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# Epigenetic regulation of behavior in *Drosophila melanogaster*

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Animal behavior is arguably among the most intricate and complex phenotypes. Not only are the molecular processes underlying most behavioral phenotypes exceedingly complex, but these processes are also variable, to allow for behavioral adjustments in response to external and internal conditions. The adaptation of behavior to the current circumstances (i.e. plasticity) can be crucial for survival as a single behavioral strategy may only be beneficial under the right conditions. Accordingly, the molecular processes that regulate behavior need to allow for a certain degree of plasticity, within an optimal range of behavioral responses. Over the last decade an extensive number of studies from insects to humans has highlighted the importance of epigenetic gene regulation in the fine-tuning of behavioral responses. Here we discuss recent behavioral epigenetics work using the fruit fly, *Drosophila melanogaster*.

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## Introduction

The *Drosophila melanogaster* model has been at the forefront of behavioral genetic research for many decades [1]. However, research on the little fly currently lags behind mammalian models when it comes to behavioral epigenetics (Figure 1). This is likely because mammalian behavioral epigenetics research has benefited from technologies that allow easy and cost effective measurements of DNA cytosine (CpG) methylation, which is only found at trace levels in *D. melanogaster* and, so far, has no known developmental or behavioral functions [2–4]. Nonetheless, other key epigenetic mechanisms are known to have important functions in fly behavior.

In the last five years, epigenetic regulation at the level of gene transcription (e.g. histone modifications) and post-transcriptional regulation (e.g. small regulatory RNAs and RNA methylation) has been found to regulate a variety of *D. melanogaster* behaviors, such as learning and memory, feeding and food related behaviors, sleep and circadian rhythms, and sex-related behaviors (Figure 2). These behaviors are regulated by all or at least several epigenetic mechanisms rather than a single mechanism in isolation, creating the complex regulatory landscapes necessary for the fine-tuned responsiveness of the animal to external and internal states.

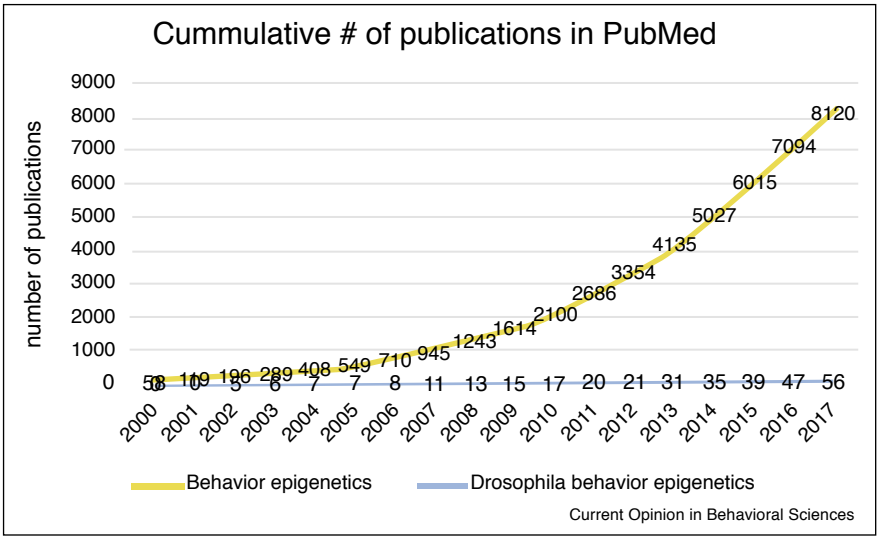
## Epigenetic regulation of learning and memory

Fruit flies have been used extensively to study the molecular mechanisms underlying learning and memory. As such, it is perhaps no surprise that this area of study also contains the largest number of articles on epigenetic regulation of behavior. The most common learning and memory paradigms in *Drosophila* are courtship conditioning assays, where males learn to avoid courting non-receptive females, and odor-avoidance assays where flies of either sex are taught to avoid a 'bad' odor. These assays allow us to test for distinct aspects of memory formation (learning, short-term (STM), medium term (MTM), and long-term (LTM) memory) that are associated with different molecular processes and epigenetic regulators. For instance, the histone methylase (HMT) *G9a* affects learning, STM and LTM [5], while the histone demethylase (HDM) *KDM5* affects STM and LTM, but not learning [6].

