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Tarun Gupta

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METHYL-CpG BINDING PROTEINS MEDIATE OCTOPAMINERGIC
REGULATION OF COMPLEX BEHAVIORAL TRAITS

BY

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Each animal has devised extraordinary and baroque mechanisms to achieve behavioral and physiological flexibility in the context of its environment, genetic and neuronal complement, and biomechanical constraints. It will only be by looking for general principles across species that we will find the more general rules that govern life in its many shapes and forms.

- Adapted from a quote by Eve Marder

**ABSTRACT**

An organism’s survivability in the natural world is contingent to its ability to respond rapidly and appropriately to various cues and challenges in its physical and social environment. The dynamicity of various environmental and social factors necessitates plasticity in morphological, physiological and behavioral systems – both at the level of an individual organism and that of a species. For more than century, natural selection of existing genetic variation in populations has helped us understand such plasticity across generations. However, recent years have seen a re-emergence of somewhat contentious quasi-Lamarckian framework with which organisms can reliably transmit acquired traits to subsequent generations in response to changes in external conditions. Whether or not it can be categorized as such, a stable transgenerational transmission of acquired alterations in epigenetic code, including methylation patterns and small RNA molecules, associated with behavioral and physiological, and I use the term here loosely, ‘adaptations’ for up to three generations has indeed been demonstrated in a number of species. The focus on methyl-binding proteins in this dissertation is guided by a motivation to advance our understanding of such epigenetic systems in one of the most extensively used model systems in biological and biomedical research – *Drosophila*.

In contrast to the vast body of literature on the genetics, physiology, ecology, and neurobiology of *Drosophila*, methylation and methylation-associated processes represent one of the few relatively unexplored territories in this system. This certainly hasn’t been for the lack of trying (see section 1.8). Consistent with their role in other species,
Drosophila MBD proteins have been implicated in dynamic regulation of chromatin architecture and spatiotemporal regulation of gene expression. However, methylation-dependence of their functions and their contribution to the overall organismal behavior remains equivocal.

In this dissertation, I explore the role of the conserved methyl-CpG binding (MBD) proteins in the regulation of octopaminergic (OA) systems that are associated with a number of critical behaviors such as aggression, courtship, feeding, locomotion, sleep, and learning and memory. In chapter II, I, along with my colleagues, demonstrate functional conservation of human and Drosophila MBD-containing proteins. We show – (a) that a well-characterized human protein – MeCP2 – can regulate amine neuron output in Drosophila through MBD domain, (b) that endogenous MBD proteins in Drosophila regulate OA sleep circuitry in a manner similar to human MeCP2, and (c) that human and Drosophila MBD proteins may share a select few genomic binding sites on larval polytene chromosomes. In chapter III, we describe a novel function of these chromatin modifiers in the regulation of social behaviors, including aggression and courtship. Returning to the issue of methylation, we demonstrate an interaction effect between induced-DNA hypermethylation and MBD-function in context of aggression and inter-male courtship.

Species– and sex–specific behaviors such as courtship and aggression rely on an organism’s ability to reliably discriminate between species, sexes and social hierarchy of interacting partners, and adjust to the dynamic shifts in sensory and behavioral feedback cues. At the level of an individual organism, such behavioral flexibility is often achieved by modulating the strength and directionality of neural network outputs which endows a limited biological circuit the capacity to generate variable outputs and adds richness to the repertoire of behaviors it can display (Marder, 2012). The role of MBD proteins discussed in this dissertation highlights a mechanism that couples chromatin remodeling and OA neuromodulation in context-dependent decision-making processes.
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In 2009, I was taking a class in neuroendocrinology when I came across a study about reproductive behaviors in voles. The study mentioned different species of voles – some that displayed monogamous pair-bonding and others that were rather promiscuous. I learned that by altering the distribution of just one gene – Arginine vasopressin receptor (AVPR1a) – in the ventral pallidum area of the brain, a seemingly complex reproductive behavior i.e. monogamous pair bonding can be altered to promiscuous and vice versa (Ophir et al, 2008). I learned later that polymorphisms in the same gene have been associated with pair-bonding and quality of marital relationships in humans as well (Walum et al, 2008). The idea that manipulations of single or very small set of genes may result in dramatic alterations in complex social behavior brought me to Sarah Certel who was exploring underlying mechanistic correlates of such plasticity in context of dynamic social behaviors.

Sarah graciously took me under her mentorship and granted me the freedom and support, both intellectual and material, in my scientific pursuits. Throughout my studies in the Certel Lab, she has been a kind, patient, supportive, and an empathetic mentor who genuinely cares for her students, understands the challenges of graduate student life and enables a harmonious work environment. For instance, she put in a lot of effort helping each of us prepare for each and every presentation we have ever delivered in various meetings nationally or within our department. She allowed and encouraged me to seek professional opportunities outside my regular bench time enabling my participation as a TEDx speaker and as a reviewer within the UGP program. When she found out that the department’s contribution to the premium costs for the health insurance offered by the University to graduate students is insufficient, she even attempted to find ways to provide supplemental coverage for us. Overall, she is a great mentor and a fantastic human being.

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1 CHAPTER I

INTRODUCTION

This dissertation investigates the role of Methyl-CpG binding (MBD) proteins in the regulation of complex, multivariate behavioral traits in *Drosophila*. Specifically, it describes how endogenous MBD proteins regulate octopamine neuron function in context of dynamic reproductive and aggressive social interactions. These studies are complemented by temporal assessment of alterations in neural circuit output for high-throughput profiling of domain-specific functional interactions. This introductory chapter will (1) provide a brief overview of the genesis and organization of the central nervous system in *Drosophila* (sections 1.1 to 1.4), (2) review the octopaminergic system in context of behavioral traits and social interactions examined in this dissertation (sections 1.4 to 1.6), and (3) discuss the controversy surrounding DNA methylation in *Drosophila* along with a few recent confirmatory studies that provide some context and rationale behind the exploration of MBD protein function in this model organism (section 1.7).

1.1 DROSOPHILA AS A MODEL SYSTEM

Ever since Morgan’s pioneering experiments on sex-linked inheritance in 1909, *Drosophila* has played a pivotal role in advancing our understanding of some of the most fundamental processes in biology. As a result, there is an extensive knowledgebase spanning over a century covering almost all aspects of the biology of this organism. This has led to the emergence of an extraordinary versatility and specificity of genetic tools available for fly models; allowing spatiotemporally controlled manipulation of gene expression at the resolution of a single neuron. Coupled with the emergence of centralized stock distribution centers, high resolution imaging and sequencing systems along with high-throughput behavioral assays, *Drosophila* offers an unprecedented degree of ease and sophistication in the exploration of genetic, cellular and neurobiological basis of organismal development, physiology, and behavior. As a testament to their utility as a model system, these flies have been frequent visitors to the International space station (ISS) over last three decades for studies on the effects of microgravity on the development of the nervous system, ageing, and host immunity.
According to the latest genome assembly and annotation report (2015/10/19; release 6.08 - GenBank: 1186808), *Drosophila melanogaster* genome is 143.7Mb in size with 30,443 known proteins, and an estimated 17,651 genes currently mapped to the genome. Of these, at least 585 fly genes represent functional homologues of 714 distinct genes associated with disease in humans representing ~77% of all known disease causing genes, many of which are involved in neurological disorders (Reiter et al., 2001).

In terms of behavioral complexity, despite a relatively small brain, *Drosophila* exhibits an extraordinary repertoire of dynamic multivariate behaviors, many of which can be examined in a high-throughput manner with automated analytical methods. Furthermore, most neurotransmitter and neuromodulator systems associated with these behaviors are conserved between flies and higher mammals, including humans. For instance, the noradrenergic system – the primary neural cluster examined in this dissertation – shows functional conservation across species for its role in the regulation of arousal, wakefulness, aggression and formation and retrieval of memories. In this dissertation, I will attempt to capitalize on such sequence and functional conservation in an attempt to unravel mechanistic underpinnings of some of these complex processes by manipulating single or a small subset of genes selectively in a targeted set of neurons.

### 1.2 Genesis of the Nervous System

Before we begin our discussion of the role of aminergic neurons in the regulation of complex behavioral traits, it is fitting to provide the reader with a brief and general introduction to the development and the organization of the nervous system in *Drosophila*. After all, the transformation of a single cell in to a sophisticated calculating brain has long been an object of curiosity and wonder for many of us. *Drosophila* development has been studied intensely for more than six decades and this very brief summary doesn’t even begin to scratch the surface of the vast amount of literature on this subject. With that disclaimer out of the way, let me attempt to summarize the genesis and
the organization of the nervous system, and introduce you to this powerful model system of scientific inquiry.

*Drosophila*, like all dipterans, undergoes a holometabolous mode of development with four distinct stages: (a) egg or embryo, (b) larvae, (c) pupae, and (d) adult. Starting from the first nuclear division in the zygote to the hatching of the first instar larvae, embryogenesis in *Drosophila* has been categorized into 17 distinct stages (Hartenstein and Campos-Ortega, 1985). During the first two hours after fertilization (stage 1-4), the zygote undergoes a series of 13 nuclear divisions resulting in a syncytial blastoderm with an estimated 5000 nuclei arranged around the periphery of the oocyte plasma membrane (Foe and Alberts, 1983; Gilbert, 2000). Subsequently, these nuclei undergo cellularization by invagination of the plasma membrane. The cellular blastoderm is then reorganized into three germ layers (ectoderm, mesoderm and endoderm) that give rise to all tissues and organs, including the brain (Gilbert, 2000). Around embryonic stage 9-11 (between ~3.5-7 hours after fertilization), a subset of ectoderm cells delaminate to form ~100 individual, scattered neural progenitor cells called neuroblasts (Younossi-Hartenstein et al., 1996; Urbach and Technau, 2003). These neuroblasts divide asymmetrically to produce two daughter cells. The apical daughter cell retains the properties of a neuroblast while the basal daughter cell forms a ganglion mother cell (GMC). In most cases, the GMC undergoes one final division to produce two neuronal cells and in some cases, glia (Jan and Jan, 2001). These divisions result in the formation of ~3000 primary neurons organized into distinct, structurally cohesive clonal units based on their respective neuroblast lineages, and segregated equally into two hemispheres (Ito et al., 1997; Lai et al., 2008; Spindler and Hartenstein, 2010). By embryonic stage 16 (i.e. ~13-16 hours after first nuclear division), these primary neurons begin to differentiate and project the primary axonal tracts away from the outer rind of the cell bodies and into the central brain, giving rise to early neuropil connectivity (Younossi-Hartenstein et al., 2006; Larsen et al., 2009). These early innervations are established in response to specific chemo- and contact-guidance cues in the extracellular milieu that attract or repel these innervations along their migratory pathway (Schmucker et al., 2000). Later during second and third larval instars, neuroblast cells divide again and give rise to the secondary clonal
lineage that uses primary axonal bundles and glial boundaries as structural scaffolds for projecting secondary axonal tracts (Spindler and Hartenstein, 2010). These primary and secondary clonal lineages and their innervations undergo subsequent refinement, degeneration, reorganization and maturation through the course of development as well as in an activity-dependent manner (Albright et al., 2000). A large number of neurons are also added during the pupal stage. Some of these embryonic and larval neurons and their projections persist through profound morphological and physiological changes during metamorphosis well into the adult nervous system (Shepherd and Smith, 1996; Truman, 1992; Truman and Bate, 1988; Truman, 1990).

1.3 ORGANIZATION OF CENTRAL NERVOUS SYSTEM

The central nervous system in *Drosophila* is composed of a dorsal bi-hemispheric brain (supraesophageal ganglion) connected to a composite ventral ganglion (*fig 1.1*) (Power, 1943). The supraesophageal ganglion and the anterior part of the larval ventral ganglion – the suboesophageal ganglion (SOG) – constitute the central brain in adult *Drosophila*. The central brain is roughly 500 µm wide, 250 µm tall and 200 µm thick and contains an estimated 135,000 neurons (Alivisatos et al., 2012). In contrast to the vertebrate neuronal architecture, most of these neurons are unipolar, with cell bodies confined to the outer cortical layer and single neurites projecting towards the neuropil (Hartenstein et al., 2008). Neurons from different clonal lineages project onto specific regions of the neuropil contributing to the modular or segmental organization of the brain structure and connectivity (Younossi-Hartenstein et al., 2003; Ito and Awasaki, 2008). Such compartmentalization is quite apparent in the structural demarcation (by glial sheaths) of certain brain areas such as antennal lobe (al), mushroom bodies (mb) or the central complex (cc) (*fig 1.2*). Although a detailed review of the structural organization of *Drosophila* brain is beyond the scope of this brief summary, it is useful for the reader to orient herself with respect to some of the major neuroanatomical features of the brain, especially those that are discussed later in chapters II and III of this dissertation. These include, but are not limited to, the subesophageal ganglion (seg/sog), mushroom bodies (mb), antennal lobe (al), and ventrolateral protocerebrum (vlp). These structural features are highlighted in the figure 1.2 below.
Figure 0.1: Lateral view of the central nervous system in Drosophila

Figure 0.2: Anterior surface of an adult Drosophila brain.
Dorsal Layer – VL: vertical lobe of mushroom body; SMP, SIP, SLP: superior medial, intermediate, and lateral protocerebrum respectively; LH: lateral horn
Middle Layer – ML: medial lobe of mushroom body; CCX: central complex; IP: inferior protocerebrum; MB: Mushroom body; LAL: lateral accessory lobe; AOTU: anterior optic tubercle
Ventral Layer: SEG: subesophageal ganglion (also, SOG); AL: Antennal Lobe; PENP: periesophageal neuropil; VLP: ventrolateral protocerebrum
(Source: Volker Hartenstein, Drosophila Brain Lineage Atlas (DBLA))
1.4 SPECIFICATION OF NEURONAL IDENTITY

The differentiation of neuronal identity, in terms of neurotransmitter release, is specified according to their clonal lineage as well the extracellular environment (Huff et al., 1989; Taghert and Goodman, 1984). Neuronal identity is inherent to the gastrulation-stage neuroblasts which, shortly after their formation, are committed to the production of specific monoamines (Huff et al., 1989). Transcriptional activity is first initiated in the embryo after 11th nuclear division in a stage 4 syncytial blastoderm. As early as stage 16, monoamines such as dopamine and serotonin can be detected in the embryos (Lundell and Hirsh, 1994).

