 2016; Lee et al., 2000). Increased exposure of the MB to 20-hydroxyecdysone (20E) *in vivo* caused increased total neurite length and total number of branches compared to unexposed MB neurons (Kraft et al., 1998). Additionally, genetic mosaic screening identified a defective *ultraspiracle (usp)* allele that caused MB γ lobe pruning during MB development. It was also shown that the ecdysone receptor (EcR)-B1 isoform, which heterodimerizes with USP, is specifically expressed in MB γ neurons, and is also required in MB pruning in larval development. Interestingly, mutations in downstream targets of EcR/USP showed no MB development phenotypes, which indicated an independent role for the ecdysone signaling pathway in MB neuronal remodeling aside from the standard ecdysone transcriptional cascade (Lee et al., 2000). Antagonistic regulation of EcR-B1 expression by *Hr39* and *ftz-f1* in the MB controls MB γ neuron pruning and remodeling (Boulanger et al., 2011), and overexpression of miR-34 downregulates EcR-B1 expression in differentiated MB γ neurons caused defective γ axon pruning (Lai et al., 2016). These findings suggest an important role for regulation of the ecdysone signaling pathway in proper MB development.

Recent evidence has determined that the ecdysone signaling pathway plays an important role in the regulation of *Drosophila* behaviours, including sleep (Ishimoto and Kitamoto, 2010), and courtship memory (Ishimoto et al., 2009). Administration of 20E to adult *Drosophila* increased total amount of sleep in a dose-dependent fashion. Additionally, mutations in ecdysone signaling pathway genes caused reduced sleep, which could be recovered by administering 20E in adult flies (Ishimoto and Kitamoto, 2010). In courtship conditioning experiments, it was observed that total body levels of 20E increased in response to increased training time (Ishimoto et al., 2009). Increased 20E levels also correlated with

increased CREB-dependent transcription, which is critical in the formation of long-term memory (Ishimoto et al., 2009). Studies have also shown that the nuclear G-coupled ecdysone receptor DopEcR plays an important part in regulating courtship memory in the mushroom body through the cAMP pathway (Ishimoto et al., 2013). It was shown that loss of courtship memory in *rutabaga* mutants could be recovered by overexpression of DopEcR. Conversely, loss-of-function mutations in DopEcR restored loss of memory phenotypes observed in *dunce* mutants (Ishimoto et al., 2013). Ecdysone signaling was confirmed to regulate the cAMP pathway, as acute feeding of 20E caused an increase in DopEcR-mediated elevation of cAMP levels in the MB (Ishimoto et al., 2013). These findings indicate that the ecdysone signaling pathway plays an important role in regulating *Drosophila* behaviour, as well as development, in the MB.

Loss of courtship memory is observed in core SWI/SNF genes that interact with the ecdysone signaling pathway (*brahma* and *Snr1)*, and in knockdowns of the PBAP-specific genes, *polybromo* and *e(y)3.* It is well understood that the BAP- and PBAP-specific subunits direct the Brahma ATPase to different, but occasionally overlapping, transcriptional start sites (Mohrmann et al., 2004). Based on these findings, I propose that interactions between the PBAP complex and the ecdysone signaling pathway are critical in the regulation and maintenance of *Drosophila* courtship memory. Although ecdysone is an insect-specific steroid, a human ortholog of *EcR*, NR1I3, has been identified as a possible ID gene, and was shown to be part of a genetic interaction network with the human SWI/SNF gene, SMARCB1 (Kleefstra et al., 2012). There is also evidence of other steroids, such as cortisol (Het et al., 2005) and gonadal hormones (Luine, 2008), playing an important role in neuronal plasticity and memory in mammals. Therefore, it is possible that the conserved role for the SWI/SNF complex in the brain could be in activating transcription of steroid-dependent signalling pathways.

## 4.3. Limitations

Despite pre-screening using a lethality assay, some inconsistencies were observed in courtship conditioning results between knockdowns of the same SWI/SNF gene using different RNAi stocks. For example, inconsistent memory phenotypes were observed in long-term memory experiments in knockdowns of *moira*, despite observed lethality in both RNAi lines.

For some of these cases, the discrepancies could be caused by off-target effects or insufficient knockdowns in the MB. To better quantify the effectiveness of RNAi-mediated knockdown in these lines, future studies could use qPCR to quantify mRNA expression levels, or use immunohistochemistry to stain for the protein product of each of these genes in the MB. Overall, inconsistencies between RNAi lines were not a major constraint on the ability to interpret the data. In fact, consistent results were observed in most cases (seven of 11 genes tested with multiple RNAi lines in short-term memory experiments, and nine out of 11 genes in long-term memory experiments).

For four SWI/SNF genes, experiments were only conducted using one RNAi line because of ineffective knockdown observed in the lethality assay (Table 3), or in the case of *polybromo,* because knockdowns using other available stocks were incapable of eclosing adequate number of progeny for normal courtship conditioning experiments. Future studies should aim to validate the loss of memory phenotypes observed in knockdowns of *Bap55*, *BCL7-like*, *CG10555,* and *polybromo* using a second RNAi line. For each of these genes, additional RNAi lines are available for purchase from the Bloomington Drosophila Stock Center and from VDRC, and could be used to knockdown these genes in the MB to validate loss of memory phenotypes observed in courtship conditioning experiments.

The randomization test that was used to calculate differences in LI between knockdowns and control groups has been shown to be an effective method of detecting reductions in memory in various studies (Kamyshev et al., 1999; Keleman et al., 2007). However, the power of this statistical test to detect significant reductions in memory decreases with increased variability in CI in a cohort, and in cohorts with low numbers of tested flies. For example, a very strong reduction in LI is observed in short-term memory experiments on MB-specific knockdowns of *Snr1* (32372: $LI_{rel}$=0.026) (Figure 10A), but the randomization test did not identify a significant reduction in LI (P=0.058) because only 20 naïve and 20 trained flies were tested due to high levels of mortality in males collected for testing (Appendix B). A similar observation can be made for short-term memory experiments on knockdowns of *e(y)3* (32346), in which no significant reduction in LI (P=0.081) was observed based on the randomization test despite $LI_{rel}$=0.482, which can also be explained by low numbers of tested flies resulting from increased mortality, leading to increased variability within naïve and trained groups

(Figure 10A, Appendix). The limitations associated with the randomization test impacts only a small percentage of the data, and can largely be explained through fine scrutiny of the results.

Loss of memory phenotypes were observed due to adult-specific knockdown of SWI/SNF genes in the MB, indicating an acute role for this complex in cognitive function in *Drosophila*. However, due to insufficient learning in some of the control genotypes used (Figure 13A, B), courtship conditioning results of adult-specific knockdown of SWI/SNF genes in which the attP2, attP2 (Dicer2), GD, or KK controls were the appropriate control genotypes were not included. Poor learning in these genotypes could be due to increased GAL4 expression at 29ºC compared to the 25ºC temperatures used for knockdowns in Objective 1. GAL4 expression increases with increasing temperature, and it is understood that high levels of untargeted GAL4 can have a negative effect on *Drosophila* (Kramer and Staveley, 2003). Additional RNAi lines for some SWI/SNF genes that use the effective mCherry-RNAi or the attP40 background as their appropriate control are available for purchase from the Bloomington Drosophila Stock Centre. Further investigation into the adult-specific role of the SWI/SNF complex in *Drosophila* memory could use these lines to study SWI/SNF genes not already included and validate the results observed with a second RNAi line. These available stocks were not initially selected because of the desire to use RNAi transgenes inserted in different genetic backgrounds. Repeating adult-specific experiments in the future is crucial because it is possible that some genes that were not included in adult-specific experiments but show loss of memory phenotypes in GAL4-mediated knockdowns, such as *brahma*, may play an acute, adult-specific role in the regulation of memory, as well.

Alternatively, adult-specific knockdown in the MB could be conducted using a different system for spatial and temporal control of gene expression, called GeneSwitch, which allows for ligand-induced activation of UAS transgenes (Osterwalder et al., 2001). To create GeneSwitch drivers, the GAL4 DNA-binding domain is combined with the progesterone receptor transcriptional activation domain, which requires binding of the RU486 (mifepristone) ligand to become transcriptionally active (Nicholson et al., 2008; Osterwalder et al., 2001). As with regular GAL4 constructs, GeneSwitch also allows for tissue-specificity using an upstream tissue-specific enhancer or promoter. The disadvantage of using the GeneSwitch system compared to the $GAL80^{ts}$ system is that GeneSwitch drivers must be created rather than simply

using GAL80$^{ts}$ expression to control an existing GAL4 construct. Fortunately, MB-overexpressing GeneSwitch drivers are available from the Bloomington Drosophila Stock Center (such as: BL59953) or have been used in other publications (Mao et al., 2004; Nicholson et al., 2008). To drive adult-specific knockdown of SWI/SNF genes in the MB, crosses of SWI/SNF RNAi lines and MB-specific GeneSwitch GAL4 flies could be raised on standard *Drosophila* media at 25ºC. Male flies can be collected at eclosion and placed in individual wells in a 96-well chamber filled with RU486-supplemented *Drosophila* media at 25ºC for five days to activate GeneSwitch GAL4 expression, and drive adult-specific knockdown of SWI/SNF genes in the MB. Although the GeneSwitch GAL4 MB-expression drivers drive GAL4-UAS activity in slightly different regions from the R14H06-GAL4 driver used in this study to knockdown SWI/SNF genes beginning during development, the GeneSwitch GAL4 system allows for constant temperature conditions, thus providing an alternative method of adult-specific control of RNAi-mediated knockdown that eliminates negative effects caused by high temperatures. Another limitation with using GeneSwitch drivers is that administration of RU486 occurs through feeding, and therefore it is impossible to determine if individual flies consumed the same amount of the activating ligand as one another, thus introducing another variable. It is important to test this ligand-driven technique to determine the efficacy of using this alternate method of temporal control before using it for adult-specific experiments.