Similarly, the histone deacetylase (HDAC) *Grunge* regulates learning and LTM [7], while the HDACs *Rpd3* (*HDAC1*) and *HDAC4* affect only LTM [8,9]. In contrast, the histone acetyl transferase (HAT), *Tip60*, does not affect learning or LTM, but plays a role in STM [10]. Interestingly, *HDAC4*'s function in LTM is at least in part independent of its HDAC enzymatic activity [8,11], highlighting the fact that epigenetic regulators might not always act through their catalytic functions.

Histone modifiers such as HMTs, HDACs and HATs regulate the transcription of genes, but epigenetic

**Figure 1**




Differences in behavioral epigenetics publications in fruit flies and all organisms. The cumulative number of publications in PubMed from 2000 to 2017 for behavioral epigenetics in all animals (keywords: behavior; epigenetics) and behavioral epigenetics in fruit flies (keywords: behavior; epigenetics; *Drosophila melanogaster*). The number of publications in the general field of epigenetics (yellow line) has increased exponentially in the last 18 years, while the number of publications in fruit flies (blue line) has only increased moderately indicating that there is much research to be done in *Drosophila* behavioral epigenetics.

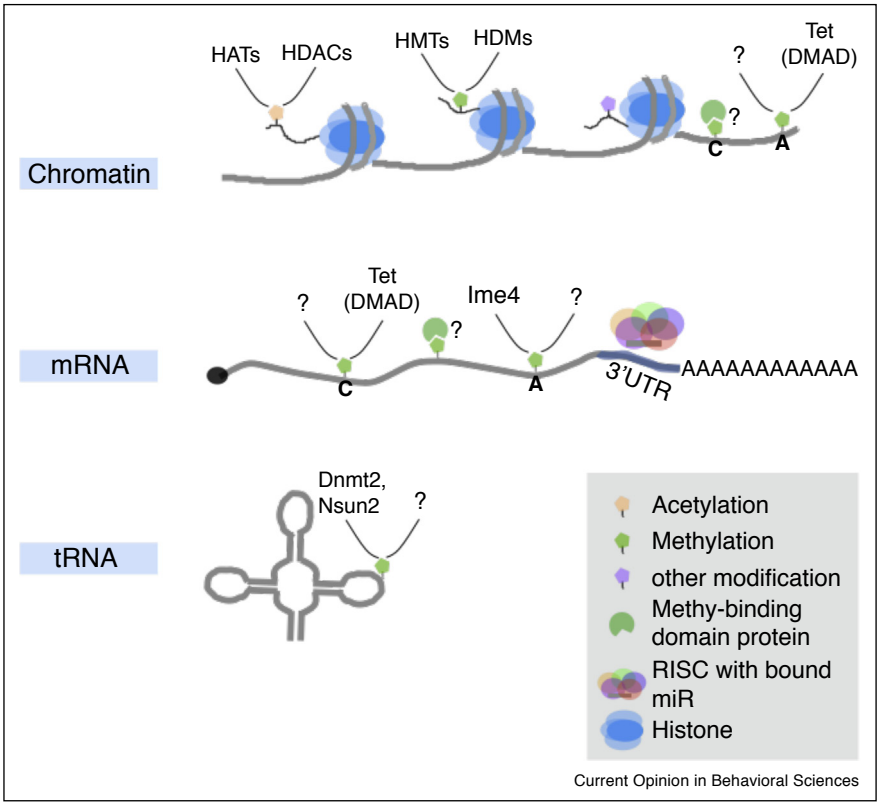
mechanisms can also act post-transcriptionally. microRNAs (miRs) are small non-coding RNAs, which bind to a mRNA's 3′UTR using sequence complimentary binding sites. miRs largely act by decreasing translation rate or promoting RNA degradation. miRs and proteins associated with miR processing have been implicated in a variety of behaviors in *Drosophila*, including several aspects of memory formation. Components of the miR-binding RNA induced silencing complex (RISC) affect both LTM and long-term habituation [12,13]. Furthermore, individual miRs are necessary for different phases of olfactory memory formation and their effects differ because each miR has specific downstream targets. For instance, miR-980 suppresses learning and MTM by affecting the expression of the autism-related gene *A2bp1* [14], while miR-276a promotes odor-avoidance LTM via regulation of a dopamine receptor [15]. A screen of 134 miRs identified miR-9c, miR-31a, miR-305a, miR-974 as positive regulators of MTM, and miR-980 as a negative regulator of MTM [16]. Interestingly, miR-974 has opposite effects on MTM, depending on its expression pattern. When miR-974 is knocked-down pan-neuronally, MTM is suppressed, but when knocked-down only in olfactory receptor or mushroom body neurons MTM is enhanced. Finally, there is some evidence that RNAs involved in memory formation are not only regulated via miRs but also by methylation of RNAs themselves [17].