1.5 DROSOPHILA OCTOPAMINERGIC SYSTEM

Octopamine (OA) is a biogenic, sympathomimetic amine that was first discovered in the Octopus salivary glands more than 60 years ago (Erspamer and Boretti, 1951). It is synthesized from the precursor tyrosine which is decarboxylated by Tyrosine decarboxylase (neuronal dTdc2 and non-neuronal dTdc1) to form tyramine (TA) (Cole et al., 2005). TA may act independently as an agonist to TA receptors or hydroxylated by tyramine β-hydroxylase (Tβh) to OA (Monastirioti et al., 1996). As a result, the tdc2 promoter is commonly used within the UAS-Gal4 binary expression system for selectively labeling and manipulating OA/TA neurons in the central brain of Drosophila. Coupling this approach with the traditional immunohistochemistry methods, an estimated 137 OA/TA neurons have been identified in the adult brain (Busch et al., 2009).

There are 3-isomers of OA (-para, -meta, and -ortho) and only p-OA is present in significant amounts in Drosophila (Farooqui, 2012). OA is structurally and functionally related to norepinephrine and fulfills similar physiological roles in invertebrates (fig.1.3). One of the salient features of adrenergic systems is the “flight or fight” response during altercations with competitors or potential predators. As discussed at length in section 1.6, OA plays a similar role in the regulation of complex agonistic interactions in Drosophila. As with most amines,
OA is associated with an array of physiological roles and behaviors in the capacity of a neurotransmitter, neuromodulator and neurohormone. These include flight, locomotion, sleep, olfaction, foraging, ovulation, courtship, and learning and memory. A comprehensive description of such functions is beyond the scope of this brief review and interested readers should refer to the excellent review by (Farooqui, 2012).

OA signal transduction is mediated by a family of seven-transmembrane G-protein coupled receptors (GPCRs). On this basis of sequence, structural and functional similarities with vertebrate adrenergic receptors, OA receptors (OARs) in *Drosophila* are categorized into three major classes (Maqueira et al., 2005) –

a) *DmOCTα* receptors are similar to α₁-adrenergic receptors; downstream signaling involves an increase in both Ca^{2+} and cAMP second messengers. The OAMB receptors belong to this category.

b) *DmOCTβ* receptors are similar to β-adrenergic receptors, and are further divided into 3 pharmacological subclasses. Downstream signaling in these receptors is mediated by an increase in cAMP levels, but not Ca^{2+} levels.

c) *DmTYRI* receptors are similar to α₂-adrenergic receptors and display an agonist specific downstream signaling. These receptors have been discussed in detail elsewhere (Farooqui, 2012; Roeder, 2005).
1.6 Octopaminergic Regulation of Complex Behavioral Traits

Octopaminergic (OA) system plays a significant role in the regulation and modulation of a number of dynamic multifactorial behavioral traits that invariably necessitate interactions with various internal and external factors. These interactions are quite evident in social contexts where organisms continually negotiate access to territory, resources, mating partners and social status with each other. Organisms negotiate this social space by acquiring and integrating various cues about their own genetic, epigenetic, nutritional, metabolic and hormonal states with information about the sex, species, dominance hierarchy, and reproductive status of its interacting partner(s). This multimodal integration allows an organism to respond to various internal and external stimuli in a context-dependent manner by generating an array of specific, mutually non-overlapping behavioral programs. For instance, depending on the sex and the history of previous encounters with the interacting organism, males in many species display agonistic behaviors when interacting with other males and canonized courtship rituals when interacting with conspecific females. That is, there exists a context-dependent behavioral switch between mutually non-overlapping behaviors of aggression and courtship. For any organism, it’s important that these behaviors are directed in response to appropriate cues, and inhibited when such cues are absent. Unregulated aggression towards potential mating partners, for instance, may be maladaptive. Therefore, one of the central goals in neuro-ethology is to understand how these behavioral choices are made. What are the mechanistic underpinnings of context-dependent decision-making?

The dynamic regulation of aggression and courtship behaviors provides us with a useful framework with which to examine general mechanics of multimodality integration, sensory motor processing, and decision-making in a social setting. Across species, biogenic amines such as serotonin, dopamine, and octopamine are key neuromodulators that promote or regulate innate behavioral sequences associated with aggression and reproductive behaviors as well as modulate them in an experience-dependent manner (Zhou et al., 2012; Szczuka et al., 2013; Kravitz and Fernandez, Maria de la Paz, 2015; Miczek et al., 2002). Here I’ll briefly describe the role of octopaminergic system in generation and modulation of these complex behavioral traits in *Drosophila*. 
1.6.1 Aggression

Male competition for access to resources and mating partners is one of the key features of sexual selection that results in the evolution of often extravagant and sexually-dimorphic morphological, physiological and behavioral systems (Darwin, 1871; Vehrencamp et al., 1989; Hack, 1997; Arak, 1983; Emlen, 2001). Exactly a hundred years ago in 1915, Sturtevant first described aggression-like behavioral sequences in *Drosophila ampirophila* males. While courting the same female, Sturtevant reported, males “often grow very excited, especially if she is unwilling to stay quiet. In such cases they may sometimes be seen to spread their wings, run at each other, and apparently butt heads. One of them soon gives up and runs away. If the other then runs at him again within the next few minutes he usually makes off without showing fight.” (p. 353) (Sturtevant, 1915). These behavioral sequences have since been extensively characterized and documented in a number of *Drosophila* species, including *D. melanogaster*, both in their ecological context as well as in the laboratory setting (Jacobs, 1960; Dow and von Schilcher, 1975; Hoffmann, 1987a; Hoffmann, 1987b; Pritchard, 1969; Shelly, 1999; Baier et al., 2002; Chen et al., 2002). Figure 1.4 illustrates some of these common and gender-specific behavioral patterns in male-male pairings in *D. melanogaster*.

With the ability to explore the genetic and neural landscape with targeted manipulation methods, we have come to appreciate the sophistication and complexity of these behavioral programs and the underlying mechanisms associated with them. Various genetic, hormonal, and neuromodulatory components have been identified for their role in innate expression and experience-dependent modulation of behavioral modules associated with male-male competition, territoriality, and formation of social hierarchy relationships. Interested reader can refer to Zwarts et al., 2012; and Kravitz and Fernandez, 2015 for excellent and comprehensive reviews of this subject (Kravitz and Fernandez, 2015).
Fernandez, Maria de la Paz, 2015; Zwarts et al., 2012). Many of these systems show functional conservation across species in context of aggression (Yanowitch and Coccaro, 2011). Here, I will attempt to briefly highlight the role of octopaminergic (OA) system in this context.

The role of biogenic amines, including OA, in Drosophila aggression was first reported in 2002 by Baier and co-workers (Baier et al., 2002). Since then, a number of different studies from our lab and others have examined the role of OA in socially naïve and experienced flies. While many of these studies use different protocols and scoring schemes thereby making direct comparisons difficult; in general, inhibition of OA signaling correlates with reduced aggression and lunge frequency (Baier et al., 2002; Zhou et al., 2008; Certel et al., 2007; Hoyer et al., 2008). Absence of OA in $T\beta h^{M18}$ mutants that lack tyramine $\beta$-hydroxylase ($T\beta H$) – the rate limiting enzyme in OA biosynthesis – has been reported to cause a delay in onset to aggression as well as an overall decrease in lunging, holding, boxing and tussling behaviors (Baier et al., 2002; Zhou et al., 2008; Certel et al., 2007; Hoyer et al., 2008). In contrast, pharmacological stimulation of OA signaling and neuronal activation of OA-neurons restores aggression in OA-null ($T\beta h^{M18}$) mutants. A distinct subset of ~2-5 OA neurons in the SOG area of the posterior brain is critical for such rescue in $T\beta h^{M18}$ males (Zhou et al., 2008).

Furthermore, such enhanced OA signaling only increases aggression in socially experienced males, and not in socially naïve males (Zhou et al., 2008; Certel et al., 2010). That is, OA system may not only mediate expression of innate behaviors but also facilitate modulation of such canonical behavioral sequences in an experience-dependent manner. Such modulation hints at interactions between OA systems and mushroom bodies – the primary centers for learning and memory and modality integration in Drosophila. In fact, blocking the synaptic output from mushroom bodies (MB) result in complete abolition of aggressive behaviors (Baier et al., 2002), and OAMB-receptor neurons in the MB respond robustly to male-specific, aggression-mediating pheromone cis-vaccenyl acetate (cVA) (Zhou et al., 2012; Datta et al., 2008).
OA exhibits multilayered effects in wiring and regulation of circuitry and sensorimotor programs associated with aggression and reproductive behaviors. For instance, a subset of OA neurons may act as second order transducers of chemosensory information required for species and sex identification (see section 1.6.3). OA also acts as a key mediator in transmitting effects of sleep deprivation on aggressiveness in *Drosophila* (Kayser et al., 2015). Sleep deprived males display reduction in aggression and reduced reproductive fitness – both rescued by pharmacological administration of OA agonists (Kayser et al., 2015). Additionally, OA signaling plays a critical role in transmitting behavioral effects of *Wolbachia* infection in *Drosophila* brain; which significantly reduces total OA levels and initiation of aggressive encounters in males by down-regulating the expression of two key OA biosynthetic genes – *tdc2* and *Tβh* (Rohrscheib et al., 2015).

### 1.6.2 Courtship

OA system has also been implicated in the regulation of male courtship behaviors. Like aggression, courtship behaviors in *Drosophila* are innate, modular, sequential and dynamically-modulated (fig 1.5).

Within the aggression paradigm, OA-null (*Tβh*<sup>M18</sup>) and OA-hypomorphic (*Tβh*<sup>M1F372</sup>) males increasingly transition to courting the other male, instead of fighting and spend significantly greater time in male-male courtship compared to control pairs (Certel et al., 2010). Certel *et al* (2010) identified a small subset of OA neurons (two neurons in the VUM1 cluster...)

*Figure 0.5: Stereotypical courtship sequences in Drosophila* (steps 1-6); and the timing of *fruM*-mediated determination of sexually-dimorphic courtship circuitry during development (Source: Yamamoto et al., 2014)
and one in VUM2 cluster; VUM: ventral unpaired median) in the SOG area that co-express the male form of fruitless (fruM) – a key component of sex-determination pathway that specifies the sex-specific courtship circuitry in Drosophila (Certel et al., 2010). Selective feminization of OA neurons by turning on the transformer (tra) – a female-determinant gene upstream of fruitless in sex-determination pathway (Salz, 2011) – also recapitulates the homosexual courtship phenotype observed in OA-null males (Certel et al., 2010).

Not unlike aggression, multiple lines of evidence suggest that social-experience can override and modify the innate stereotypical and sequential behaviors within the courtship program (Siegel and Hall, 1979; Siwicki et al., 2005); and octopamine plays a role in that as well (Chartove et al., 2015). When Drosophila males are rejected by previously mated and unreceptive females, sexual rejection often leads to associative learning in the form of suppression of future courtship attempts even when paired with receptive, virgin females (Siegel and Hall, 1979; Kamyshev et al., 1999). The clues about mechanistic underpinnings of such associative social learning are found in sexually dimorphic pheromonal profiles. In Drosophila males, 9-pentacosene (9-P) acts as an aphrodisiac signal, whereas 11-cis-vaccenyl acetate (cVA) act as an anti-aphrodisiac signal (Jallon et al., 1981). Mating results in alteration of female pheromonal profile and mated females begin to display male-specific volatile pheromone cVA (Ejima et al., 2007; Ejima, 2015). During courtship conditioning, males learn to associate 9-P aphrodisiac signal (CS) released by all females with the suppression effects of rejection behavior (US) and possibly with anti-aphrodisiac cVA (US) displayed by mated females (Siwicki et al., 2005; Ejima et al., 2007). Removal of OA (TβhM18) or inactivation of OA neurons impairs courtship conditioning whereas transient activation of OA neurons in TβhM18 males mimics the aversive effects of courtship conditioning rescuing the OA-null phenotype (Zhou et al., 2012). This process is mediated by OA transmission to OAMB-expressing Kenyon cells that send projections to αβ lobes of the mushroom bodies (MB) (Zhou et al., 2012). Interestingly, however, induced-octopamine release during courtship training in non-OA-deficient lines also mitigates the effects of rejection or impairs courtship conditioning, suggesting a dose-dependent effect of OA on courtship memory (Chartove et al., 2015).
1.6.3 Consolidation of Behavioral Object Choice

An impaired OA signaling results in enhanced uncertainty in decision-making between aggression and courtship behaviors (Certel et al., 2007). A recent study from our group demonstrated that OA neurons facilitate context-dependent decision-making by downstream processing of chemosensory information relayed by gustatory Gr32a neurons (Andrews et al., 2014). These foreleg neurons gather pheromonal information by tapping the female abdominal wall early during the courtship and relay this information via axonal projections to the OA neurons in the suboesophageal ganglion (SOG) (Andrews et al., 2014; Miyamoto and Amrein, 2008; Stocker, 1994). These chemosensory cues are subsequently integrated with the inputs from acoustic, visual and mechanosensory modalities and a decision is made with respect to the modulation of male behavioral choice (Krstic et al., 2009; Griffith and Ejima, 2009). These observations suggest a role for OA in coordination of sensory information in male behavioral choice in complex social interactions.