## 4.4. Research implications and future directions

Although the *Drosophila* nervous system is less complex than the mammalian nervous system, the molecular mechanisms of memory regulation are highly conserved across species (Dunning and During, 2003; Frank and Greenberg, 1994). Most studies on the *Drosophila* SWI/SNF complex have focused on its role in organismal development and *in vivo* transcriptional regulation (Chalkley et al., 2008; Marenda et al., 2004; Terriente-Félix and de Celis, 2009), while the specific role of the SWI/SNF complex, especially the PBAP complex, in the nervous system has largely been ignored. In this study, I have identified SWI/SNF complex genes that play an important role in the regulation of *Drosophila* courtship memory. The specific mechanism through which the SWI/SNF complex regulates memory in the MB is still unknown. Future studies should aim to identify the specific transcriptional targets affected

by knockdown of SWI/SNF complex members in the MB to better understand the precise pathway through which the SWI/SNF complex affects courtship memory.

In this study, loss of memory was observed in MB-specific knockdowns of *Drosophila* orthologs of known mammalian SWI/SNF genes, providing evidence towards a role for these genes in the nervous system. Since the SWI/SNF complex is highly conserved between *Drosophila* and mammals, it is possible that these genes play an important role in the regulation of memory and neural plasticity in mammals. Targeted studies on these genes, each of which have been rarely been studied, should aim to confirm their role as members of the *Drosophila* SWI/SNF complex, and further describe the role for these genes in the nervous system and in cognitive function. Loss of memory phenotypes were also observed in MB-specific knockdowns of *e(y)3* and *Bap55*. The mammalian orthologs of *e(y)3* (BAF45a) and *Bap55* (BAF53a/b) have been shown to be important in the switch in subunit composition of the npBAF complex to the nBAF complex (Lessard et al., 2007; Yoo et al., 2009), and the nBAF-specific gene BAF53b has been shown to play a role in the acute regulation of memory in mice (Vogel-Ciernia et al., 2013). Future studies should focus on the consequence of manipulating these genes in mammalian models to better understand the importance of this subunit switch in the context of memory.

## 4.5. Conclusions

There is still much to be understood about the role of the SWI/SNF complex in the nervous system. In this project, I have revealed an important role for the *Drosophila* SWI/SNF complex in the MB for the proper regulation of short- and long-term memory. Loss of memory phenotypes were observed in adult-specific knockdowns of several SWI/SNF genes in the MB, indicating that the SWI/SNF complex plays an acute role in the regulation of courtship memory. Based on the observed loss of memory in MB-specific knockdowns of *brahma, Snr1,* and *e(y)3,* and the importance for these same genes in interactions with ecdysone signaling, I hypothesize that the *Drosophila* PBAP complex might interact with parts of ecdysone signaling cascade to regulate transcription and cell signaling that is critical in the formation of memory. Furthermore, loss of memory phenotypes observed in *Drosophila* orthologs of known human SWI/SNF genes *(BCL7-like, CG7154, CG9650, CG10555)* indicate a novel role for

these rarely-studied genes in the MB in cognitive function. The findings from this project also reveal the first indication that the SWI/SNF complex plays an important adult-specific role in memory regulation, indicating that this complex may contribute the regulation of the cAMP signaling pathway and CREB-mediated transcription that are essential in the consolidation and retrieval of memory. Loss of memory was observed in six *Drosophila* SWI/SNF genes (*brahma, Bap60, Bap111, moira, CG9650, Snr1*) that are orthologs of human SWI/SNF genes (Table 1) that have been identified as ID genes (Figure 3). As a result, these findings provide critical initial steps in the understanding of mutations in ID genes in cognitive function, and may lead to the better understanding of the mechanisms in the nervous system that are affected by mutations in SWI/SNF genes that cause ID.

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# 6. Appendices

**Appendix A: List of all *Drosophila* stocks used in this project.**

All *Drosophila* stocks were obtained from either the Bloomington Drosophila Stock Centre (BDSC) or the Vienna Drosophila Resource Centre (VDRC).

| **Toolkit & RNAi control stocks** | | | | |
|---|---|---|---|---|
| **Stock name** | **Stock No.** | **Obtained from:** | **Genotype** | **Description** |
| Act5C-GAL4 | 25374 | BDSC | *y[1] w[*]; P{Act5C-GAL4-w}E1/CyO* | Expresses GAL4 ubiquitously under control of Act5C promoter. |
| R14H06-GAL4 | 48667 | BDSC | *w[1118]; P{y[+t7.7] w[+mC]=GMR14H06-GAL4}attP2* | Expresses GAL4 at or near *rutabaga* (FBgn0003301) with mushroom body specificity. |
| tubP-GAL80$^{ts}$ | 7019 | BDSC | *w[*]; P{w[+mC]=tubP-GAL80[ts]}20; TM2/TM6B, Tb[1]* | Temperature sensitive GAL80 under the control of alphaTub84B promoter. Used to build tubP-GAL80$^{ts}$/R14H06-GAL4 driver. |
| UAS-Dicer2 | 24644 | BDSC | *P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; Pin[1]/CyO* | Expresses Dicer-2 under UAS control. Used to build fly lines that co-express Dicer-2 and SWI/SNF RNAi. |
| UAS-Dicer2 | 24645 | BDSC | *P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; Df(3L)Ly, sens[Ly-1]/TM3, Sb[1]* | Expresses Dicer-2 under UAS control. Used to build fly lines that co-express Dicer-2 and SWI/SNF RNAi. |

| | | | | |
|---|---|---|---|---|
| UAS-Dicer2 | 24650 | BDSC | *w[1118]; P{w[+mC]=UAS-Dcr-2.D}2* | Expresses Dicer-2 under UAS control. Used to build fly lines that co-express Dicer-2 and R14H06-GAL4. |
| UAS-EGFP | 6658 | BDSC | *y[*] w[*]; P{w[+mC]=UAS-2xEGFP}AH3* | Expresses EGFP under UAS control. |
| mCherry-RNAi | 35785 | BDSC | *y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM20-mCherry}attP2* | Hairpin targeting mCherry. Used as control for VALIUM20 TRiP RNAi collection. |
| attP2 | 36303 | BDSC | *y[1] v[1]; P{y[+t7.7]=CaryP}attP2* | Background stock. Used as control for VALIUM10 TRiP RNAi collection. |
| attP40 | 36304 | BDSC | *y[1] v[1]; P{y[+t7.7]=CaryP}attP40* | Background stock. Used as control for attP40 site TRiP RNAi stocks. |
| GD | v60000 | VDRC | *w[1118]* | Isogenic host strain. Used as control for GD RNAi library. |
| KK | v60100 | VDRC | *y,w[1118];P{attP,y[+],w[3`]* | Background stock with VIE-260B annotated insertion. Used as control for KK RNAi library. |

***RNAi Stocks***

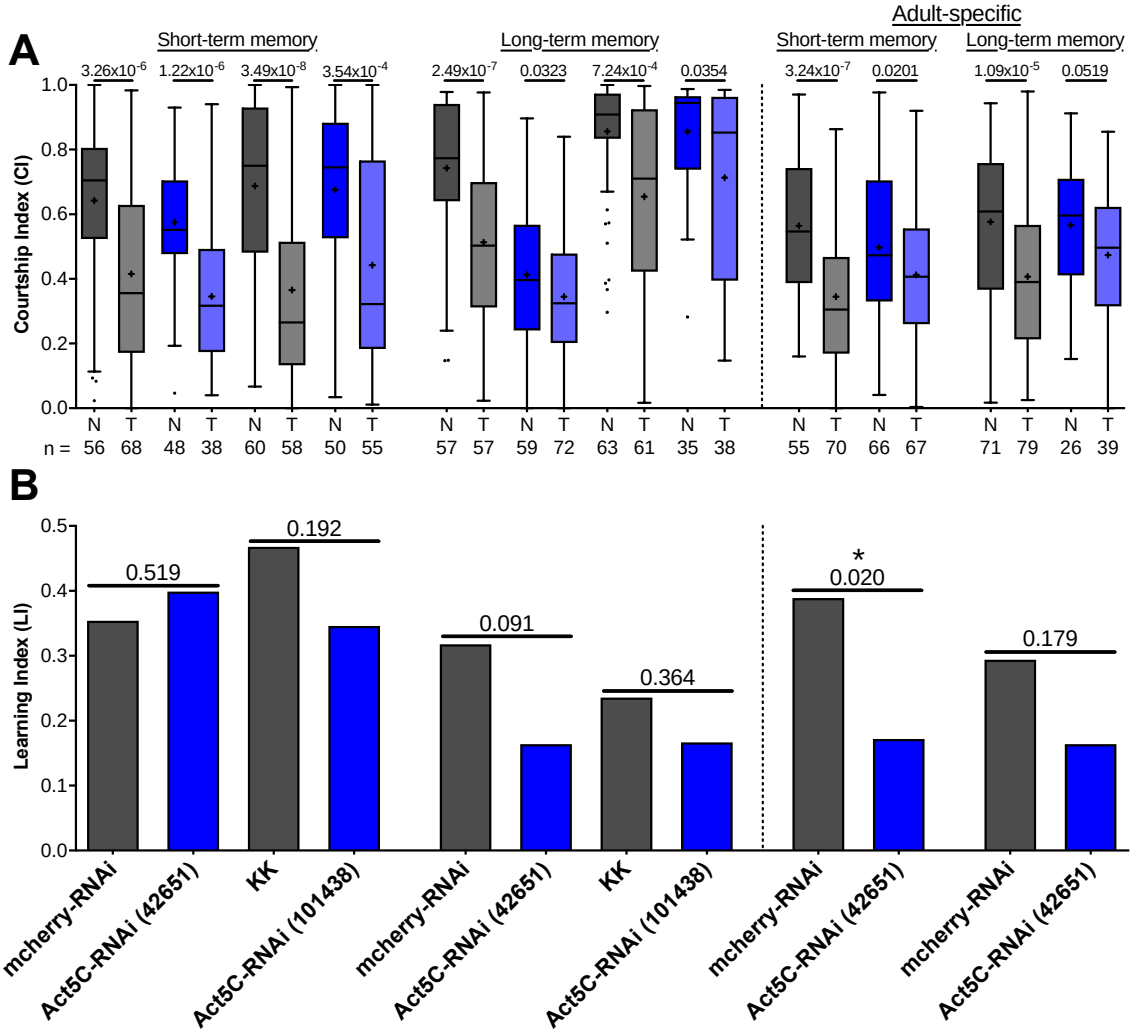
| ***Gene name*** | **Stock No.** | **Obtained from:** | **Genotype** | **Appropriate Control** |
|---|---|---|---|---|
| *Act5C* | 42651 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS02487}attP2* | mCherry-RNAi |
| | v101438 | VDRC | *P{KK109161}VIE-260B* | KK |
| *Bap55* | 31708 | BDSC | *y[1] v[1]; P{TRiP.HM04015}attP2/TM3, Sb[1]* | attP2 (Dicer2) |
| | v24703 | VDRC | *w[1118]; P{GD11955}v24703/CyO* | GD |