In summary, a variety of epigenetic processes (e.g. histone methylation, histone acetylation and miRs) regulate different components of memory formation. While some epigenetic regulators are only involved in a specific aspect of memory formation (e.g. *Tip60* only affects STM), other epigenetic factors, such as *G9a*, regulate all aspects of learning and memory [5].

## Epigenetic regulation of food-related behavior

Most studies on epigenetic regulation of behavior in *Drosophila* are based on the phenotyping of fly mutants or the knock-down of epigenetic regulators. Although this approach is effective to find genes that are required for a given behavior, it does not provide information on natural differences in behavior of non-mutant flies. A recent study on adult foraging behavior investigated the underpinnings of distinct foraging strategies of two *Drosophila* strains and found that the differences were mediated by an interaction between genetic background and epigenetic regulation [18•]. Anreiter *et al.* found that the HMT *G9a* mediates the foraging patterns of the 'rover' and 'sitter' fly strains, by differentially regulating the expression levels from promoter 4 (pr4) of the protein kinase gene *foraging*. Rovers are more likely to explore the inside of the foraging arena and find and consume more drops of sucrose than sitters, whose movement patterns hug the edges of a foraging arena [18•,19]. In sitters, *foraging's* promoter 4 has lower *G9a*-mediated histone methylation and higher expression, and transgenic manipulation of pr4 expression converts sitter to rover behavior. Thus, *G9a* regulation of *foraging* gene expression mediates the rovers/sitter differences in adult foraging behavior. *G9a* also regulates other food-related phenotypes such as fat

**Figure 2**




Epigenetic modifications associated with behavioral regulation in *Drosophila melanogaster* include histone (chromatin) modifications and RNA modifications. While the presence of cytosine methylation in fruit fly DNA is controversial [3,55,56], adenosine methylation mediated by the Tet homolog DMAD is present on DNA [40]. Histone proteins show a variety of modifications, among these acetylation and methylation are common, and enzymes depositing or removing these marks at specific locations have been identified. Histone acetyltransferases (HATs) or histone methyltransferases (HMTs) deposit acetyl- or methyl-marks respectively, whereas histone deacetylases (HDACs) or histone demethylases (HDMs) remove them [57]. Histone modifications can be either repressive or activating, sometimes depending on context. In mRNA, direct methylation of cytosine and adenosine bases is present. The Tet homolog DMAD likely also functions here to demethylate cytosines on RNA, but the identity of the methylase is unknown [41•]. *Ime4* methylates adenosine in RNA, but it is unknown whether a demethylase exists [58]. RNAs other than mRNAs can also be modified and tRNA methylation via Dnmt2 and Nsun2 has been described [59]. Methyl-binding domain proteins have been described in fruit flies, but contrary to many other species, they might not preferentially bind to methylated DNA [48]. Small non-coding RNAs, such as microRNAs, bind to the 3′UTR of mRNAs in a sequence-specific manner under the involvement of the RISC complex and thus repress translation [60]. Besides the mechanisms described here, long non-coding RNAs, other DNA, RNA or histone modifications exist in fruit flies and can affect transcriptional and translational processes in various ways. However, to date none of these additional pathways have been studied from the perspective of *Drosophila* behavior.

storage, starvation resistance, sucrose responsiveness, and starvation-induced hyperactivity [18•,20•,21]. *G9a* mutant flies have higher fat storage and better survival under starvation [18•,20•], consistent with previous findings in mammals [22]. Curiously, a different *G9a* lack-of-function mutant was shown to have lower survival and more sensitive behavioral responses under starvation [7,23], suggesting that the effect of *G9a* on these food-related phenotypes might also be dependent on genetic background.

In addition to histone methylation, histone acetylation has also been implicated in food-related behaviors. The sirtuin HDAC *Sir2* negatively regulates fat storage levels, resistance to starvation and alcohol-related behavior [24,25]. Like *G9a* mutants, *Sir2* mutants are fatter and survive longer under certain starvation conditions [24]. Furthermore, they are less tolerant of alcohol and more sensitive to repeated alcohol exposure. Interestingly, while *Sir2* mutants also have a higher naïve preference for ethanol consumption, they fail to acquire a stronger preference for alcohol after prolonged exposure, which is typical for wild type flies [25]. Alcohol-related behavior is also regulated by several members of the JmjC-KDM family of histone demethylases [26]. Mutants of the JmjC-KDMs *KDM3* and *NO66* are more sensitive to EtOH-induced sedation, and mutants of the JmjC-KDMs *KDM3*, *HSPBAP1* and *JMJD7* do not develop a tolerance to ethanol upon prolonged exposure. Curiously the JmjC-KDM *Lid* is more sensitive to sedation but has increased

tolerance upon repeated exposure to EtOH [26], highlighting the complexity and specificity of epigenetic regulation.