Alternatively, it has been suggested that male-female courtship specificity and avoidance of male-male courtship is a learned phenomenon (Anaka et al., 2008). Under this framework, males learn to refrain from male-male courtship after experiencing antiaphrodisiac pheromones and rejection from other males (Anaka et al., 2008; Spieth, 1974; Hirsch and Tompkins, 1994). Context-inappropriate behaviors such as homosexual courtship or reduced sex specificity in courtship attempts may, therefore, suggest learning deficits in addition to, or in exclusion of, difficulties in gender recognition. A number of mutants with learning-deficits also display male-male courtship (Anaka et al., 2008; McRobert et al., 2003; Savvateeva et al., 2000). As OA is involved in the formation of courtship memory (Zhou et al., 2012; Chartove et al., 2015), it may therefore also facilitate specification of context-appropriate behaviors through learning and memory of previous social experiences in addition to its role in species and sex recognition.
1.7 Octopamine in Vertebrates

All three isomers of OA are found in the vertebrate systems, albeit only in trace amounts. However, since no specific OA receptor has yet been detected in vertebrates, most of the effects of OA in mammalian systems are considered indirect “false transmitter” effects because of OA-mediated displacement and release of other classical amines from storage vesicles (Farooqui, 2012; Borowsky et al., 2001). Interestingly, however, trace amines including OA have been implicated in a number of psychiatric disorders including depression, migraine, and schizophrenia in humans (D’andrea et al., 2006; Lindemann and Hoener, 2005; Berry, 2007). In 2001, a novel family of mammalian GPCRs called trace amine associated receptors (TAAR1) was identified that bind and respond to an array of agonists, including OA (Borowsky et al., 2001; Xie and Miller, 2008). TAAR1 receptors are distinct from invertebrate OA/TA receptors and are expressed in adrenergic and dopaminergic brain nuclei (Xie et al., 2007; Lindemann et al., 2008). Interested readers can refer to Miller G., 2012 (Miller, 2012) for a more comprehensive review of distribution and function of TAAR1 receptors. In 2012, D’Andrea and co-workers reported OA-mediated modulation of nitric oxide (NO) production in rat astroglial cells through β2-adrenoceptors (D’Andrea et al., 2012). If OA binding and functional activity through β2-adrenoceptors in mammalian systems is further substantiated, this will likely mark a paradigm shift in the way trace amines like OA are viewed in terms of their physiological role in vertebrates.
Cytosine methylation ($m^5C$) is a key process in the spatiotemporal regulation of gene expression (see footnote\(^1\)). However, DNA methylation has had a bit of a controversial history in *Drosophila*. DNA methylation is phylogenetically highly variable (Jeltsch, 2010). All examined land plants and vertebrates retain extensive DNA methylation and presence of *de novo* DNA methyltransferases (fig 1.6) (Jeltsch, 2010; Goll and Bestor, 2005; Suzuki and Bird, 2008).

While many invertebrates including representatives of molluscs, cnidarians, and echinoderms exhibit stable methylation patterns through different stages of development, presence or absence of methylation in many other species, however, including *C. elegans*\(^2\), *Drosophila*, and yeast remained inconclusive for decades (Tweedie et al., 1997; Rae and Steele, 1979; Bird et al., 1979).

After serving as a textbook example of organisms that are free of methylation for decades (Rae and Steele, 1979; Urieli-Shoval et al., 1982; Patel and Gopinathan, 1987), genomic methylation was conclusively detected in *Drosophila* embryos in the year 2000 by bisulphite-based sequencing methods (Lyko et al., 2000). Methylation was found to be enriched primarily during early embryonic stages (0.4% in 1-2hr old embryos) with

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\(^1\) While 5C-methylation is predominant form of methylation in vertebrates, a number of protists, bacteria, and lower eukaryotes contain methyl-groups at the 4\(^{th}\) position of cytosine (m4C), and more frequently at the 6\(^{th}\) position of adenine residues (N6A) (Wion and Casadesús, 2006). N6A-methylation plays a key role in methylation-sensitive restriction-digestion based bacterial defense systems. Recently, however, 6A-methylation was also discovered in *Drosophila* (Zhang et al., 2015) where it is proposed to act as an epigenetic modifier.

\(^2\) N\(^6\)A methylation was also recently detected in *C. elegans* (Greer et al., 2015) although cytosine methylation has not yet been determined.
gradual reduction during later stages (0.1% in 15–16 h old embryos; see footnote ³) (Lyko et al., 2000). However, no methylation was detected in the adult genome (but see Achwal et al., 1984). As a result, the general understanding was that adult *Drosophila* genome lacks detectable m⁵C and methylation is restricted primarily to the embryonic stages. That line of thinking was contradicted after more than a decade when an estimated 2 x 10⁴ methylated cytosine bases were conclusively detected in adult *Drosophila* genome using highly sensitive liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) based methods (Capuano et al., 2014). This level of methylation represents only ~0.034% of the fly genome (below the threshold of earlier bisulphite based methods); in contrast, 7.6% of mice genome and 2.3% of *E.coli* genome is methylated (Capuano et al., 2014). In contrast to global distribution of methylation in vertebrate genomes (Tweedie et al., 1997), methylation in *Drosophila* is typical of fractional distribution in invertebrates, albeit towards the lower end of the spectrum. Despite relatively sparse distribution, ⁵C-methylation in *Drosophila* is associated with at least 23% reduction in the expression of transcription factors and anatomical structure development genes suggesting functional equivalence with mammalian cytosine methylation (Takayama et al., 2014).

Another peculiar feature of methylation in *Drosophila* is selective enrichment on non-CpG motifs, particularly CpT and CpA dinucleotides (Lyko et al., 2000). Non-CpG (CpH; H = A/C/T) methylation, however, is by no means unique to *Drosophila*. CpH methylation has been reported in mammalian systems including the human brain, adult mouse cortex, and dentate gyrus neurons (Lister et al., 2013; Varley et al., 2013; Guo et al., 2013). Mice dentate gyrus neurons contain as much as 25% of overall methylation on CpH dinucleotides (Guo et al., 2013). In context of MBD-function, there are indications that CpH methylation is just as relevant to MeCP2 function and regulation of gene expression as methylation in CpG context. Methylated CpH moieties are associated with the repression of gene expression in cultured neurons and show binding to MeCP2 both

³ Adenine methylation (N6A) also exhibits high levels of enrichment during early embryonic stages and undergoes a strong reduction during subsequent stages of development (45 min old embryo: ~0.07%, 6mA/dA; 4-16hr old embryo: ~0.001%, 6mA/dA) (Zhang et al., 2015).
in vitro and in vivo (Guo et al., 2013). One of the notable findings pertains to the
concurrent emergence of neuronal CpH methylation and postnatal onset of Rett syndrome
(Guo et al., 2013). In this context, Drosophila is especially relevant to the investigation of
CpH-mediated functional interactions with MBD–containing proteins.

1.9 Methyl-CpG Binding Proteins
As a result of the recent confirmation of cytosine (and adenine) methylation in
Drosophila, the focus has once again shifted to the functional relevance of such sparsely
distributed methylation tags; and the role, if any, endogenous methyl-CpG binding
(MBD) proteins play in translating these epigenetic marks to appropriate functional
states. Proteins containing a methyl-CpG-binding domain (MBD) bind methylated DNA
and translate the methylation pattern information into appropriate cellular differentiation
states through alterations in chromatin structure and assembly. The correct readout of
epigenetic marks is of particular importance in the nervous system where abnormal
expression or compromised MBD protein function, can lead to disease and
developmental disorders.

Many of these proteins exert these effects in a methylation-dependent manner.
However, not all methyl binding proteins contain a canonical methyl-CpG binding
domain (MBD), and not all MBD-containing proteins have been identified to interact
directly with the methylated DNA. As a result, based on their constituent domain
structures and motifs, methyl binding proteins can broadly be categorized into 3 major
super-families (Hung and Shen, 2003; Parry and Clarke, 2011):

a) MBD containing proteins (e.g. MeCP2),
b) Methyl-CpG binding zinc-finger proteins (e.g. Kaiso), and
c) SET and RING finger–Associated domain (SRA) – containing proteins.

The mCpG-binding zinc-finger proteins and SRA-containing proteins vary significantly
from the MBD-containing proteins in their structural properties and binding affinities for
methylated DNA. For instance, Kaiso zinc-finger proteins can bind a pair of methylated
CpG dinucleotides (mCGmCG) and with even greater affinity – unmethylated DNA
(Daniel et al., 2002). The SRA-containing proteins, on the other hand, bind hemi-
methylated DNA through a base-flipping mechanism (Arita et al., 2008) while the MBD
domain of MeCP2 binds hydrated surface (and not the methylated cytosines per se) of
symmetrically methylated CpG pairs (Ho et al., 2008). This dissertation primarily focuses
on the category-I MBD-containing proteins of the MeCP2-type.

At the time of writing this dissertation, the UniProtKB/Swiss-Prot release
2015_12 contains at least 43 MBD-containing proteins from a number of different
species including Arabidopsis, C. elegans, D. melanogaster and pseudoobscura,
Xenopus, mice, rats, chicken, macaques, and humans. Based on their composition and
presence of additional domains, the MBD superfamily of proteins is classified into three
subsequent categories:

a) MBD_MeCP2
b) Histone methyltransferases (HMT_MBD)
c) Histone acetyltransferases (HAT_MBD)

The HMT family of MBD proteins includes SETDB1 and SETDB2 lysine-methyl
transferases that are involved in tri-methylation of H3K9 – a key histone modification
associated with formation of heterochromatin (Völkel and Angrand, 2007). These
proteins contain SET domains – named after Drosophila genes Su(Var)3-9, Enhancer of
zeste E(z), and trithorax (trx) – in addition to the methyl-binding domain (Clough et al.,
2007). The HAT family of MBD proteins includes BAZ2A and BAZ2B histone
acetyltransferases (see footnote4). These are characterized by the presence of PHD-type
zinc-finger domains and bromodomain that associate with acetylated lysine and
chromatin remodeling complexes such as nucleolar remodeling complex (NoRC) (Hung
and Shen, 2003; Dhalluin et al., 1999). Finally, the MeCP2_MBD family of proteins is
characterized by MeCP2 and MBD1-6 proteins illustrated in fig 1.7. The subsequent
chapters in this dissertation primarily concerns with the MeCP2_MBD family of proteins
where it is discussed at length.

4 Toutatis protein in Drosophila belongs to HAT category of MBD proteins and positively regulates expression of pro-
nearal genes (Vanolst et al., 2005).
Both HMT and HAT family of MBD proteins lack a “canonical” MBD domain characteristic of MeCP2 that binds methylated cytosine residues (Hung and Shen, 2003; Roloff et al., 2003; Hendrich and Tweedie, 2003). At the same time, presence of a canonical MBD-domain does not guarantee association with m$^5$Cs as many members of the MeCP2_MBD family do not bind methylated DNA (Hendrich and Tweedie, 2003; Laget et al., 2010). Therefore, one must exercise caution while contextualizing the observations related to Drosophila MBD proteins in subsequent chapters of this dissertation.
1.10 References


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2 CHAPTER II

FUNCTIONAL CONSERVATION OF MBD PROTEINS

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2.1 ABSTRACT

Methyl-CpG-binding domain (MBD) proteins are characterized by the ability to bind methylated DNA and translate the methylation pattern information into appropriate functional cellular states through alterations in chromatin structure and assembly. The correct readout of epigenetic marks is of particular importance in the nervous system where abnormal expression or compromised MBD protein function, can lead to disease and developmental disorders. Recent evidence confirms presence of ⁵C – and ⁶A – methylation across various developmental stages in Drosophila (Capuano et al., 2014; Zhang et al., 2015). As a result, the focus has once again shifted to the functional relevance of such sparsely distributed methylation tags; and the role, if any, endogenous MBD proteins play in translating these epigenetic marks to appropriate functional states. Are Drosophila MBD proteins required for neuronal function? Additionally, as MBD-containing proteins have diverged and evolved, does the MBD domain retain the molecular properties required for conserved cellular function across species?

To address these questions in a systematic manner, we started out by exploring the role of a better characterized human MBD-family protein – MeCP2 (methyl-CpG binding protein 2) in Drosophila. We expressed MeCP2 in distinct subsets of amine neurons and quantified alterations in sleep circuit output as an endpoint behavioral readout for spatiotemporally restricted functional interactions. MeCP2 gain-of-function resulted in phase-specific sleep loss and sleep fragmentation. Cell-type specific baseline behavioral data was then used to dissect domain-specific interactions by systematically removing specific domains from the full-length protein. Intact methyl-CpG binding (MBD) domain was found to be a critical player for MeCP2-induced alterations in sleep...
architecture. Partial truncation of transcription repression domain (TRD) and complete removal of C-terminal regions (CTD) did not rescue MeCP2 gain-of-function phenotype.

Subsequently, we explored the role of the MBD-family proteins endogenous to *Drosophila* i.e. dMBD-2/3 and dMBD-R2. To examine if human MeCP2 and *Drosophila* MBD proteins are targeting common neuronal functions, we knocked-down dMBD levels in conjunction with hMeCP2 overexpression in a 2X2 factorial design. A significant interaction (dMBD × hMeCP2) effect was observed between relative dMBD and hMeCP2 expression on combined measures of sleep. Chromosomal binding experiments indicate dMBD-R2 and MeCP2 localize on a small set of shared genomic loci. Our results demonstrate that *Drosophila* MBD-containing family members are required for neuronal function and suggest the MBD domain retains considerable functional conservation at the whole organism level across species.

**Keywords:** methyl-CpG Binding Protein 2 (MeCP2), MBD proteins, *Drosophila*, sleep, octopamine, methylation
2.2 INTRODUCTION

Gene expression and even more fundamentally, chromatin architecture, is controlled by a number of different chemical modifications to the DNA and histone proteins. In plants, vertebrates and more recently *Drosophila*, one of these key modifications is an added methyl group at position 5 of cytosine bases (5mC) (Capuano et al., 2014, Gehring, 2013, Schubeler, 2015, Takayama et al., 2014, Varriale, 2014, Zilberman, 2008). Most methyl-CpG binding domain (MBD)-containing proteins bind methylated DNA and function to translate the chemical modification into appropriate cellular states (Bogdanovic and Veenstra, 2009, Fatemi and Wade, 2006, Sasai and Defossez, 2009). By interacting with diverse partners, MBD-containing proteins regulate the differentiation and function of a cell by maintaining or altering chromatin structure, interpreting genomic imprinting, gene-specific transcriptional activation/repression and controlling RNA splicing (Chahrour and Zoghbi, 2007, Lyst and Bird, 2015, Samaco and Neul, 2011). Due to this wide array of nuclear functions, MBD-containing proteins and in particular, the MBD family member, methyl-CpG-binding protein 2 (MeCP2), have been described as a genome-wide modulator of gene expression and cellular differentiation (Cohen et al., 2011, Della Ragione et al., 2012, Skene et al., 2010, Yasui et al., 2013). Alterations in MeCP2 levels, either through loss-of-function mutations or gene duplication, results in the postnatal neurodevelopmental disorders, Rett Syndrome (RTT) and MeCP2 duplication syndrome. MeCP2 dysregulation is also an important component of neuropsychiatric and neurological disorders ranging from Alzheimer’s and Huntington’s to depression and drug addiction (Ausio et al., 2014, Hutchinson et al., 2012, Lv et al., 2013, Ramocki et al., 2009, Zimmermann et al., 2015).