| | | | | |
|---|---|---|---|---|
| *Bap60* | 32503 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00507}attP2* | mCherry-RNAi |
| | 33954 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00909}attP2* | attP2 |
| *Bap111* | 26218 | BDSC | *y[1] v[1]; P{TRiP.JF02116}attP2* | attP2 (Dicer2) |
| | 35242 | BDSC | *y[1] sc[*] v[1]; P{TRiP.GL00129}attP2* | mCherry-RNAi |
| *Bap170* | 26308 | BDSC | *y[1] v[1]; P{TRiP.JF02080}attP2* | attP2 (Dicer2) |
| | v34582 | VDRC | *w[1118]; P{GD10922}v34582/TM3* | GD |
| *BCL7-like* | 35714 | BDSC | *y[1] sc[*] v[1]; P{TRiP.GLV21079}attP2* | mCherry-RNAi |
| | v20410 | VDRC | *w[1118]; P{GD9322}v20410* | GD |
| *brahma* | 31712 | BDSC | *y[1] v[1]; P{TRiP.HM04019}attP2* | attP2 (Dicer2) |
| | 34520 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00050}attP2* | mCherry-RNAi |
| | v37720 | VDRC | *w[1118]; P{GD4507}v37720* | GD |
| *CG10555* | 50606 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMC02408}attP2* | mCherry-RNAi |
| | v105802 | VDRC | *P{KK111183}VIE-260B* | KK |
| *CG7154* | v107992 | VDRC | *P{KK100498}VIE-260B* | KK |
| | v37670 | VDRC | *w[1118]; P{GD4426}v37670* | GD |
| *CG9650* | 40852 | BDSC | *y[1] v[1]; P{TRiP.HMS02019}attP40* | attP40 |
| | v104402 | VDRC | *P{KK108364}VIE-260B* | KK |
| | v23170 | VDRC | *w[1118]; P{GD13222}v23170* | GD |
| *e(y)3* | 32346 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00337}attP2* | mCherry-RNAi |
| | v105946 | VDRC | *P{KK112108}VIE-260B* | KK |

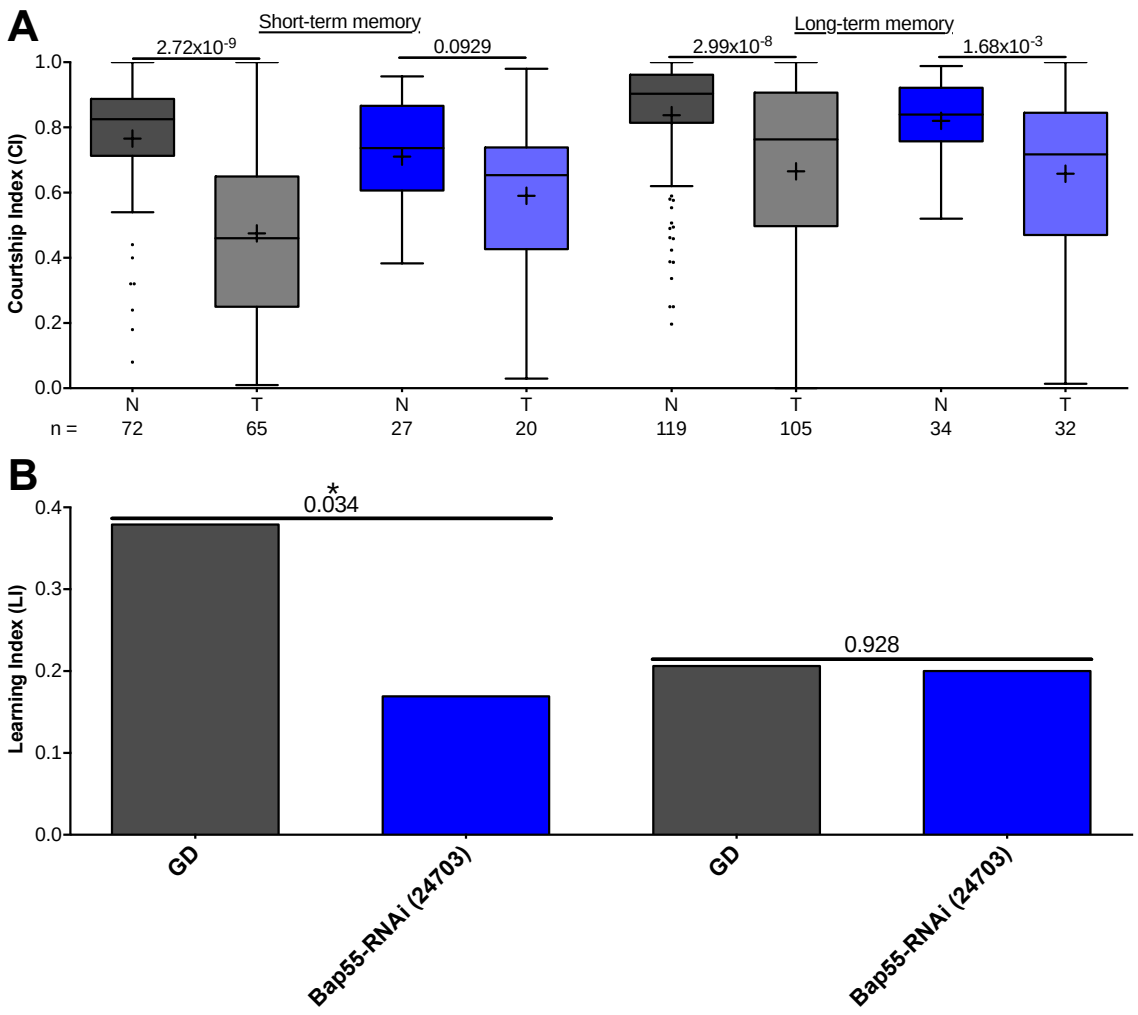
| | | | | |
|---|---|---|---|---|
| *moira* | v110712 | VDRC | *P{KK102003}VIE-260B* | KK |
| | v6969 | VDRC | *w[1118]; P{GD1257}v6969* | GD |
| *osa* | 38285 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS01738}attP40* | attP40 |
| | v7810 | VDRC | *w[1118]; P{GD1502}v7810* | GD |
| *polybromo* | 32840 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00531}attP2* | mCherry-RNAi |
| *Snr1* | 32372 | BDSC | *y[1] sc[*] v[1]; P{TRiP.HMS00363}attP2* | mCherry-RNAi |
| | v12644 | VDRC | *w[1118]; P{GD4140}v12644* | GD |

**Appendix B: Effect of individual knockdowns of SWI/SNF subunits in the MB on courtship memory.**

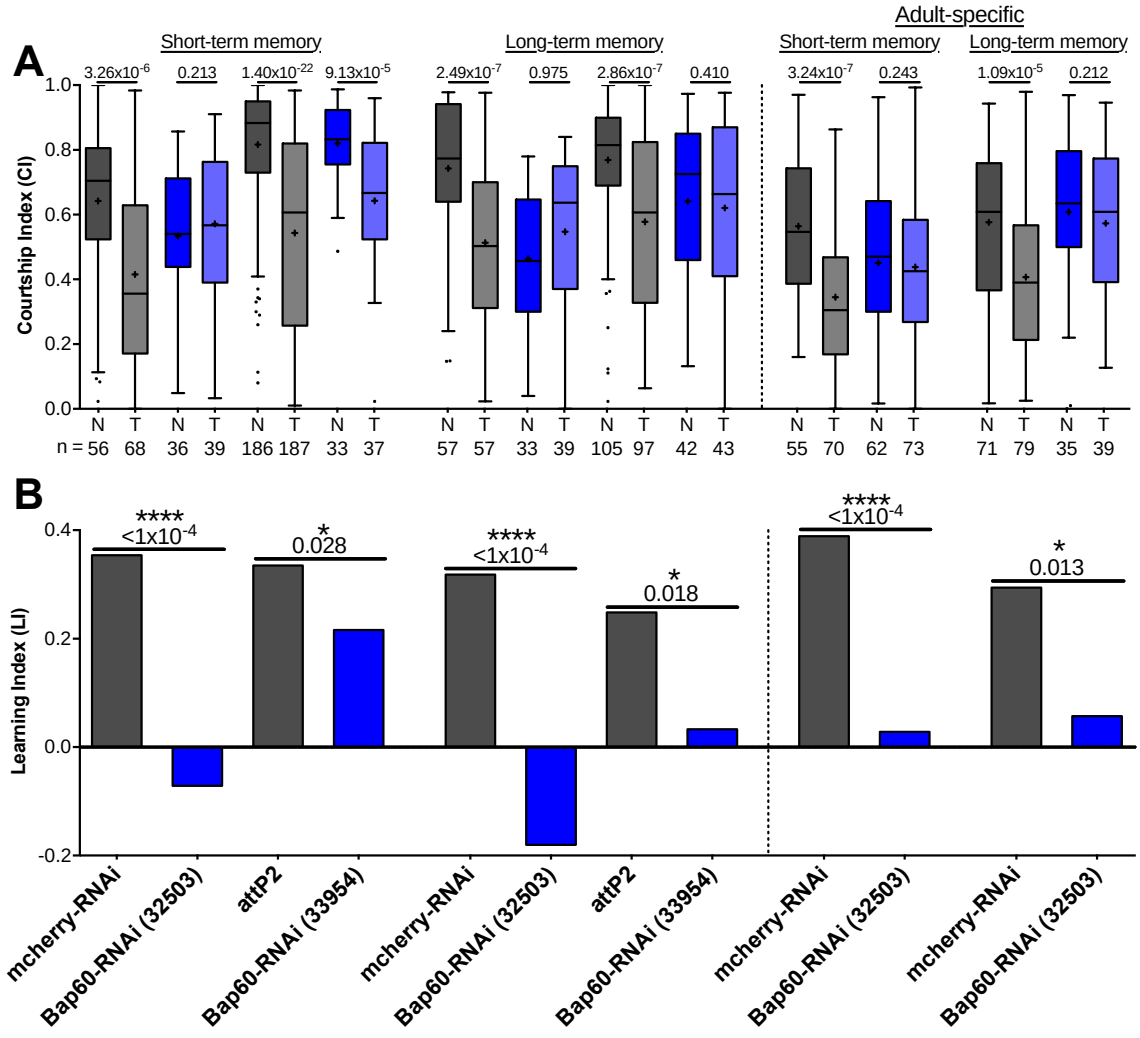
A) Boxplots represent distribution of CIs for each condition tested. Total number of flies tested for each cohort are represented on the "n=" row below the x-axis. Differences between naïve (N) and trained (T) flies for each genotype and condition were conducted using a one-tailed Mann-Whitney test. Exact P-values are indicated above each comparison. B) LIs derived from CIs (A) for each knockdown and condition compared to its respective control group. Comparisons between knockdowns and controls were conducted using a randomization test. Exact P-values are indicated above each comparison.