Although there are far fewer studies on epigenetic regulation of food-related behaviors than on learning and memory, it is clear that food-related behaviors are also regulated by a variety of epigenetic processes. Foraging behavior, starvation resistance, and alcohol preference are regulated by both histone methylation and acetylation, and different HMTs, HDACs and KDMs affect different aspects of these behaviors.

## Epigenetic regulation of sleep and circadian behavior

Sleep and circadian behavior has been extensively characterized in flies; in fact the molecular mechanisms controlling circadian rhythms were first discovered in fruit flies [27]. Several of the epigenetic regulators discussed in the context of memory formation and food-related behaviors also regulate sleep and circadian behavior. One significant area of overlap is the regulation of alcohol induced behavior and circadian rhythms. Shalaby *et al*. found that 11 JmjC-KDMs, some of which have been implicated in regulating the response to ethanol (*KDM3*, *NO66*, *JMJD7*, *lid*), also affect different aspects of circadian behavior and sleep [28]. While *lid* and *KDM* affect circadian rhythmicity, *KDM2* and *JMJD5* affect period length and strength of the rhythms, *JMJD5*, *NO66*, *JMJD7*, *KDM4A* and *KDM4B* affect sleep, and *JMJD5*, *NO66*, *KDM4A* and *KDM4B* affect general activity levels. As with alcohol-induced behavior, the different JmjC-KDMs sometimes have opposing effects on sleep and circadian rhythms. For instance, *KDM4A* and *KDM4B* mutants sleep more and are less active whereas *JMJD5* and *NO66* mutants sleep less and are more active [28].

Similarly, the HAT *Tip60*, which plays a role in STM, also regulates sleep [29], and *HDAC4*, which plays a role in LTM, also regulates circadian function [30]. Loss of *Tip60* in the *Drosophila* pacemaker cells (sLNvs) disrupts sleep–wake cycles and over-expression of *Tip60* in sLNvs protects from amyloid precursor protein (APP) induced neurodegeneration in a *Drosophila* model for Alzheimer's disease [29]. Reduced *HDAC4* function disrupts locomotor activity levels and circadian rhythms, potentially by affecting expression of the clock gene *period* [30]. Together this data strongly suggests that many of these histone modification genes exhibit pleiotropic (multiple) functions in the regulation of behavior.

As is the case with learning and memory, miRs have been shown to be involved in circadian behavior via manipulations of the miR maturation pathway or proteins associated with the RISC complex [31,32]. Several core components of the circadian system are strongly associated with the miR binding protein AGO1 [31]. Specifically, the clock genes *Clock* and *clockwork orange* are regulated by the miRs *bantam* and *let-7* respectively and both miRs affect circadian rhythms and period length [31,33]. miR-279, on the other hand, affects the fly's circadian rhythm via *Unpaired*, a component of the JAK-STAT pathway downstream of the circadian clock [34]. Within glial cells, and specifically astrocytes, miR-263b and miR-274 affect circadian rhythms via yet unidentified target genes [35•].

A second group of miRs affects circadian phase and morning and evening peak activity without affecting period length or rhythmicity [36,37,38•]. miR-124 likely acts via components of BMP pathway and might change the morphology of *Pdf*-expressing neurons [36,37]. Contrary to histone modifications, there is no reported overlap between the miRs involved in circadian function and food-related behavior or memory formation, suggesting that individual miRs may act on specific suites of phenotypes. Nevertheless, miR-92a affects circadian rhythms and sleep by targeting the HDAC sirt2 [38•], and knockdown of *sirt2* rescues sleep defects in miR-92a knock-out flies. This suggests an interplay between miRs and histone acetylation, which together regulate circadian rhythms and sleep [38•].

Lastly, a recent study found that the DNA hydroxymethylase *Tet (DMAD)*, which in mammals preferentially demethylates DNA, is required for normal larval locomotion and circadian rhythms in adult flies [39]. Because of the apparent lack of functional DNA cytosine methylation in flies, *Tet* is thought to function through de-methylation of RNA cytosine and DNA adenosine in *Drosophila* [40,41•]. These findings are noteworthy because they underline the functional complexity of epigenetic regulators, many of which not only have alternative targets (such as RNA instead of DNA), but also secondary functions (such as the role of *G9a* in stabilizing regulatory complexes) [42].