Despite the proposed global nature of its nuclear function, MeCP2 expression is tightly regulated in a spatiotemporal manner. In the adult nervous system where MeCP2 can be found at levels nearly as abundant as the histone octamer, MeCP2 immunoreactivity can differ between brain regions as well as among neurons of the same population (LaSalle et al., 2001; Shahbazian et al., 2002). Furthermore, MeCP2 expression is regulated by the circadian clock resulting in diurnal oscillations in MeCP2 function (Martinez de Paz et al., 2015). However, in a laboratory setting, many of the
existing set of assays used for examining functional consequences of MeCP2
dysregulation only provide a brief snapshot in the temporal order of functional
interactions. A more comprehensive characterization framework necessitates accounting
for temporal variability in function through various circadian and developmental phases.
That is, characterization of cell-type and domain-specific interactions of MBD proteins
and their relationship with the overall circuit output requires assaying a phenotype that is
rigorously quantifiable through various temporal phases in defined subsets of cells over
the course of an organisms’ life in a high-throughput manner. Therefore, we used
continuous sleep-wake profiling methods for temporal assessment of MBD function.

Sleep is also a relevant behavior at the molecular and phenotypic levels in terms
of MeCP2 pathophysiology. One prevalent phenotype among children with alterations in
MeCP2 function and a common feature of neurodegenerative disease and
neuropsychiatric disorders is sleep abnormalities (Angriman et al., 2015, Kakkar and
Dahiya, 2015, McCarthy and Welsh, 2012, Musiek et al., 2015). Such sleep impairments
include delays in the onset of sleep, alterations in total sleep duration, and frequent bouts
of waking resulting in a fragmented sleep pattern (Cortesi et al., 2010, Nomura, 2005,
Piazza et al., 1990, Souders et al., 2009, Young et al., 2007). Furthermore, it has become
increasingly clear that epigenetic factors play fundamental roles in transcriptional and
post-transcriptional regulation within the circadian clock network (Liu and Chung, 2015,
Qureshi and Mehler, 2014). For example, in mice changes in day length alters promoter
DNA methylation within the suprachiasmatic nucleus (SCN) – the master circadian
oscillator (Azzi et al., 2014); an observation also supported in humans, where
methylation levels have been observed to display 24-hr rhythmicity (Angriman et al.,
2015, Kakkar and Dahiya, 2015). In Drosophila, diurnal oscillations of several non-
coding RNAs are regulated by the clock gene, period (Hughes et al., 2012). In mice, two
miRNAs – miR134 and miR132 – have been implicated in circadian regulation; one of
which – miR134 – is highly enriched in the brain and processed under the control of
MeCP2 (Alvarez-Saavedra et al., 2011, Cheng et al., 2014, Gao et al., 2010).

Sleep and arousal are regulated by multiple neurotransmitters including
octopamine, dopamine, γ-aminobutyric acid (GABA), and serotonin (5HT) through
different but interacting circuits (Cirelli, 2009, Crocker and Sehgal, 2010, Potdar and Sheeba, 2013). Therefore, we manipulated distinct subsets of aminergic neurons through a series of experiments and asked, if the functional output of these neurons is altered in a distinct, quantifiable manner. Our results indicate cell-type-specific and phase-specific alterations in sleep duration and architecture. Sleep-deficits were accompanied with a significant reduction in latency to sleep initiation suggesting an increased homeostatic drive for recovery of lost sleep. To separate the role of disrupted amine production from disrupted neuron function, we expressed MeCP2 in OA neurons that completely lacked OA and established that MeCP2-induced deficits in nighttime sleep are mediated, at least partly, in an OA dependent manner. Partial truncation of transcription repression domain (TRD) and removal of C-terminal domains (CTDα & CTDβ) could not rescue MeCP2-induced alterations in sleep-wake patterns. However, males expressing hMeCP2Δ166 allele, in which the N-terminal region (NTD) and methyl-CpG binding domain (MBD) are truncated, displayed no alterations in quality or duration of sleep. These observations suggest an integral role for MBD in MeCP2 functional interactions.

Second, as the *Drosophila* genome contains two proteins with extended homologies to vertebrate MBD family members; and in consideration of the recent confirmation of cytosine methylation in *Drosophila*, we asked if reducing endogenous dMBD2/3 and dMBD-R2 proteins could also alter the function of OA neurons. As with hMeCP2 expression, targeted knockdown of dMBD2/3 and dMBD-R2 in OA neurons caused sleep fragmentation. If OA neuron function is altered due to the targeting of similar or overlapping set of genomic targets by hMeCP2 and the endogenous MBD proteins, then reducing dMBD2/3 or dMBD-R2 in conjunction with hMeCP2 expression should suppress or reduce the severity of hMeCP2-mediated sleep deficits. Our results indicate the phase-specific sleep deficits that occur due to hMeCP2 are partially rescued with a concomitant reduction in MBD-R2. Finally, we labeled 3rd instar larval polytene chromosomes and found that hMeCP2 and MBD-R2 accumulate together at distinct chromosomal bands. Taken together, our results demonstrate that *Drosophila* MBD-proteins can alter neuron output suggesting functional conservation of MBD proteins across species.
2.3 MATERIALS AND METHODS

2.3.1 Drosophila Stocks:

Canton-S, UAS-Red Stinger (BL 8545, BL 8546), UAS-mCD8:GFP (BL 5130), UAS-MBD-R2-IR (BL 30481) and UAS-dMBD2/3-IR (BL 35347) were obtained from the Bloomington Stock Center (Bloomington, IN). The UAS-MeCP2, UAS-MeCP2^R294X, UAS-MeCP2^R106W, and UAS-MeCP2^A166 lines were generously provided by Juan Botas (Cukier et al., 2008). dTdc2-Gal4 was obtained from Jay Hirsh (Cole et al., 2005), th-Gal4 was provided by Sirge Birman (Friggi-Grelin et al., 2003), and trh-Gal4 was a gift from OlgaAleksenko (Alekseyenko et al., 2010).

2.3.2 Husbandry:

All fly stocks were maintained in a temperature (25 °C) and humidity-controlled (~50%) environment on a standard cornmeal based medium (agar, cornmeal, sugar, yeast extract, Triton-X). During development and post-eclosion, all flies were entrained to standard 12hr-12hr light:dark (L:D) conditions under 1400 ± 200 lx fluorescent light intensity.

Transgenic control males were generated by crossing Canton S females with males from the respective UAS- or gal4- lines. Before experimentation, male pupae were isolated and aged individually in 16X100mm borosilicate glass tubes containing standard food medium described above.

2.3.3 Behavioral Analysis:

For activity and sleep monitoring, 2-3 day old socially naive males were transferred to 65x5mm glass tubes with 15mm food on one end and a cotton plug on the other. Flies were transferred under CO₂ anesthesia and allowed 24-hr to recuperate and acclimatize to new housing conditions before data collection. The locomotor activity counts were recorded for both control and experimental males using Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) for a period of 10 consecutive days at 1-min bin acquisition mode. Count data for the first and the last day were truncated to remove mechanical noise. Data from 8 consecutive days was analyzed further using Counting Macro 5.19.5 (CM) program generously provided by R. Allada (Northwestern University, Evanston, IL). Various indices of sleep including temporal organization, duration and latency of sleep and the number and length of sleep bouts were analyzed as described.
Sleep was defined as complete inactivity for a period of 5 consecutive minutes (Shaw et al., 2000). Graphs were generated with Graphpad Prism and Adobe Illustrator CS5.

### 2.3.4 Immunohistochemistry and imaging:

Adult male brains were dissected and fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 40 minutes and labeled as described previously (Certel et al., 2010). The following primary antibodies were used: rabbit anti-MeCP2 (1:30, Cell Signaling Technologies), mouse anti-MeCP2 (1:500, Abcam), rat anti-CD8 (1:100, Molecular Probes), monoclonal rabbit anti-GFP (1:200, Molecular Probes), mouse nc82 (1:100) and anti-MBD-R2 (1:200) (Prestel et al., 2010). Secondary antibodies include Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 594-conjugated goat anti-rabbit, Alexa Fluor 647-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated goat anti-rat cross-adsorbed antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Brain samples were mounted in a drop of Vectashield™ (Vector Laboratories Inc, Burlingame, CA) and Images were collected on an Olympus Fluoview FV1000 laser scanning confocal mounted on an inverted IX81 microscope and processed with Image-J 1.33 (NIH) and Adobe Photoshop (Adobe, CA).

### 2.3.5 Polytene Chromosome Immunofluorescence:

For *Drosophila* polytene chromosomal preparation and immunofluorescence, third instar larvae raised at raised at 25°C and dissected in 0.1% Triton X-100 solution in phosphate buffer saline (PBS). Salivary glands were placed in 250µm of solution 2 (3.7% paraformaldehyde, 1% Triton X-100 in PBS) for 30-45 seconds. Solution 2 was replaced with solution 3 (3.7% paraformaldehyde, 50% acetic acid) for another 2 minutes. Salivary glands were pipetted along with 20µl of solution 3 on siliconised glass cover slips and picked up onto a poly-L-lysine coated slide (Sigma), tapped to aid chromosomal spreading and frozen in liquid nitrogen. Cover slips were removed and slides were processed for IF as described previously (Capelson et al., 2010). Mouse α-MeCP2 was used at 1:100 and rabbit anti-dMBDR2 at 1:200 (a gift from Dr. Peter Becker). Secondary antibodies include Alexa Fluor 594-conjugated goat anti-rabbit and Alexa Fluor 647-conjugated donkey anti-mouse for spectral non-overlap with DAPI (1µg/ml) which was
used as a DNA counterstain. Polytenic samples were mounted in a drop of Vectashield™
and imaged as described previously. Images were processed for background subtraction
and contrast enhancement with contrast-limited adaptive histogram equalization
(CLAHE) in ImageJ. Theoretical PSF (point spread function) was calculated for images
used for colocalization analysis followed by an iterative 2D deconvolution for each
channel (macro code and algorithm parameters are available upon request). Pearson’s
correlation coefficient (PCC) and Manders colocalization coefficient (MCC) were
estimated and then PCC was statistically evaluated against randomized images using
Costes’ randomization methods (Costes et al., 2004). Percentile based thresholding was
applied to segment polytenic chromosomes from the background for MCC calculations
within the JaCoP plugin for ImageJ.

### 2.3.6 RT-qPCR:

Expression levels of dMBD2/3 and dMBD-R2 genes were measured quantitatively by
RT-qPCR. Heads from socially naive 3-5 day old adult males from control and
experimental groups were extracted under CO2 anesthesia and frozen immediately in sets
of three in 1.5-ml Eppendorf tubes kept in dry ice. Total RNA from each pool (~35 heads
/pool) was isolated by Tri-Reagent, (Molecular Research Center, Cincinnati, OH). RNA
samples were DNase treated and reverse transcribed as described previously (Hess-
Homeier et al, 2014). qPCR reactions were carried out in quadruplicate for each gene and
genotype on an Agilent Stratagene Mx3005P platform using following thermal protocol:
95°C – 10min; 40 X (95°C – 30sec; 53°C – 1min; 72°C – 1min) followed by 0.5°C
stepwise increment from 65°C to 95°C. Cdc2c (cyclin-dependent kinase 2) reference
gene was used for data normalization. Expression levels were calculated using the ΔCT
method. dMBD-R2 expression was quantified from the total head RNA using following
primer pair, with forward primer spanning exon2-exon3 junction: F: 5’-
GGCCAGTTTGGATATAGCATCCC-3’, and R: 5’-
GCACGATAACAGTGGGTTCCTGG-3’. For dMBD2/3, exon-exon junction primers
were not designed in order to target all transcript variants. Following primers were used
for dMBD2/3: F: 5’-AGAAGCGACTGGAACTAC-3’ and R: 5’-
CGGTCTGTCGTCATTGGG-3’. For cdc2c reference gene, pre-designed exon-
spanning primer pair PP1255 was used from the *FlyPrimerBank*:

F: 5′-CGAGGGCACCTACGTTATAGT-3′
R: 5′-CGCCTTCTAGCCGAATCTTTTTG-3′.

### 2.3.7 HPLC:

For HPLC analysis, brains from socially naive 3-5-day old adult males from control and experimental groups were dissected in ice-cold PBS (137 mM NaCl/2.7 mM KCl/10 mM Na2HPO4/1.8 mM KH2PO4, pH 7.4) and frozen immediately in sets of three in 1.5-ml Eppendorf tubes at -20°C. To measure OA levels from the central brain, the photoreceptors were removed in all dissections. Each pool (n=15) of brains were homogenized in 150µL of ice-cold 0.05M perchloric acid containing 30 ng/mL DBA and chilled on ice before analysis. Immediately before analysis, the samples were centrifuged at 14,100g for 20 min at 4°C. The supernatant was removed and 50µL injected into the HPLC. Amine levels were measured with an ESA CoulArray Model 5600A HPLC with electrochemical detection equipped with a C18 column (Varian), and a 200µl loop (Rheodyne). The flow rate was set at 0.8 ml/min. The mobile phase was composed of 10% acetonitrile (Fisher, HPLC grade), 14.18g monochloroacetic acid, 4.80g NaOH (pH adjusted to 3.0-3.5 with glacial acetic acid), and 0.301g sodium octyl sulfate (SOS) in 1000mL of sterile, polished water and filtered with 0.2µm filter. The electrodes were set at -50, 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 920 mV. OA was detected in the 600-mV channel. Retention times and concentrations of the amines were determined by comparison to a standard composed of 80, 160, 320, 800, and 1200pg of octopamine hydrochloride in 0.1 M perchloric acid containing 30ng/mL DBA. The data from three groups of pooled males (n=15 in each pool) were averaged. Peaks were identified based on elution times.