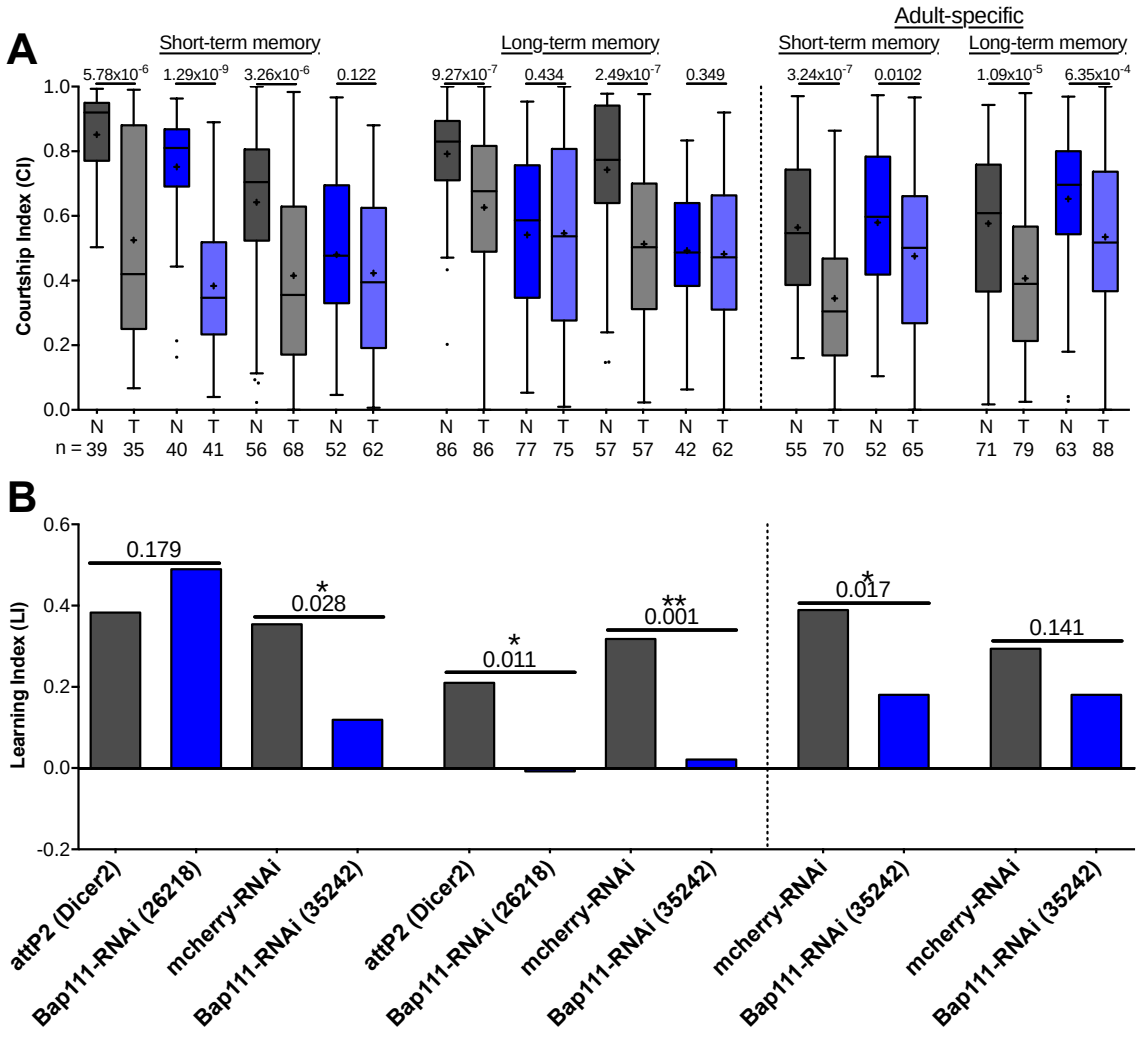

**Appendix B-1: Effect of knockdown of *Act5C* in the MB on courtship memory.**



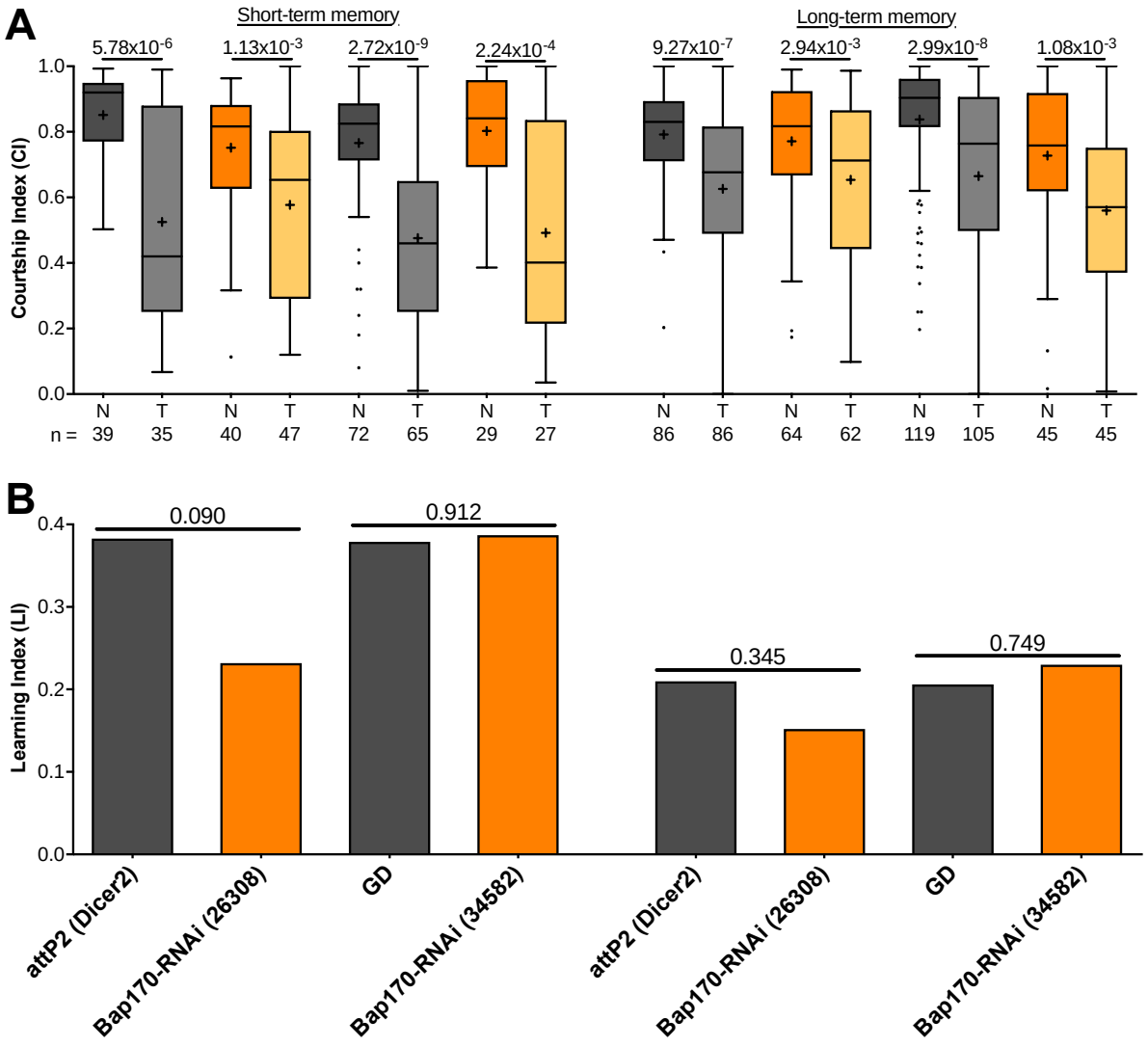

**Appendix B-2: Effect of knockdown of *Bap55* in the MB on courtship memory.**



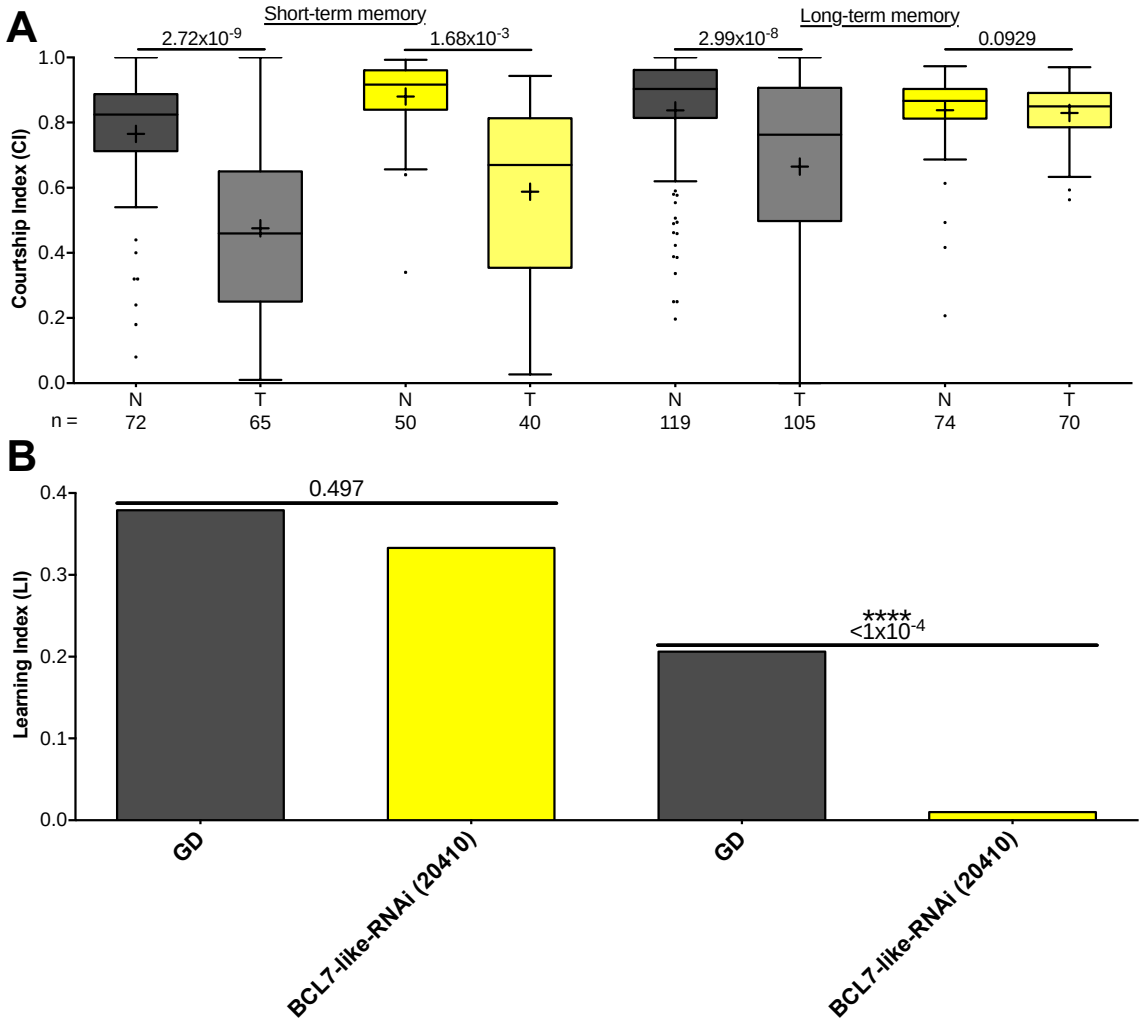

**Appendix B-3: Effect of knockdown of *Bap60* in the MB on courtship memory.**



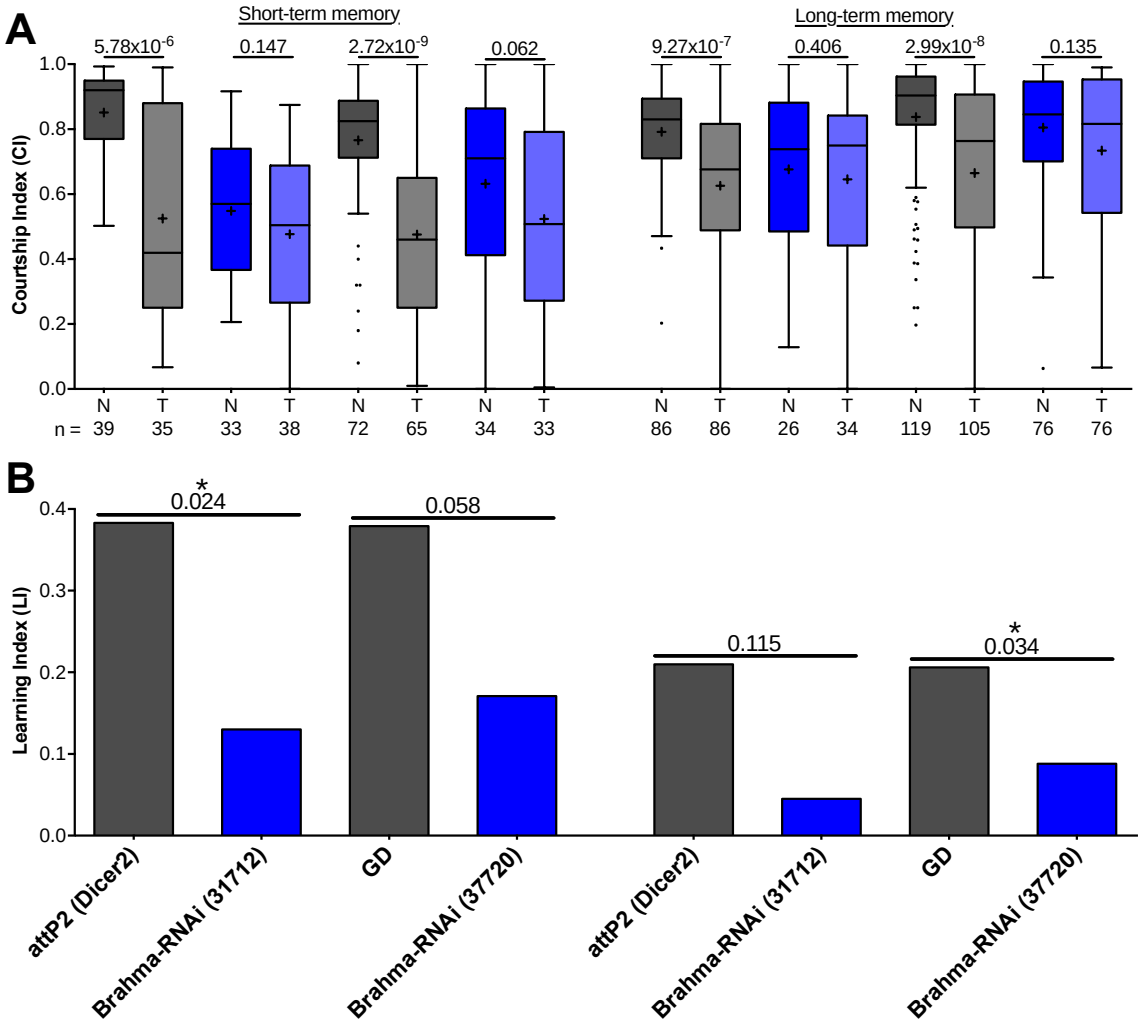

**Appendix B-4: Effect of knockdown of *Bap111* in the MB on courtship memory.**



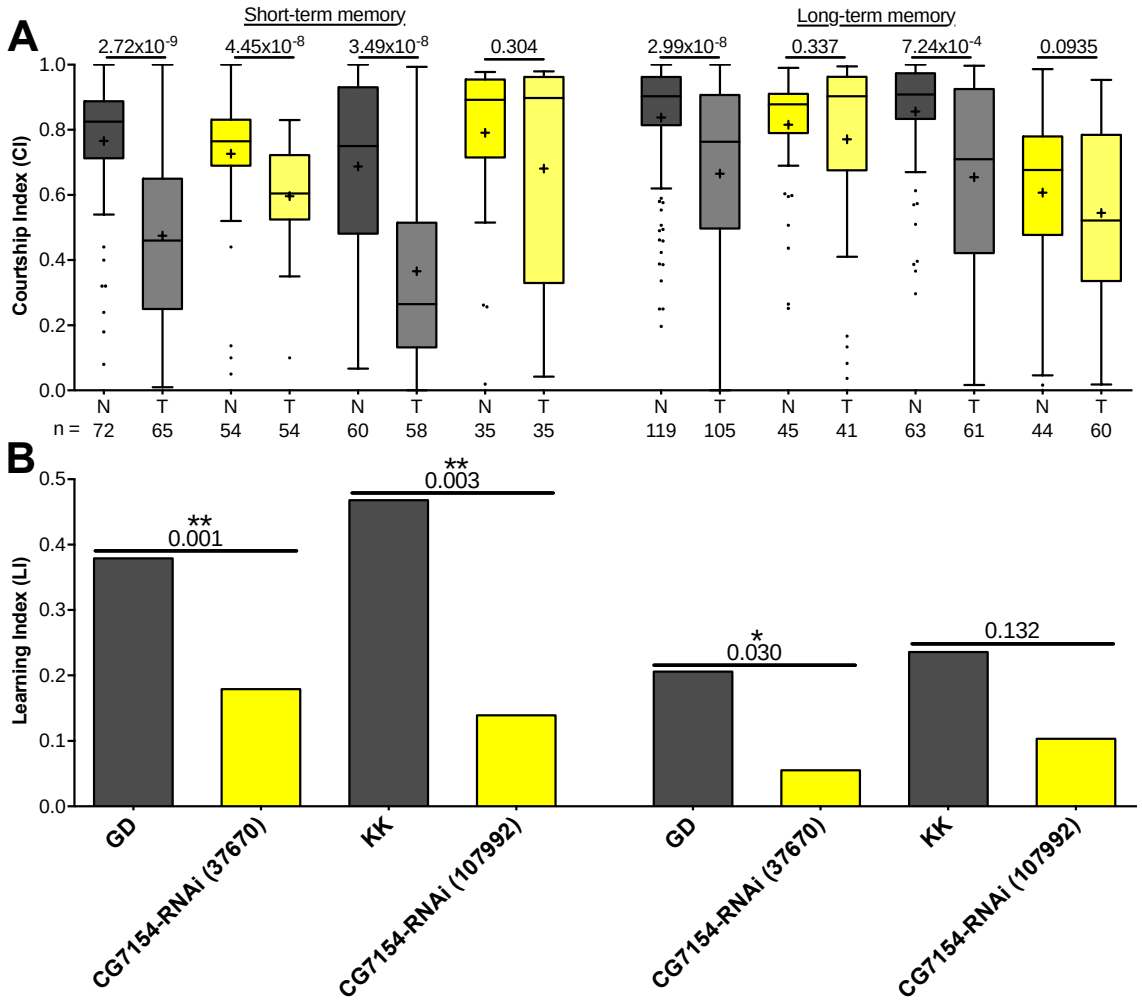

**Appendix B-5: Effect of knockdown of *Bap170* in the MB on courtship memory.**



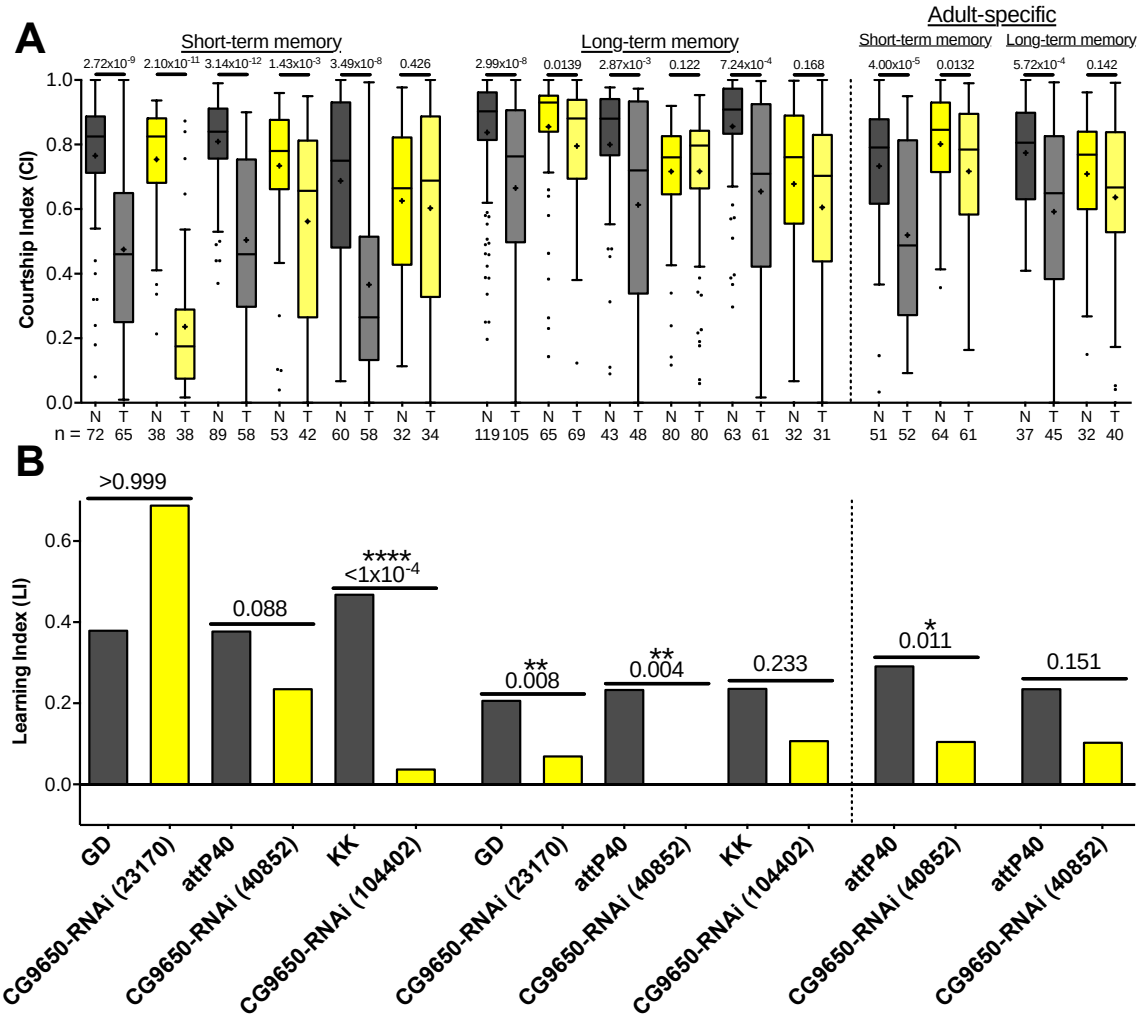