## Sex determination and sex-specific behavior

Although sex determination is not a behavior per se, the processes involved in it are tightly linked with sex-specific behaviors such as mating and courtship. A major determinant of sex-specific behavior in flies is the transcription factor *fruitless* (*fru;* for review see [43]), which regulates male courtship behavior by forming alternate complexes with either the HDAC *Rpd3* (which also regulates LTM) or the heterochromatin associated protein *Su(var)205* [44]. Males lacking *fru* court other males, and while mutations in *Rpd3* further enforce this abnormal courtship behavior, mutations in *su(var)205* normalize it, suggesting that male courtship is regulated through antagonistic histone pathways [44]. Similarly, mutants of the histone demethylase *kdm4* have decreased *fru* expression and increased courtship-like responses to other males

[45]. To date only a single study implicates miRs in courtship behaviors [46], miR-124, which also regulates circadian behavior, affects the responsiveness of both males and females to miR-124 mutant males [46]. miR-124 targets the sex-determination pathway gene *transformer*, which is expressed in a female-specific and a shared isoform. In miR-124 mutants, the female-specific isoform is increased in males and the level of specific cuticular hydrocarbons (*Drosophila* pheromones) is changed, reducing the responsiveness of females to mutant male courtship and increasing the courtship of males towards mutant males.

Interestingly, the methyl-CpG-binding domain (MBD) containing proteins *MBD-R2* and *MBD2/3*, play a role in male courtship behavior as well. Downregulation of these MBDs in octopaminergic neurons increases male-male and inter-species courtship and decreases aggression towards con-specific males [47•]. These findings are intriguing because MBD proteins are predominantly known for binding methylated cytosines in DNA in most species. Nevertheless, both proteins also bind to other targets, such as HDACs (*MBD2/3*; [48]) and unmethylated DNA (*MBD-R2*; [49]), and there is evidence that *MBD2/3* fails to bind artificially methylated DNA *in vitro* [48], suggesting that they might act via alternative pathways to binding methylated cytosines in DNA.

## Conclusions

The fly behaviors discussed here are regulated through a combination of epigenetic mechanisms. Most studies have focused on adult behavior and their regulation by epigenetic mechanisms, but larval behavior can be affected as well, pointing to additional roles for epigenetic mechanisms during development [50].

Both histone methylation and acetylation play roles in regulating a variety of food-related behaviors, including foraging strategies, response to starvation, and reward and addiction. Interestingly, a recent study found that epigenetic changes (histone methylation and acetylation) induced by a high paternal sugar diet can be passed on to their offspring to affect food-related phenotypes in the next generation, and that this might happen through conserved pathways from flies to humans [51].

Besides histone modifications, miRs and RNA methylation function in regulating memory formation and circadian behaviors. Many of the epigenetic modifiers of learning and memory discussed here also have cognitive functions in humans. For instance, *G9a* underlies a form of intellectual disability known as Kleefstra Syndrome [52], *HDAC4* is associated with the brachydactyly mental retardation syndrome [53], and *KDM5* and *NSUN2* are both associated with intellectual disability [17,54].

Although many epigenetic modifiers have wide-reaching effects on behavior (e.g. G9a regulates memory formation and feeding behavior; JmCs affect alcohol-induced behavior, circadian behavior and sleep), most mutants of these genes are viable, with relatively normal development. This highlights the fact that rather than being necessary for development, many epigenetic modifiers play a complex and intricate role in fine tuning phenotypes, perhaps in response to internal and external cues. In the future, it will be crucial to dissect the interplay and hierarchy between different epigenetic mechanisms in shaping behaviors and how they respond to specific environmental contexts.

Finally, we would like to argue that although fruit flies are still underrepresented in behavioral epigenetic research, they are an excellent system to study the effects of epigenetic regulation on behavior. The many qualities that have made flies such excellent models for genetic research during the last decades will undoubtedly also be of great advantage for behavioral epigenetics studies. The ease of handling flies and the readily available genetic tools for most *Drosophila* epigenetic modifiers (e.g. null mutants, knock-down, over-expression, tagged proteins) make it possible to dissect causal relationships between epigenetic regulation and behavior, moving beyond gene-behavior associations. Finally, many well-understood fruit fly behaviors are epigenetically regulated often through pathways that are conserved in other organisms, including humans.

## Conflict of interest statement

Nothing declared.

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