### 2.3.8 Statistical Analysis:

One-way ANOVA with Holm-Sidak's multiple comparisons test was used to evaluate effects of genotype on various sleep parameters in three or more groups. Multiplicity-adjusted p-values are obtained for each pairwise comparison and only the most conservative/numerically higher values were reported. Data was examined for gaussian distribution and homogeneity of variance using D'Augustino Pearson omnibus normality
test and Brown-Forsythe test respectively. Data were log-transformed or central limit theorem was assumed for datasets with n>30 in case of violations of assumptions of normality. Otherwise, non-parametric Kruskal-Wallis with Dunn’s post-hoc test was used. Generalized ESD test (Rosner, 1993) was used to examine outliers. Results are expressed as either mean±s.e.m. or mean±c.i. as indicated in the text. Empirical cumulative distribution (CDF) for sleep bouts were plotted using the `ecdf` function in MATLAB (The MathWorks, Natick, MA).

Ordinary two-way Multivariate ANOVA (MANOVA) was carried out in SPSS23 using the general linear model (GLM) procedure to explore interactions between the effect of hMeCP2 and dMBDs on linear composite of various measures of sleep. Multivariate outliers were detected for all sleep parameters based on a chi-square distribution using Mahalanobis distance (MD). Cases with MD>18.47 (critical $\chi^2$ value assessed at $p < .001$, df = 4) were identified as outliers and removed. Box-Cox transformed dependent variables (i.e. total sleep, waking activity, consolidation index, and number of sleep bouts) were auto-scaled for the purposes of scale standardization and univariate outliers were identified using $+3.0$ z-score criterion. Multi-collinearity was checked against the variance inflation factor (VIF; threshold=5). As our dataset contained an unbalanced design (unequal sample size across groups), and violated the assumption of homogeneity of covariance matrices, Pillais’ trace criterion (which is most robust to such violations) was reported. These results were cross-validated by employing a non-parametric or permutation MANOVA (NPMANOVA / PERMANOVA) in PASTv3.09 (Hammer et al., 2001) which is insensitive to such violations (Anderson, 2001).

### 2.3.9 Homology modeling:

The SWISS-MODEL template library (SMTL version 2015-04-15, PDB release 2015-04-17) was searched with Blast (Altschul et al., 1997) and HHBlits (Remmert et al., 2012) for evolutionary related structures matching the target MBD amino acid sequence for both MBD-R2 and MBD2/3. The templates with the highest quality predicted from features of the target-template alignment were then selected for model building. Models were built based on the target-template alignment using Modeller (Sali and Blundell, 1993) within the UCSF Chimera package (Pettersen et al., 2004). The model
quality/reliability was assessed using the z-DOPE (Shen and Sali, 2006) and GA341
(Melo et al., 2002) scoring functions through ModEval Model Evaluation Server
(http://modbase.compbio.ucsf.edu/evaluation/).
2.4 Results

2.4.1 MeCP2 expression in OA neurons results in reduced and fragmented sleep patterns

Examining sleep output in fruit flies provides an ideal paradigm for investigating the role of MBD proteins in neuronal function for several reasons. First, numerous behavioral parameters can be quantified in a large cohort of genetically identical control and experimental populations (Bellen et al., 2010, Venken and Bellen, 2014). Second, behavioral output can be measured at the single minute level, which provides a formidable temporal resolution of function, and finally this functional output is responsive to changing environmental stimuli thus requiring a dynamic readout of the neuronal nuclear state.

To determine if MeCP2 expression in distinct amine neurons can alter sleep-wake circuitry function, we used the Gal4-UAS gene expression system and previously generated UAS-hMeCP2 transgenic lines (Cukier et al., 2008). As norepinephrine and OA regulate sleep levels by promoting wakefulness (Crocker and Sehgal, 2008, Mitchell and Weinshenker, 2010, Robbins, 1997), we expressed hMeCP2 (the MeCP2e2 isoform) in OA/tyramine (TA) neurons via the tyrosine decarboxylase2 (tdc2)-gal4 driver (Cole et al., 2005) (fig 2.1a-a’) and quantified sleep-wake patterns, sleep onset, duration, and the quality of sleep over a 10-day period using a standard automated high-throughput activity monitoring system (Ho and Sehgal, 2005) (Drosophila Activity Monitor, Trikinetics, Waltham, MA).

Adult males expressing hMeCP2 in OA neurons exhibited specific deficits in sleep quantity and quality including a significant reduction in total sleep as compared to transgenic controls (tdc2-gal4/+, UAS-hMeCP2+/+) and the nuclear protein expression control (tdc2-Gal4;UAS-dsRed) (fig 2.1b). Further examination of sleep patterns indicated that these deficits spanned over roughly 6-8 hours (Zeitgeber hours ZT04-10 and ZT14.5-22) distributed through both day and night (fig 2.1c, d). A reduced propensity for an anticipated increase in activity was observed during light-dark transition hours (fig 2.1c). The reduction in the amount of sleep was accompanied with an increase in the number of sleep bouts (fig 2.1e) and a rather significant decrease in the consolidation
index (C.I.) suggesting altered sleep architecture (*fig 2.1f*). Consolidation index is a weighted measure of average bout length corrected for potential structural bias in data from unusually short bouts (Pfeiffenberger, 2010). This difficulty in maintaining sleep was also evident by plotting sleep bout data using the empirical cumulative distribution function (ECDF) (*fig 2.1g*). The ECDF demonstrates that MeCP2 gain-of-function in OA neurons shifts the temporal structure of sleep bouts to a more fragmented state. That is, longer consolidated bouts of sleep are replaced with a greater proportion of relatively shorter bouts of sleep in experimental males but not in controls. Experimental males also displayed a significant reduction in the latency to initiate sleep (*fig 2.1h*), suggesting the need for recovery after sleep loss and homeostatic relevance of the observed sleep deficits. This sleep loss induced by hMeCP2-expression in OA neurons did not shorten the average lifespan of the experimental males; on the contrary, the Kaplan-Meier survival plot indicated a modest increase in the median survival age (*fig 2.2*).

In addition to controlling for nuclear protein expression, we further verified the specificity of the sleep defects observed in *tdc2-gal4;UAS-hMeCP2* adults by asking if hMeCP2 expression in serotonin neurons would alter sleep architecture differently (*fig 2.3a*). While the overall amount of sleep was not changed (*fig 2.3b*), males expressing hMeCP2 in 5HT neurons via the *tryptophan hydroxylase (trh)-Gal4* line (Alekseyenko et al., 2010) did exhibit sleep loss similar to hMeCP2 effects in OA neurons towards the latter hours of the dark phase (ZT19-22.5; *fig 2.3 c, d*). However, the nighttime sleep deficits caused by hMeCP2 expression in 5HT neurons were not accompanied by structural changes in measures of sleep quality such as consolidation index or average number of sleep bouts (*fig 2.3 e, f*). At the same time, significant structural alterations in sleep architecture were observed during the day with no concomitant changes in daytime sleep duration (*fig 2.3 c, e-f*). The conserved nighttime sleep reduction suggests that hMeCP2 expression may alter a specific aspect of sleep circuit that is shared by different aminergic neurons, yet other sleep impairments are cell-specific.
2.4.2 OA is required for a subset of MeCP2-mediated sleep deficits

Since MeCP2 overexpression in OA neurons resulted in relatively broad ranging effects on sleep duration and quality, we investigated if these effects are mediated through alterations in OA neurotransmitter function. Activation or suppression of OA-neuron activity or OA biosynthetic machinery results in diametrically opposite effects on sleep-wake behavior (Na et al., 2012). Increased expression of tyrosine decarboxylase 2 (tdc2) – a rate-limiting enzyme in OA biosynthetic pathway in neurons – results in a decrease in the amount of sleep. On the other hand disruption in OA biosynthetic pathway through mutations in tyramine β-hydroxylase (tβh) results in an increased duration of sleep (Crocker and Sehgal, 2008). Therefore, one possible explanation for this particular sleep deficit is that the expression of genes required for OA biosynthesis is altered by MeCP2 overexpression. To address this question, we quantified OA levels extracted from the heads of control and experimental males using High Performance Liquid Chromatography (HPLC). Heads were removed during the period of daytime sleep reduction, ZT04-10, to determine if the OA levels were altered. OA concentrations per head did not differ between control (tdc2-gal4/+; and UAS-hMeCP2/+) and experimental (tdc2-gal4;UAS-hMeCP2) males (fig 2.4a). Although we cannot rule out the possibility of OA level differences in specific neurons contributing to sleep deficits, these results demonstrate that a global reduction in OA production does not occur as a result of hMeCP2 expression in OA neurons.

Although hMeCP2 expression in OA neurons does not alter OA production, it is possible, however, that the observed sleep deficits require OA function. To test this possibility, we expressed hMeCP2 in flies that completely lack OA due to a null mutation in tyramine-β-hydroxylase (Tβh<sup>hM18</sup>), the rate-limiting enzyme in OA biosynthesis (Monastirioti et al., 1996). Not unlike wildtype males expressing hMeCP2, OA null males expressing hMeCP2 also exhibited hourly specificity in sleep reduction (fig 2.4b-d). However, the nighttime sleep deficit (ZT 14-17.5) quantified in figure 2.1 is completely rescued in hMeCP2-expressing males that lack OA (fig. 2.4 b, c). This result suggests OA is required to translate the hMeCP2-mediated neuronal defects into a reduction in nighttime sleep during specific hours. Not all hMeCP2-mediated sleep deficits, however,
rely on OA-neurotransmitter function, as alterations in the consolidation index and sleep
bout number (fig 2.4 e, f) were similar between hMeCP2-expressing males irrespective of
the presence or the absence of OA.

In contrast to the rescued dark phase sleep deficits, the daytime sleep reduction
observed during ZT04-10 in tdc2-Gal4;UAS-hMeCP2 adults persisted in males that lack
OA (fig 2.4c). A possible explanation for any sleep reduction is a concomitant increase in
activity. As Tβh converts tyramine (TA) to OA, the absence of this enzyme results in an
accumulation of TA (Monastirioti et al., 1996; Crocker and Sehgal, 2008). To determine
if the periods of sleep reduction observed in males lacking OA are due to elevated TA-
induced increases in locomotion rather than hMeCP2 expression (Hardie et al., 2007,
Monastirioti, 1999), we quantified the activity levels in these males. Changes in waking
activity were not observed in the absence of OA (fig 2.4g). Finally, hMeCP2 expression
in the nucleus of octopamine neurons may provide some protection against the OA
deficient circuit alterations as the increase in sleep observed in OA null males is returned
to control levels in the same males now expressing hMeCP2 (Tβh^{M18} tdc2-gal4;;UAS-
hMeCP2) (fig 2.4d, dark gray vs. yellow column).
2.4.3 The C-terminal region of hMeCP2 is not sufficient to generate sleep deficits in OA neurons

One approach to understanding the potential targets of multi-domain containing proteins is to link protein domain(s) with a corresponding phenotype. Therefore, we investigated which conserved domains are essential in generating the observed sleep impairments by expressing hMeCP2 alleles that lack the CTD and separately, the MBD (Cukier et al., 2008). Due to the relatively sparse distribution of 5mC methylation in Drosophila, we first postulated that hMeCP2 exerts its affects through methylation-independent interactions mediated by the C-terminal transcriptional repression domain (TRD) and the C-terminal domain (CTD). The TRD functions as a recruitment center for several transcriptional and epigenetic regulators including components of the transcription repression machinery such as Sin3a, HDAC1, and HDAC2 (Ghosh et al., 2010, Nan et al., 1998); while the CTD (residues 295 to 486) contains one or more chromatin binding regions (Ausio et al., 2014, Roloff et al., 2003). Together the TRD and CTD domains have been implicated in nucleosomal clustering, array compaction and oligomerization, and gene repression (Nikitina et al., 2007). To remove the C-terminus, we expressed the early truncating mutation encoded by the hMeCP2\(^{R294X}\) allele which is found in ~5-6% of RTT patients (Laccone et al., 2001, Wan et al., 1999). In the resulting R294X protein, the TRD is partially truncated and the CTD is completely removed (fig 2.5a) (Wan et al., 1999). The Gal4-driven protein expression of UAS-hMeCP2\(^{R294X}\) was previously verified by western blot analysis (Cukier et al., 2008).

If the sleep deficits observed in males expressing hMeCP2 in OA neurons were mediated through the C-terminus, we would predict sleep would be normal in males expressing hMeCP2\(^{R294X}\). However, removing TRD and CTD function, did not eliminate the daytime sleep reduction observed in tdc2-gal4;UAS-hMeCP2 males, and only a partial recovery in the nighttime sleep deficits occurred (ZT14.5-22, figure 2.5 b,c). Males expressing R294X exhibited a decrease in the latency to initiate sleep (fig 2.5d) and changes in sleep architecture (fig 2.5 e-g) in a manner similar in males expressing full-length hMeCP2. Specifically, the number of sleep bouts and weighted average bout lengths exhibited by tdc2-gal4;UAS-hMeCP2\(^{R294X}\) males remained significantly different.
than controls (fig 2.5 e,f). These results indicate that the hMeCP2-induced changes that drive sleep alterations in the OA neuronal population do not occur primarily through the CTD and TRD domains.

### 2.4.4 The N-terminus and MBD domain are necessary for MeCP2-induced alterations in sleep architecture

We next asked if the majority of the sleep deficits observed in tdc2-gal4;UAS-hMeCP2 males are due to the conserved MBD domain. To test this question, we used the UAS-hMeCP2Δ166 line to express a truncated hMeCP2 allele that lacks the N-terminal and MBD domain (Cukier et al., 2008) (fig 2.6 a,b). We found the sleep deficits caused by hMeCP2 expression including the amount of sleep, latency to sleep, sleep bout number, and sleep bout length were absent in tdc2-gal4;UAS-hMeCP2Δ166 males (fig 2.6 c-h). This lack of sleep defects could be explained if the Δ166 protein was not expressed, however we demonstrated hMeCP2Δ166 accumulates in the nucleus of tdc2-gal4;UAS-hMeCP2Δ166 adult brains by immunohistochemistry (fig 2.6 b). Also, previous studies demonstrated hMeCP2Δ166 localizes on distinct chromosomal bands along polytene chromosomes, phosphorylated at amino acid S423, and is able to cause Drosophila neuronal morphology and dendritic defects (Cukier et al., 2008, Vonhoff et al., 2012). However, in context of sleep, it completely rescues MeCP2-induced alterations in sleep duration and quality.