**Appendix B-6: Effect of knockdown of *BCL7-like* in the MB on courtship memory.**



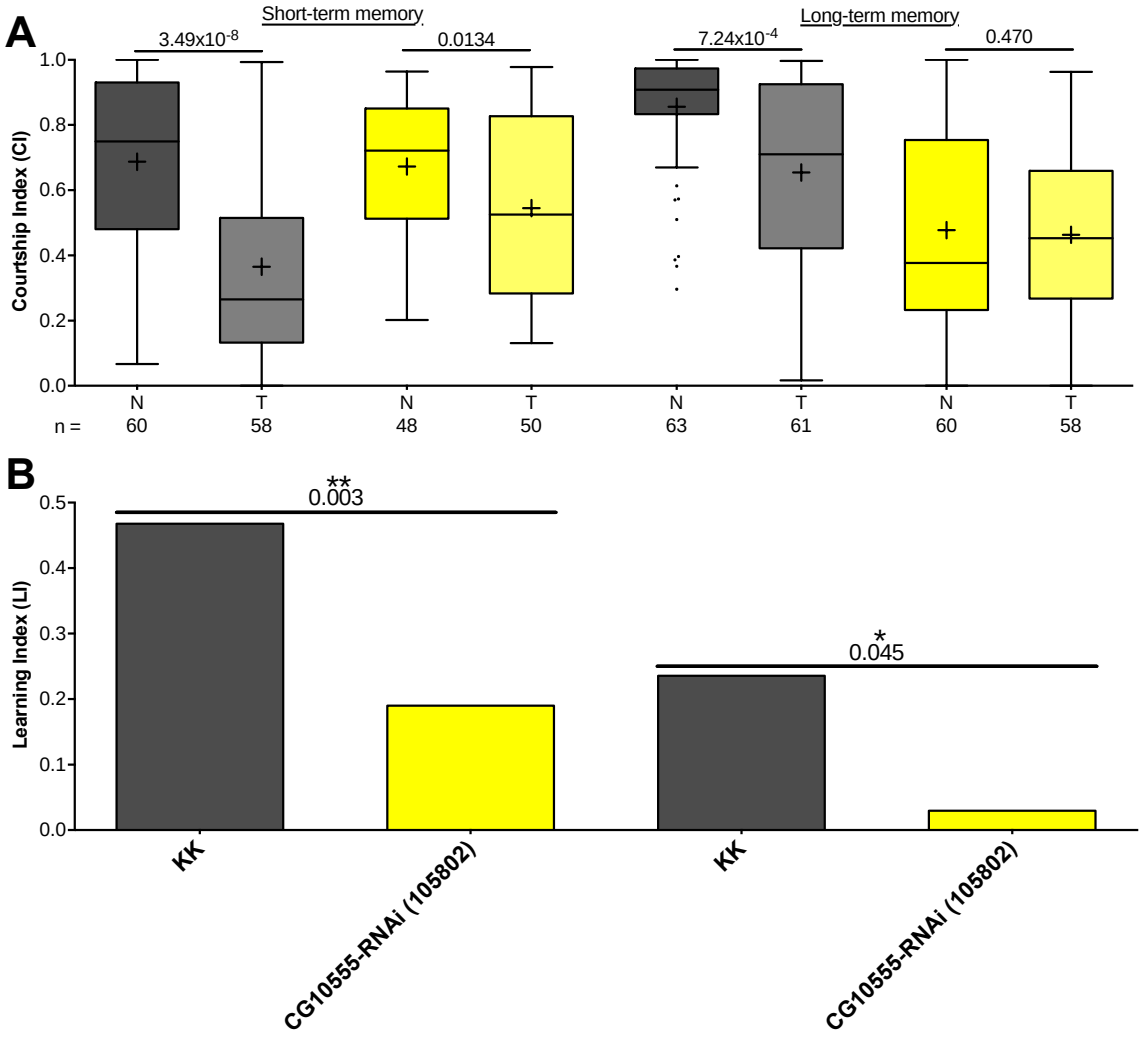

**Appendix B-7: Effect of knockdown of *brahma* in the MB on courtship memory.**



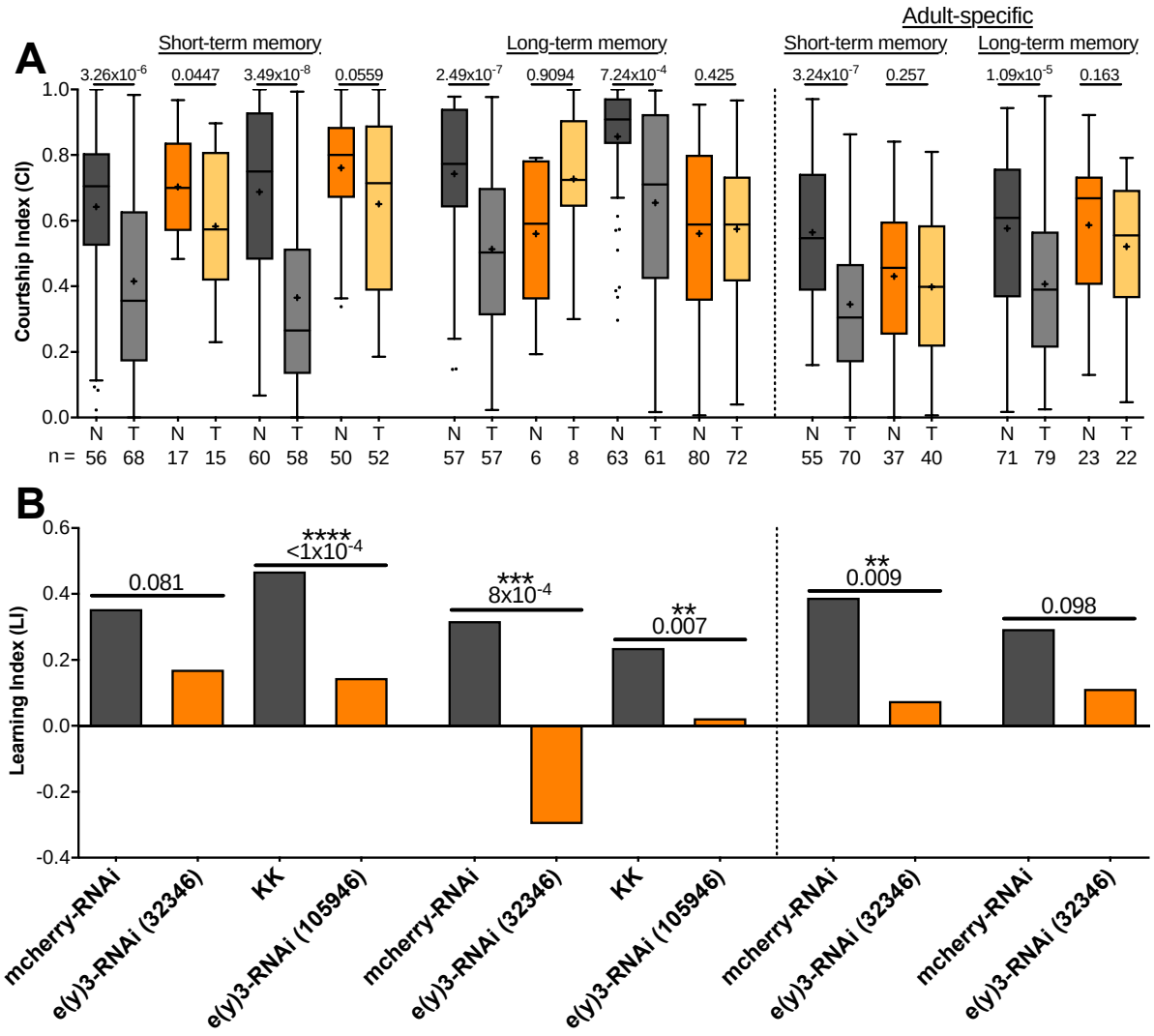

**Appendix B-8: Effect of knockdown of *CG7154* in the MB on courtship memory.**



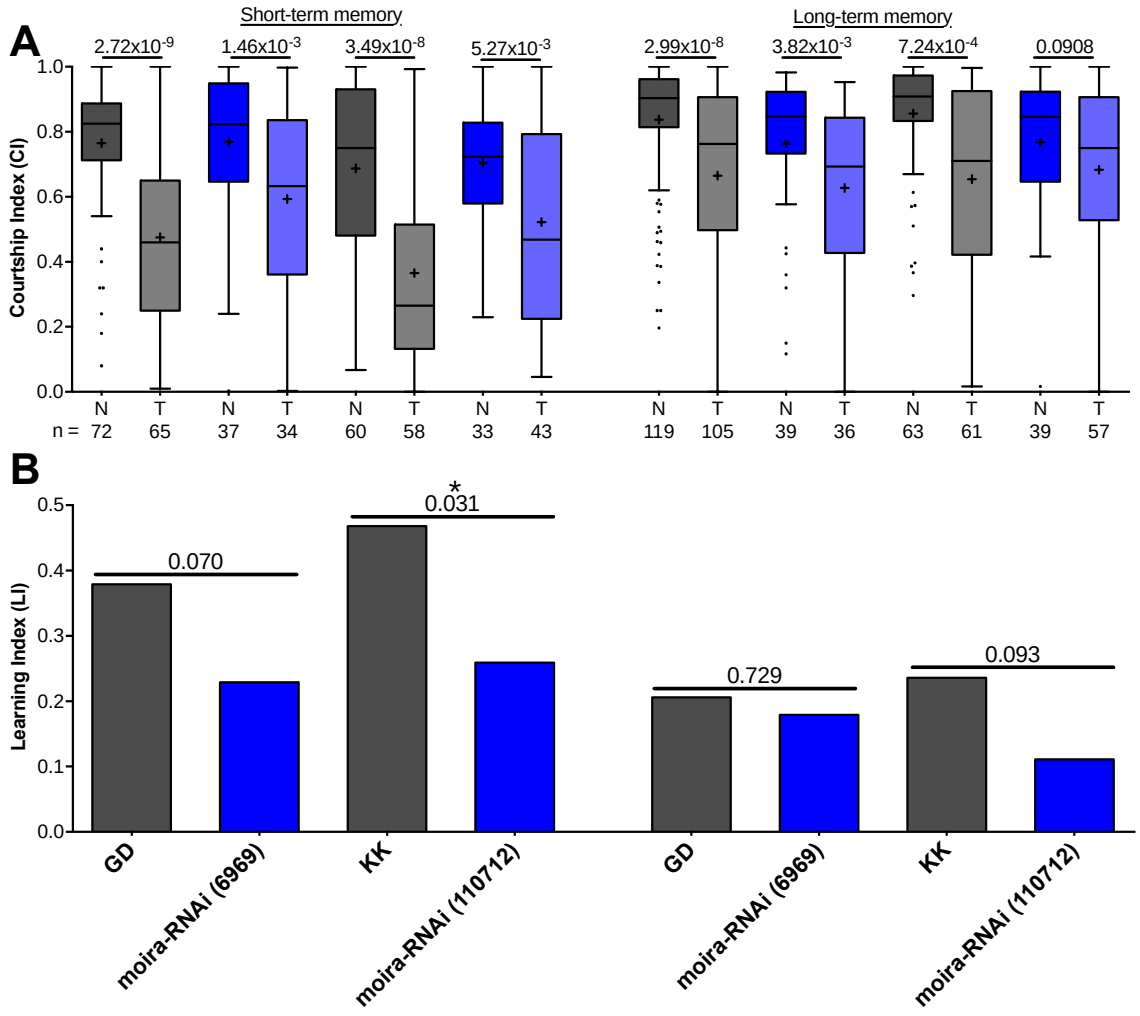

**Appendix B-9: Effect of knockdown of *CG9650* in the MB on courtship memory.**



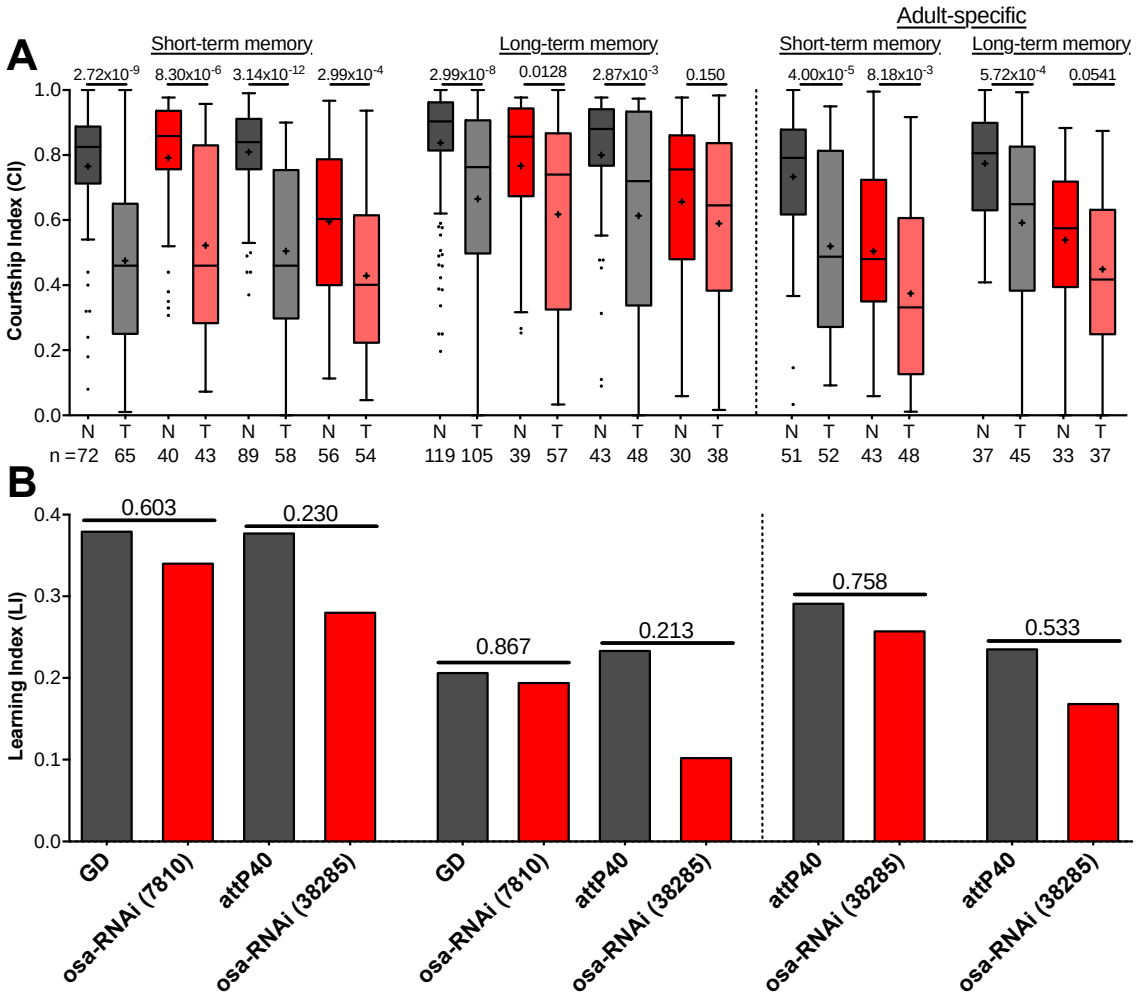

**Appendix B-10: Effect of knockdown of *CG10555* in the MB on courtship memory.**



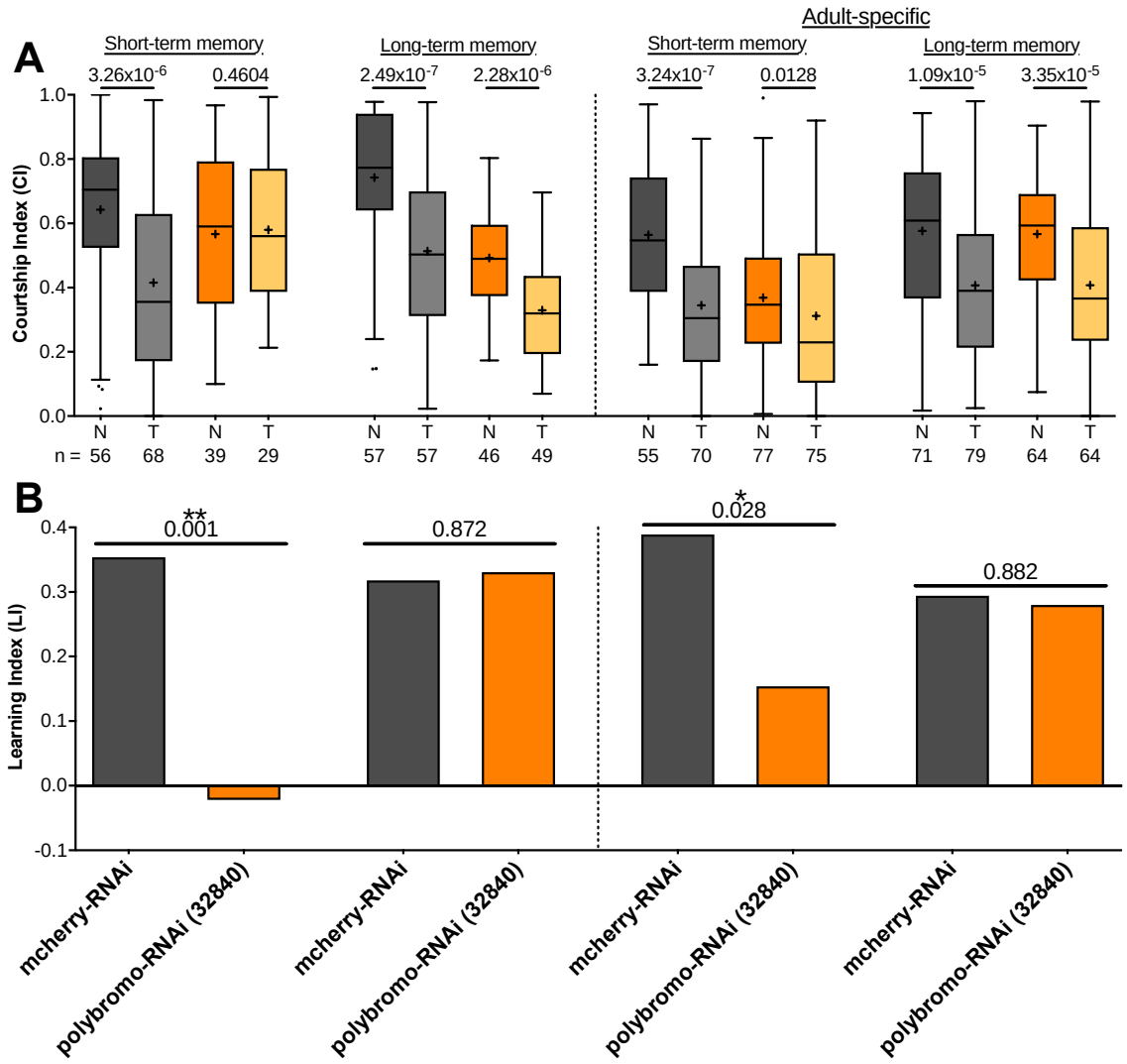

**Appendix B-11: Effect of knockdown of *e(y)3* in the MB on courtship memory.**



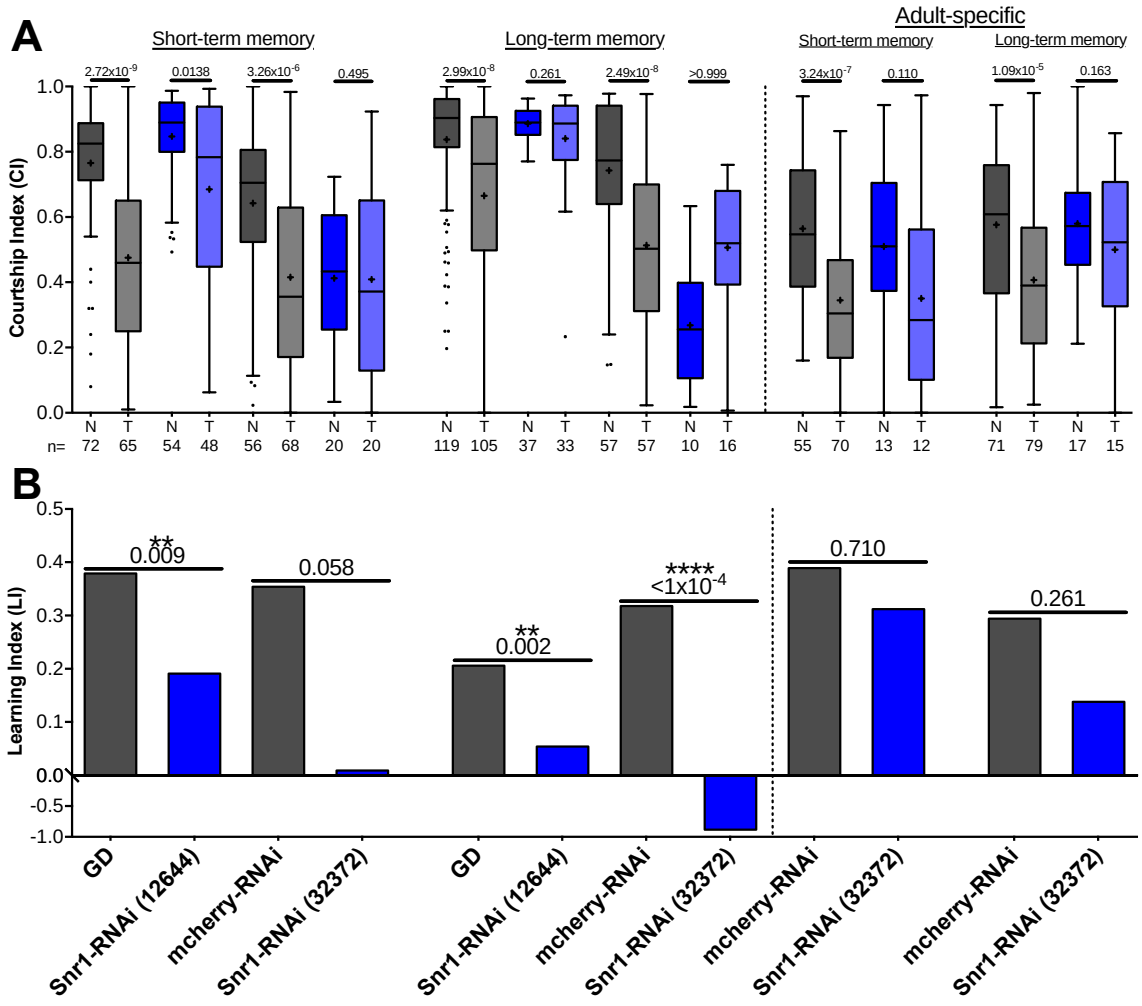

**Appendix B-12: Effect of knockdown of *moira* in the MB on courtship memory.**




**Appendix B-13: Effect of knockdown of *osa* in the MB on courtship memory.**




**Appendix B-14: Effect of knockdown of *polybromo* in the MB on courtship memory.**




**Appendix B-15: Effect of knockdown of *Snr1* in the MB on courtship memory.**

# 7. Curriculum Vitae

**Name:** Max Harrison Stone

**Post-secondary Education and Degrees:**

University of Western Ontario
London, Ontario, Canada
2015–2017 M.Sc., Biology (Developmental Biology Collaborative Program)

University of Western Ontario
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2011–2015 B.Sc., Honors Specialization in Biology

**Related Work Experience:**

Graduate Teaching Assistant
University of Western Ontario
2015–2016

**Publications:**

Koemans, T.S., Kleefstra T., Chubak M.C., **Stone M.H.**, et al. Functional Convergence of Histone Methyltransferases EHMT1 and KMT2C Involved in Intellectual Disability and Autism Spectrum Disorder. PLOS Genetics (Accepted: June, 2017)

**Selected List of Presentations:**

**Stone M.H.** The SWI/SNF Complex Regulates *Drosophila* Courtship Memory Formation. Oral Presentation, Canadian Drosophila Research Conference. Banff, AB, Canada. June, 2017.

**Stone M.H.,** Kramer J.M. The SWI/SNF Chromatin Remodeling Complex is Essential in the Regulation of *Drosophila* Short-Term Memory. Oral Presentation, Biology Graduate Research Forum. London, ON, Canada. October, 2016.

**Stone M.H.,** Kramer J.M. Dysregulation of *Drosophila melanogaster* Learning and Memory Due to Knockdown of Individual SWI/SNF Complex Subunits. Poster Presentation, Canadian Conference on Epigenetics. Estérel, QC, Canada. September, 2016.

**Awards:**

Department of Biology Travel Award (Value: $75)
Department of Biology, University of Western Ontario
London, ON, Canada, 2017

Epigenetics Trainee Fellowship (Value: $10,000)
Children's Health Research Institute
London, ON, Canada, 2016

Epigenetics Conference Travel Award (Value: $750)
Children's Health Research Institute
London, ON, Canada, 2016