### 2.4.5 MeCP2-induced alterations in sleep output are dependent on the MBD domain

To determine if the MBD domain itself is required for the MeCP2-induced changes in sleep output, we expressed the severe RTT-causing missense hMeCP2R106W allele in which arginine is replaced with tryptophan at position 106. Arg106 is required for structural integrity of MBD as a part of select group of residues that comprise the hydrophobic core of wedge-shaped tertiary structure of MBD (Wakefield et al., 1999). Two β-sheet strands in MBD run parallel along the major groove of the DNA near methylated 5C and Arg106 lies in the middle of one of those β-sheets (Wakefield et al., 1999; Ballestar et al., 2000). The R106W mutation in the MBD domain alters the MBD secondary structure and impacts the MeCP2 protein by severely disrupting its ability to bind methylated DNA (~100-fold reduction); thereby, potentially altering target gene
repression and chromatin condensation (Chapleau et al., 2009; Kudo et al., 2001).

However, the methylation-independent binding remains intact (Bellestar et al, 2000; Yusufzai et al, 2000; but also see Nikitina et al., 2007 and Ghosh et al., 2008 for conflicting observations). In *Drosophila*, the R106W protein also localizes to specific sites on the polytene chromosomes, suggesting preservation of methylation-independent DNA binding activity (Cukier et al., 2008).

Males expressing hMeCP2^{R106W} in OA neurons (*tdc2-gal4;UAS-hMeCP2^{R106W}*), completely lack the sleep deficits, including all sleep reductions and fragmentation phenotypes caused by *wildtype* hMeCP2 function (*fig 2.7 a-e*). These results demonstrate that an intact MBD domain is necessary to cause the hMeCP2-mediated changes in sleep behavior. Furthermore, if the hMeCP2-induced changes were a result of non-specific methylation-independent cellular effects in OA neurons, we would expect the sleep deficits to remain as was observed in a previous study describing R106W-induced structural defects in the eye (Cukier et al., 2008). However, our results indicate methylation-dependent mechanisms *may* play a key role in hMeCP2-induced changes in OA neuron output. Recent experiments examining hMeCP2-induced motoneuron dendritic defects also reported an absence of morphology changes upon R106W expression (Vonhoff et al., 2012).

2.4.6 **OA neuron function requires the *Drosophila* MBD-containing proteins, MBD2/3 and MBD-R2**

At this point, our results describe specific hMeCP2-induced sleep deficits and establish the MBD of MeCP2 is a critical component. We next asked if endogenous MBD-containing proteins are required for amine neuron function and sleep-wake circuitry output. At least two proteins in *Drosophila* belong to the MBD family: a) dMBD-R2 and b) dMBD2/3 (*fig. 2.8*) (Hendrich and Tweedie, 2003, Roder et al., 2000). dMBD2/3 is a small protein consisting of three MBD domains (*fig. 2.9a*) in contrast; dMBD-R2 contains a THAP, TUDOR, and PHD-type Zinc finger in addition to the MBD domain (*fig. 2.10a*). dMBD2/3 and the MBD2/3Δ splice variant associate with the nucleosome remodeling and deacetylase (NuRD) complex (Marhold et al., 2004a), repress transcription in *in vitro* assays (Ballestar and Wolffe, 2001), and MBD2/3Δ preferentially
recognizes mCpG-containing DNA through its MBD (Roder et al., 2000). In addition, the expression of both dMBD2/3 and MBD2/3Δ is developmentally regulated, and is retained in adult tissues suggesting selective roles in transcriptional regulation (Marhold et al., 2004a, Marhold et al., 2004b). Unlike dMBD2/3, it has not been determined if MBD-R2 binds 5mC, however, dMBD-R2 is a part of the multi-subunit chromatin remodeling NSL (non-specific lethal) complex, which regulates gene expression at genome wide levels (Roder et al., 2000).

The human MeCP2 MBD contains 8 known DNA binding sites, half of which are lysine residues (K107, K109, R111, K119, D121, K130, R133 and E137; Conserved domain database CDD: 238690). At least five of these eight DNA-binding sites are present in the Drosophila MBD-R2 protein (R111, K119, D121, K130, R133), and four in dMBD-2/3 (R111, K119, D121, K130). These conserved sites and their location in reference to the hMeCP2 residue positions are depicted in the figure 2.8 (orange bars). In addition, a predicted homology model suggests similarity between specific secondary structural features among the MBD domains of dMBD-R2, dMBD-2/3 MBD domains and hMeCP2 (fig. 2.9b, 2.10b), as the hMeCP2 MBD domain contains three β-strands (residues: 105-110, 120-125, and 131-132) and one α-helical region (residues 135-145) (86).

Therefore, we asked if reducing dMBD-2/3 or dMBD-R2 levels using RNA interference could alter the function of neurons as measured by changes in the sleep network. To measure the RNAi effect on transcript levels, quantitative reverse transcription PCR (RT-qPCR) was performed on RNA extracted from the heads of n-syb-Gal4;UAS-MBD-R2-IR and n-syb-Gal4;UAS-MBD-2/3-IR adults. Transcript levels were reduced by 26.84% (fig. 2.9c) and 36.79% respectively (fig. 2.10c). When dMBD-R2 and dMBD-2/3 levels were reduced in OA neurons by separately expressing the UAS-MBD-R2-IR and UAS-dMBD-2/3-IR lines under control of the tdc2-gal4 driver, we found that fragmentation of sleep architecture occurred in both tdc2-Gal4;UAS-MBD-2/3-IR and tdc2-Gal4;UAS-MBD-R2-IR males. This fragmentation was manifested as an increase in the number of sleep bouts along with a decrease in the consolidation index (figs. 2.9 e-f, 2.10 f-g). Males with reduced dMBD-R2 levels in OA neurons exhibited an increase in
the amount of total sleep (fig. 2.10d), while sleep levels were not significantly altered upon dMBD-2/3 reduction (fig. 2.9d). The increase in total sleep exhibited by tdc2-Gal4;UAS-MBD-R2-IR adults was not due to subpar fitness as these males were more active during waking periods than controls (Fig. 2.10e).

A third variable, the latency to initiate sleep was also unchanged (data not shown for dMBD2/3-IR and fig. 2.10 h). The absence of latency and sleep deficits upon dMBD-2/3 manipulation could simply be due to the incomplete reduction of dMBD-2/3 mRNA (73.16%); alternatively, dMBD-2/3 may not play a critical role in regulating the expression of specific sleep-related genes. However, the changes in sleep architecture are the same whether hMeCP2, dMBD2/3-IR or MBD-R2-IR are expressed in OA neurons (figs. 2.1 f-g, 2.9e-f, 2.10 f-g). These results demonstrate that a reduction in Drosophila MBD-containing proteins can alter neuronal and whole organismal behavior; and provide an avenue for examining the selectivity of gene expression and chromatin biology changes in a defined neuronal subset.

2.4.7 Reducing MBD-R2 rescues hMeCP2-mediated phase-specific sleep deficits

The observation that total sleep increased with a reduction in dMBD-R2 levels is the opposite of the sleep deficits observed in hMeCP2 overexpression lines. As both proteins function as modifiers of gene expression, it led us to speculate that dMBD-R2 knockdown and hMeCP2 overexpression could function antagonistically by modifying gene expression in opposite directions. If hMeCP2 and dMBD-R2 are functioning at overlapping set of gene loci or genomic regions, then we predict a complete or partial rescue of phase-specific sleep alterations in dMBD-R2-deficient lines with concurrent hMeCP2 expression. We tested this hypothesis by generating tdc2-gal4;UAS-hMeCP2/UAS-MBD-R2-IR adults and found that a reduction in MBDR2 levels rescued hMeCP2-induced deficits in day and night sleep profile (fig. 2.11a).

To test whether the effect of relative dMBD expression on sleep architecture varies in the presence or absence of hMeCP2, a two-way multivariate analysis of variance (MANOVA) was performed. This factorial MANOVA tested for main effects as well as interactions between dMBD and hMeCP2 induced sleep alterations by comparing various
measures of sleep as a linear composite across factors. Using Pillais’ trace and 0.05 criterion for significance, a significant interaction (dMBD2/3 × hMeCP2) effect was observed between relative dMBD2/3 and hMeCP2 expression on combined measures of sleep \( F(3, 194) = 30.665, p < 0.0001; V = 0.322; \text{Obs. Power} = 1.00, \text{fig. 2.11 b-c}. \)

Likewise, the effect of dMBD-R2 levels on sleep architecture also varied depending on hMeCP2 levels. That is, a significant interaction (dMBD-R2 × hMeCP2) effect was observed between relative dMBD-R2 and hMeCP2 expression on combined measures of sleep \( F(3, 190) = 28.192, p < 0.0001; V = 0.308; \text{Obs. Power} = 1.00; \text{fig. 2.11 d-e}. \) This interaction effect explained 32.2% of multivariate variance of sleep composite in dMBD2/3-deficient males and 30.8% of multivariate variance in dMBDR2-deficient males (V = partial \( \eta^2 \)).

### 2.4.8 MBDR2 colocalizes with MeCP2 on select chromosomal sites

To examine at a genomic level if hMeCP2 and MBDR2 can associate together at chromosomal locations, we expressed hMeCP2 in polytene salivary gland chromosomes using the 48B10-Gal4 driver. Isolated larval polytene chromosomes from 48B10-Gal4;UAS-hMeCP2 larvae were labeled with MBD-R2 and MeCP2 antibodies. As expected, MBD-R2 localizes extensively at multiple sites on polytene chromosomes likely due to its role as a general facilitator of transcription and as a component of the non-specific-lethal and male-specific-lethal complexes (Pascual-Garcia et al., 2014, Prestel et al., 2010). However, hMeCP2 and MBDR2 are detected together at a number of chromosomal sites (fig. 2.12, arrows, n=6) suggesting the possibility of common gene loci or chromatin organization targets. As a whole, our results indicate the conserved MBD domain even among disparate MBD-containing proteins such as hMeCP2 and dMBD-R2 is capable of conferring shared neuronal phenotypes, likely through shared genomic binding sites.
In this study, we tested the hypothesis that MBD-containing proteins retain considerable functional conservation by measuring neuronal output through an automated, reproducible sleep assay. Sleep impairments are a major feature in a substantial number of neurodegenerative and neuropsychiatric disorders (Piazza et al., 1990; Clements et al., 1986; Richdale and Schreck, 2009). However more fundamentally, this data can be viewed as a relevant behavioral representation of circuit dysfunction in general, which is a common theme in neurodevelopmental syndromes including RTT (Cortesi et al., 2010, Shepherd and Katz, 2011). A powerful advantage of using *Drosophila* sleep to analyze the functional differentiation of circuits and neurons is the ability to measure behavior continuously through various temporal phases at a single minute resolution. This formidable temporal resolution in combination with amine neuron-specific manipulation allowed us to analyze the functional consequences of alterations in relative MBD levels and domain-specific mutations. Not only does this approach allow for functional monitoring through various circadian and developmental phases, temporal windows of interest identified through this assay can facilitate a more empirical selection of functionally-relevant timeframes for sampling and further mechanistic investigations. For example, our results demonstrate that adults expressing hMeCP2 in OA neurons sleep less; however, this sleep loss is not a general phenomenon but rather occurs during specific day and nighttime intervals. In a similar manner, hMeCP2 expression in 5-HT neurons also results in a loss of nighttime sleep. However, with the fine temporal resolution, we can identify sleep deficit intervals that are both unique and overlapping when compared to hMeCP2 expression in OA neurons. Finally, in a previous study we determined that hMeCP2 expression in astrocytes non-cell-autonomously alters the sleep network only during distinct nighttime hours (Hess-Homeier et al., 2014).

How might hMeCP2 expression in amine neurons reduce sleep amounts and sleep quality? At the DNA level, MeCP2 binds to the promoters of enzymes involved in amine synthesis including L-dopa decarboxylase (Ddc) (Urdinguio et al., 2008) and MeCP2 levels themselves oscillate under the control of circadian clock (Martinez de Paz et al., 2015). Previous studies have demonstrated that a loss of OA promotes sleep (Crocker and
Sehgal, 2008) and our HPLC studies indicate global OA levels in the brain are not reduced upon hMeCP2 expression. However, it is possible that the MeCP2-induced reduction in nighttime sleep is mediated through an increase in OA signaling. This hypothesis is consistent with previous observations as overexpression of Tdc2 or genetically activating OA neurons significantly decreases nighttime but not daytime sleep (Crocker and Sehgal, 2008). It is further supported by complete rescue of hMeCP2-mediated nighttime sleep deficits (ZT14-17.5) in OA-null lines in our study (fig. 2.4 c). Additionally, components of the arousal circuitry respond to OA wake-promoting signals including the large-lateral ventral neurons (l-LNvs) neurons (Crocker et al., 2010). When hyper-excited, OA receptor-expressing l-LNv neurons reduce both sleep duration and quality (Kula-Eversole et al., 2010, Shang et al., 2008). In our experiments, MeCP2 expression could potentially increase OA neuron activity by modulating presynaptic function either through changes in levels of OA biosynthetic enzymes, components of OA transport and release, or conserved RNA-binding proteins such as Lark, which regulate neuronal excitability in the circadian system (Ishimoto et al., 2012).

As many MBD family members have a conserved DNA-binding surface that shows high affinity for methylated DNA, a key question is whether individual proteins bind differentially to distinct regions within the genome. Variations in the affinity for binding methylated targets include double-stranded vs. single-stranded, sequence dependent vs. sequence independent, and CpG vs. non-CpG (CpH; H=A/C/T) methylation (Baubec et al., 2013, Fatemi and Wade, 2006, Guo et al., 2014). Recently, a role for MeCP2 binding to CpH sites and regulating the expression of genes enriched for neuronal function has been described (Chen et al., 2015). Non-CpG methylation has been reported in vertebrate neurons (Fatemi and Wade, 2006, Guo et al., 2014, Pinney, 2014), and in Drosophila where the methylation is enriched on non-CpG motifs, particularly CpT and CpA dinucleotides (Boffelli et al., 2014, Capuano et al., 2014, Takayama et al., 2014). Although the levels of such methylation are low and sparsely distributed, it is conceivable nonetheless that MeCP2 could translate endogenous CpH methylation into changes in gene expression. This idea is especially compelling as we demonstrated that an intact MBD-binding domain is required for all hMeCP2-induced sleep deficits (fig. 2.7). Furthermore, males with reduced levels of dMBD2/3, which binds methylated
DNA, exhibited overlapping sleep quality deficits (fig. 2.9). In this context, *Drosophila* may provide an ideal in vivo system to examine the functional consequences of CpH-mediated MBD protein interactions as future studies can address the significance of CpH methylation at candidate genes that control circadian rhythm and aspects of sleep.

In conclusion, epigenetically modifying chromatin structure in response to different stimuli may be a key mechanism in generating shifts in gene expression not only at successive stages of neuron development but successive stages of neuron function. Such functional changes may include responses to pheromones (predators or conspecifics), odors (food resources), or light (sleep) all critical aspects of reproduction and survival in any organism. In this study, we examined the consequences of a hypomorphic reduction of endogenous MBD proteins in a relevant neuronal subpopulation to provide a whole organism readout of changes in neuron function that should be interpretable at the chromatin level in future studies due to ever-increasing advances at the intersection of circadian biology and epigenetics. Our results provide the first demonstration that *Drosophila* MBD proteins are required for neuron function in context of sleep, and that MBD-containing proteins indicate conservation in the cell-specific functions of epigenetic translators.
**Figure 2.1** hMeCP2 expression in OA neurons reduces sleep in adult males

(a) and (a') show representative images of tdc2-gal4, uas-gfp, uas-hMeCP2 (control) and tdc2-gal4, uas-hMeCP2 (experimental) brains stained with phalloidin-Dylight 647. (b) Bar graph showing total sleep (min/24 hr) with quantification of drug treatment. (c) Graph of minutes of sleep over a 24-hour cycle (ZT0-ZT23) with control and experimental conditions. (d) Graph of average sleep (min/30 min bin) at ZT4-10 and ZT14.5-22 with control and experimental conditions. (e) Average sleep bouts (24 hr) with quantification of drug treatment. (f) Consolidation index (24 hr) with quantification of drug treatment. (g) Empirical CDF of bout length with quantification of drug treatment. (h) Latency to sleep with quantification of drug treatment.
Figure 2.1: hMeCP2 expression in OA neurons reduces sleep in adult males

(A-A″) hMeCP2 expression (red) in OA neurons from an adult tdc2-gal4/UAS-mCD8:gfP; UAS-MeCP2/+ male (anti-GFP, green; mAb nc82, labels neuropil regions, blue). (B-H) Sleep profiles of individual adult males averaged over 8 days from control and experimental groups. Controls: tdc2-gal4/+ (white), UAS-MeCP2/+ (light grey), tdc2-gal4/+; UAS-dsRed/+ (dark grey) and experimental: tdc2-gal4/+; UAS-MeCP2/+ (red). (B) Total sleep per 24-hr day is reduced in experimental males as compared to controls (P_{adj}=0.0013; one-way ANOVA with Holm-Sidak’s multiple comparison test). (C) Eduction graph displaying 30 minute bins of averaged sleep (daytime/light phase: white bar; nighttime/dark phase: black bar, shaded grey). tdc2-gal4/+; UAS-MeCP2/+ males displayed a reduction in the average amount of sleep during both day and night (arrows) as compared to controls. These deficits are quantified in (D) for Zeitgeber hours ZT04-10, (P<0.0001; two-tailed Mann Whitney test) and ZT14.5-22, (P<0.0001; two-tailed Mann Whitney test). (E-G) Sleep fragmentation in males expressing MeCP2 expression in OA neurons. As compared to controls, the average number of sleep bouts per day (E) is increased (P_{adj}<0.0001) and weighted average bout length measured by the consolidation index (F) is reduced significantly in experimental males (P_{adj}<0.0001). (G) The empirical cumulative distribution function (ECDF) demonstrating experimental males exhibit a greater proportion of short sleep bouts as compared to controls. (H) Latency to initiate sleep (the delay in minutes from the lights OFF to the time to the first sleep bout) is significantly reduced in tdc2-gal4/+; UAS-MeCP2/+ males as compared to controls (P_{adj}=0.0009; one-way ANOVA with Holm-Sidak’s multiple comparison test). Data are shown as means ± standard error of the mean (SEM).
Figure 2.2: Kaplan-Meier survival curve in males expressing hMeCP2 in OA neurons

A Kaplan-Meier survival distribution of experimental males, *tdc2-gal4; UAS-hMeCP2* males and transgenic controls (standard log-rank test, $P<0.0001$). Dotted boundaries around the curves representing standard error (SE)
Figure 2.3: Adults expressing hMeCP2 in 5HT neurons exhibit a reduction in nighttime sleep.
Figure 2.3: Adults expressing hMeCP2 in 5HT neurons exhibit a reduction in nighttime sleep only (A) hMeCP2 nuclear expression (green) in 5HT neurons from a trh-gal4; UAS-MeCP2/+ male brain. (B-H) The quality and amount of sleep in individual adult males averaged over an 8 day period from control and experimental groups. (B) The total amount of sleep per 24-hr day is not significantly changed in experimental males as compared to UAS-MeCP2/+ controls (P_adj=0.2051). (C) Elevation graph displaying the average amount of sleep per 30 minute bin (daytime/light phase: white bar; nighttime/dark phase: black bar, shaded grey) in control and experimental males. trh-gal4/+; UAS-MeCP2/+ males displayed a reduction in sleep during Zeitgeber hours ZT19-22.5 (arrow). These deficits are quantified in (D) P=0.0011, Mann Whitney test. (E-H) Sleep fragmentation in males expressing MeCP2 in 5HT neurons. (E) The daytime consolidation index is significantly reduced in experimental vs. control males (P_adj<0.0001). The nighttime consolidation index is not altered (P_adj=0.7262). (F) The average number of daytime sleep bouts is increased in experimental males vs. controls (P_adj=0.8316), without alterations in the average number of nighttime sleep bouts (P_adj=0.0001). (G) Daytime, but not nighttime, waking activity is increased in experimental males vs. controls (P_adj<0.0001). (H) The empirical cumulative distribution function demonstrates experimental males exhibit a greater proportion of short sleep bouts as compared to controls. Data are shown as means ± standard error of the mean (SEM). Unless noted otherwise, results were analyzed by one-way ANOVA with Holm-Sidak’s multiple comparison test.
Figure 2.4: The loss of OA rescues a subset of hMeCP2-induced sleep deficits

HPLC quantification of OA levels in whole brain extracts of 3-5 day old adult males collected during ZT04-10. OA levels between control and experimental groups did not differ. (B-F) Sleep profiles of individual adult males averaged over an 8-day period from control and experimental groups. Controls: tdc2-gal4/+ (white bar), UAS-MeCP2/+ (light grey), Tβh
1468
tdc2-gal4 (dark grey) and experimental: tdc2-gal4; UAS-MeCP2 (red), Tβh
1469
1470
tdc2-gal4; UAS-MeCP2 (yellow). (B) Eduction graph displaying average amount of sleep per 30 minute bin (daytime/light phase: white bar; nighttime/dark phase: black
bar) in control and experimental males. MeCP2-induced sleep deficits (red line) are restored to control levels in $t\beta h^{\text{N18}}_{\text{tdc2-gal4}}; UAS-MeCP2$ males during ZT14-17.5 (yellow line, arrow). (C) The reduction in sleep during ZT04-10 remained in OA deficient males expressing hMeCP2. The sleep reduction during ZT14-17.5 was completely rescued in the absence of OA (multiplicity adjusted P-value for pooled controls vs. $t\beta h^{\text{N18}}_{\text{tdc2-gal4}}; UAS-MeCP2$ experimental males; P= 0.8447). (D-E) Sleep fragmentation remains in hMeCP2-expressing OA deficient males. The consolidation index (D) is reduced significantly in both experimental groups ($P_{\text{adj}} = 0.1658$) and the average number of sleep bouts is increased (E) ($P_{\text{adj}} = 0.2409$). (F) No difference was observed in the waking activity between OA deficient controls ($t\beta h^{\text{N18}}_{\text{tdc2-gal4}}$) and experimental males ($t\beta h^{\text{N18}}_{\text{tdc2-gal4}}; UAS-MeCP2/+; P_{\text{adj}} = 0.6325$). (G) As predicted, total sleep is significantly increased in the OA deficient control ($t\beta h^{\text{N18}}_{\text{tdc2-gal4}}$, black column) as compared to transgenic controls ($P_{\text{adj}} = 0.0070$). This sleep increase returned to wildtype levels upon expression of hMeCP2 in OA deficient males ($t\beta h^{\text{N18}}_{\text{tdc2-gal4}}; UAS-MeCP2$, black vs. yellow columns) ($P_{\text{adj}} = 0.6563$; one-way ANOVA with Holm-Sidak’s multiple comparison).
Figure 2.5: hMeCP2-induced sleep deficits remain in males expressing the R294X allele.

(A) Schematic depicting the structural domains MeCP2 and the loss of domains due to the R294X mutation. (B-H) The sleep profiles of control and experimental adult males averaged over an 8-day period. (B) Elevation graph displaying the average amount of sleep per 30 minute bin (daytime/light phase: white bar; nighttime/dark phase: black bar, shaded grey). Average sleep during Zeitgeber hours ZT04-10 and ZT14.5-22 are quantified in (C). Males expressing the R294X allele displayed a similar reduction in the average amount of sleep during ZT04-10 as males expressing the full-length allele (P_{adj}=0.0103). During ZT14.5-22, the average sleep deficit in males expressing R294X allele remains reduced as compared to controls (P<0.0001). This 294X-induced sleep
reduction is partially recovered in comparison to hMeCP2-expressing males (P<0.0001).

(D) Males expressing full-length or R294X alleles exhibited a reduction in the latency to initiate sleep as compared to controls (P_{adj}=0.0001). (E-G) Sleep fragmentation in males expressing the full-length MeCP2 and R294X alleles in OA neurons. (E) The average number of sleep bouts increases to a lesser extent in R294X males as compared to males expressing full-length MeCP2 (P_{adj}<0.0001) however the increase in sleep bouts of tdc2-gal4; UAS-hMeCP2^{294X} is significantly higher than controls (P<0.0001). (F) The consolidation index was reduced significantly in both full-length and R294X males as compared to controls (P_{adj}<0.0001). (G) Experimental males exhibited a greater proportion of short sleep bouts as calculated by the empirical cumulative distribution function. Data are shown as means ± standard error of the mean (SEM). Unless noted otherwise, one-way ANOVA with Holm-Sidak’s multiple comparison test was used.
Figure 2.6: Sleep fragmentation and sleep deficits are rescued in males expressing hMeCP2Δ166 allele in OA neurons

(A) Schematic diagram depicting MeCP2 structure and the loss of domains due to the Δ166 truncation. (B) hMeCP2Δ166 (green) is expressed in adult OA neurons via the tdc2-gal4 driver (tdc2-gal4; UAS-hMeCP2Δ166). (C-H) The sleep profiles of control and experimental adult males averaged over an 8-day period. (C) The latency to initiate sleep is not significantly reduced in males expressing hMeCP2Δ166 as compared to controls (P_adj=0.2611). (D) Elevation graph displaying average amounts of sleep per 30-minute bin in control and experimental males. The overall sleep profile and average sleep during Zeitgeber hours ZT04-10 and ZT14.5-22 is completely rescued in males expressing hMeCP2Δ166. (D) The average amount of sleep does not differ between controls and males expressing hMeCP2Δ166: ZT04-10, (P_adj=0.514), and ZT14.5-22, (P=0.7853). (F-H)
Sleep is not fragmented in males expressing hMeCP2Δ166 in OA neurons. (F) The average number of sleep bouts is not significantly different in tdc2-gal4; UAS-MeCP2Δ166 vs. the tdc2-gal4 and UAS-MeCP2 control (P_{adj}=0.2923). (G) The consolidation index does not differ between males expressing hMeCP2Δ166 and controls (P_{adj}=0.1308). (H) The empirical cumulative distribution function demonstrates experimental males exhibit a greater proportion of short sleep bouts as compared to controls. Data are shown as means ± standard error of the mean (SEM). The one-way ANOVA with Holm-Sidak’s multiple comparison test was used.
Figure 2.7: Disruption of the MeCP2 binding by the R106W mutation eliminates MeCP2-induced sleep deficits and fragmentation

(A-E) Sleep patterns averaged over a period of 8 days from control and experimental males. (A) Eduction graph displaying average amount of sleep per 30-min bin. The sleep patterns and sleep quality of males expressing hMeCP2\textsuperscript{R106W} in OA neurons are the same as controls. (B) The average sleep during Zeitgeber hours ZT04-10 and ZT14.5-22 does not differ between males expressing R106W and controls: ZT04-10, \( P_{\text{adj}}=0.7406 \), and ZT14.5-22, \( P=0.0974 \). (C-E) Sleep fragmentation does not occur in males expressing R106W. (C) The average number of sleep bouts in males expressing R106W is not significantly different from controls (\( P_{\text{adj}}=0.8849 \)). (D) The consolidation index does not differ from the R106W-expressing experimental males and controls (\( P_{\text{adj}}=0.9843 \)). (E) Experimental males exhibited a greater proportion of short sleep bouts as calculated by the empirical cumulative distribution function.
Figure 2.8: Multiple Sequence Alignment of select MBD-famaily proteins in Humans and Drosophila

Conservation

Consensus
Figure 2.8: Alignment and conservation of MBD-containing proteins

The structural domains of hMeCP2 with domain-specific multiple sequence alignment of select MBD-family proteins in human (h) and Drosophila (d). Identical sequences are highlighted in various shades of blue depending on the degree of conservation across groups. The histogram (yellow) represents conserved physico-chemical properties for each column of the alignment. Higher scores (max=10) for non-identical columns indicate amino acid substitutions that belong to the same physico-chemical class (Livingstone and Barton, 1993).
Figure 2.9: Reducing the levels of Drosophila dMBD2/3 in OA neurons alters sleep quality: (A) A schematic diagram depicting the size and conserved domains of dMBD-2/3. (B) A structural model of the dMBD-2/3 MBD domain (Template: MBD3 (pdb: 2mb7), sequence identity = 40.9%, GA341 score = 0.955, z-DOPe score = -0.234 (C) For semi-quantitative RT-PCR experiments, RNA from the heads of adults expressing dMBD-2/3-IR in OA neurons (n-syb-Gal4-gal4; UAS-dMBD-2/3-IR, blue column), and controls (n-syb-gal4-Gal4/+, white column; UAS-dMBD-2/3-IR/+; gray column). dMBD-
2/3 transcript levels were significantly reduced in *n-syb-Gal4-gal4;UAS-dMBD-2/3-IR* adults as compared to age-matched control adults (Ordinary one way ANOVA, $P_{adj}$=0.0026). Reactions were performed in quadruplicate. Rpl32 expression was used as the reference control to normalize expression between treatment groups (error bars indicate s.e.m.). (E-I) Sleep quality and quantity exhibited by individual males averaged over an 8-day period from control and experimental groups. **(E)** The total amount of sleep per 24-hr period in MBD2/3-deficient males does not differ from the *tdc2-gal4* control ($P_{adj}$=0.1186). **(F)** The average number of sleep bouts per 24-hr period is increased in *tdc2-gal4/+; UAS-dMBD2/3^{RNAi}/+* males as compared to controls ($P_{adj}$=0.0041). **(G)** The consolidation index is significantly reduced in MBD2/3-deficient males as compared to controls ($P_{adj}$=0.0032). **(H)** No change was observed in the latency to initiate sleep ($P_{adj}$=0.7522).
Figure 2.10: Reducing dMBD-R2 levels in OA neurons increases total sleep and causes sleep fragmentation

(A) Schematic representation of dMBD-R2 showing the conserved structural domains.

(B) A structural model of the dMBD-R2 MBD domain (Template: MeCP2 (pdb: 3c2i), sequence identity = 34%, GA341 score = 0.931, z-DOPE score = -0.213). (C) RNA from the heads of adults expressing dMBD-R2-IR in OA neurons (n-syb-Gal4-gal4; UAS-dMBD-R2-IR, blue column), and controls (n-syb-gal4-Gal4/+, white column; UAS-dMBD-R2-IR/+, gray column) were used for semi-quantitative RT-PCR experiments. dMBD-R2 transcript levels were significantly reduced in n-syb-Gal4-gal4; UAS-dMBD-R2-IR adults as compared to age-matched control adults (Ordinary one way ANOVA, \(P_{adj} = 0.0045\)). Reactions were performed in quadruplicate. Rpl32 expression was used as the reference control to normalize expression between treatment groups. (D) MBD-R2-
deficient males displayed an increase in total sleep as compared to controls (P_adj<0.0001). (E) Sleep fragmentation as measured by an increase in the number of sleep bouts (P_adj<0.0) and a decrease in the consolidation index (F) occurred in tdc2-gal4/+;UAS-dMBD-R2-IR/+ males as compared to controls (P_adj=0.001). (G) The latency to initiate sleep in MBD-R2-deficient males was not significantly different from the UAS-dMBD-R2-IR control (P_adj<0.6981). Data are shown as means ± standard error of the mean (SEM). The one-way ANOVA with Holm-Sidak’s multiple comparison test was applied.
Figure 2.11: Concomitant reduction of dMBD and hMeCP2 overexpression rescues hMeCP2-mediated sleep deficits.

A

- **Controls (Pooled; n=176)**
- **tdc2-gal4; UAS-hMeCP2/UAS-dMBD2/3R (n=29)**
- **tdc2-gal4; UAS-hMeCP2 (n=31)**
- **tdc2-gal4; UAS-hMeCP2/UAS-dMBD2R (n=19)**

(b) **Consolidation Index (24hr)**

(d) **Consolidation Index (24hr)**

(c) **Average Sleep Bouts (24 hr)**

(e) **Sleep Bouts (24 hr)**

(Relative hMeCP2 Expression)
Figure 2.11: Concomitant reduction of dMBD and hMeCP2 overexpression rescues hMeCP2-mediated sleep deficits

(A) Elevation graph displaying 30 minute bins of averaged sleep between males expressing hMeCP2 in OA neurons, males expressing hMeCP2 and dMBD (UAS-dMBD-R2-IR, blue squares and UAS-dMBD-R2-IR, yellow squares) and controls (daytime: white bar; nighttime: black bar, shaded grey). The phase-specific sleep reductions quantified in tdc2-gal4;UAS-hMeCP2 males (red square line) have been rescued to control levels with the reduction in dMBD-R2 levels (arrows). (B-C) Two-way multivariate analysis of variance (MANOVA): Using Pillais’ trace and 0.05 criterion for significance, a significant interaction (dMBD-R2 × hMeCP2) effect was observed between relative dMBD-R2 expression and hMeCP2 gain of function on combined measures of sleep (F(3, 190) = 28.192, p < 0.0001; V = 0.308; Obs. Power = 1.00). (D, E) Interaction between relative dMBD2/3 expression and hMeCP2 gain of function on combined measures of sleep (F(3, 194) = 30.665, p < 0.0001; V = 0.322; Obs. Power = 1.00).
Figure 2.12: Co-immunofluorescence analysis in larval polytene chromosomes

(A-D) Polytene chromosomes from 48B10-gal4/+; UAS-hMeCP2/+ 3rd instar larvae. Both dMBDR2 (red) and hMeCP2 (green) display extensive chromosomal binding. Co-immunofluorescence is observed at selected bands (arrowheads, PCC: r = 0.508; MCC1: 0.64, MCC2: 0.694; Costes’ randomization test: P-value=100%). Individual channels in panels (C-D) correspond to the white region of interest (ROI).
2.7 REFERENCES


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3  CHAPTER III

METHYL-CpG BINDING DOMAIN (MBD) PROTEINS MODULATE AGGRESSION AND INTERSPECIES COURTSHIP IN DROSOPHILA

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3.1  INTRODUCTION

A long-standing challenge in evolutionary biology is to understand the molecular basis of adaptive, divergent phenotypes. Between recently diverged species, processes that underlie reliable sex and species discrimination can either impede or promote reproductive isolation. For instance, chemosensory signaling, visual and acoustic feedback from the interacting partner(s) and subsequent neuromodulatory processing facilitates contextual discrimination and allows an organism to respond rapidly and appropriately to social and environmental cues. While much research has focused on the functional characterization of genes and neurons associated with these processes, relatively little is known about the genomic structural and organizational features that underlie contextual plasticity in various chemosensory, visual and acoustic faculties. Therefore, we asked how various social behaviors that rely on sexual and species discrimination are modified by epigenetic changes such as DNA methylation and chromatin remodeling. To investigate the epigenetic processes that facilitate reproductive and aggressive interactions, we altered the expression of methyl-CpG-binding domain (MBD) proteins in Drosophila within a key subset of neuromodulatory neurons.

Contextual plasticity in organismal behavior and underlying sensory faculties is achieved in part by modulating the strength of sensory information and the directionality of neural network outputs (Marder, 2012). Neuromodulators such as serotonin, dopamine, and norepinephrine are associated with the regulation of aggression and reproductive behaviors in a diverse array of species ranging from crustaceans to primates (Huber et al.,
Our group and others have previously reported on the significance of octopamine (OA, the invertebrate analog of noepinephrine) neurons in modulating the choice point between aggression and courtship in *Drosophila* (Certel et al., 2007; Baier et al., 2002). OA neurons in the subesophageal ganglion (SOG) of the adult central brain receive projections from gustatory receptor-expressing sensory neurons (GRNs) found in taste sensilla within the mouth, legs and wings (Andrews et al., 2014). These GRNs neurons detect and respond to cuticular hydrocarbons (CHC) and long carbon chain esters that carry information about the species- and sex-identity of interacting partners (Claude et al., 2010; Thisle et al., 2012; Andrews et al., 2014). Eliminating Gr32a function reduces male aggression, increases male-male courtship, and prevents the inhibition of courtship between *Drosophila* species (Fan et al., 2013). Similarly, in the absence of OA, males display reduced levels of aggression as measured by lunge number (a key behavioral pattern in the establishment of hierarchical relationships) and a delay in initiating aggression (Certel et al., 2007; 2010). Additionally, males with enhanced OA signaling or feminized OA neurons increasingly exhibit male-male courtship displays illustrating the critical role of OA neuromodulation in regulating sensory inputs concerned with sexual recognition. Therefore, we set out to explore the role of components associated with DNA methylation and chromatin remodeling in OA-mediated behavioral plasticity in context of species- and sex-specific aggression and courtship displays.

For this purpose, we examined mate choice and aggressive interactions in males with altered levels of genomic methylation and/or methyl-CpG binding domain (MBD) proteins. The function of MBD proteins has been studied extensively in vertebrates where MBD family members can regulate gene expression by binding 5-methylcytosine (5mC) and interacting with histone deacetylase (HDAC)-containing complexes, thereby linking two epigenetic repression mechanisms: DNA methylation and histone deacetylation (Nan et al., 1998). As discussed in Chapter I of this dissertation, the *Drosophila* genome encodes at least two MBD-containing proteins, dMBD-R2 and dMBD-2/3 (Roder et al., 2000; Hendrich and Tweedie, 2003). dMBD2/3 and the MBD2/3Δ splice variant associate with the nucleosome remodeling and deacetylase (NuRD) complex (Marhold,
2004) and MBD2/3Δ preferentially recognizes mCpG-containing DNA through its MBD
(Roder et al., 2000). It has not been determined if the second protein - dMBD-R2 - binds
5mC in vivo, however, dMBD-R2 is part of the multi-subunit chromatin remodeling NSL
(non-specific lethal) complex, which regulates gene expression at genome wide levels
(Roder et al., 2000).

In this chapter, we describe a novel role for endogenous dMBD proteins in the
regulation of male social behavior. We found that dMBD-deficient males exhibit
significant reduction in male aggression with a concomitant increase in male-male
courtship. We also observed an increase in inter-species courtship and a reduction in
conspecific mating in these males. Subsequently, we hypermethylated the OA neuron
genomic DNA and asked if dMBDR2-induced alterations in mate discrimination and
male behavioral choice varied across various levels of methylation. Males with a
hypermethylated genome exhibited increased male-male courtship - a phenotype that was
rescued by concurrent reduction in dMBD-R2 levels. Taken together, our results
demonstrate that epigenetic mechanisms interpreted by the Drosophila MBD-containing
proteins (MBPs) are required for contextually plastic male selective behaviors and pave
the way to address how the selective utilization of the OA neuronal genome and potential
shifts in gene expression in response to sensory stimuli are coordinated.
3.2 METHODS

3.2.1 Husbandry and Stocks:
All flies were reared on standard cornmeal-based fly food containing agar, sugar, yeast, cornmeal, distilled H₂O and anti-fungal compound Tegosept (in 95% ethanol solution).

Unless noted otherwise, during developmental and post-eclosion, flies were raised at 25°C, ~50% humidity and 12:12hr light-dark cycles (1400±200 lx white fluorescent light) in humidity and temperature controlled incubators.

*Drosophila* Stocks: Canton-S, UAS-CD8:GFP (BL 5130), UAS-MBD-R2-IR (BL 30481), UAS-dMBD2/3-IR (BL 35347) and *D. virilis* lines were obtained from the Bloomington Stock Center (Bloomington, IN). The Tdc2-Gal4 and UAS-MeCP2 lines were generously provided by Juan Botas and Jay Hirsh, respectively. Transgenic control males were generated by crossing Canton S females with males from the respective UAS- or gal4-lines.

3.2.2 Aggression Assays:

For aggression and inter-male courtship analysis, male pupae were isolated and aged individually in 16x100mm borosilicate glass tubes containing 1.5ml of standard food medium described above. Two-day old males were extracted and a dab of white or blue acrylic paint was applied on the thorax under CO₂ anesthesia for identification purposes. Total CO₂ exposure time was limited to less than 2 minutes for each fly. Flies were returned to their respective tubes for a period of at least 24 hours to allow recovery from handling and anesthesia. For aggression testing, pairs of 3-5day old, socially naïve adult males were placed in 12-well polystyrene places (VWR #82050-930) as described previously (Andrews et al., 2014).

For temperature sensitive Tub-Gal80ts experiments, flies were raised at 18-19°C through all embryonic, larval and pupal stages. Individual pupae were transferred to 16 x 100 mm glass vials and allowed to eclose in isolation. 2-3 day old adult males were transferred to 30°C for 24-36hrs for Gal80ts inactivation. 30-min prior to behavioral testing, flies were moved to 25°C for recovery. Aggression and inter-male courtship were assayed at 25°C and ~45-50% humidity levels in standard polystyrene chambers as described earlier.
Scoring: All aggression was assayed within first two hours of lights ON time (Zeitgeber hours 0-2). Each fight was recorded for a period of 90 minutes and scored manually using iMovie 9. Total number of lunges and wing threat behaviors were scored for a period of 30 minutes after the first lunge according to the criteria established previously (Certel and Kravitz, 2012; Chen et al., 2002). The delay between the assay start time and the first lunge was used for calculating the delay to aggression onset (or latency to lunge). Dominance was established after 3 consecutive lunges followed by chasing the other fly off of the food cup. In most cases, a clear dominant-subordinate relationship was characterized by a disproportionate number of lunges by the winner/dominant male. However, in select few fights, frequent dominance reversal was observed and despite high number of lunges, no clear hierarchy could be established within the scoring period.

3.2.3 Male-Male Courtship:
Inter-male courtship behavior was recorded in the form of unilateral wing extensions (or singing) within the aggression paradigm. Number of single wing extensions were recorded both prior to the first lunge as well after the onset of aggression for a period of 30 minutes. No strong correlation was observed in the combined latency to aggression and single wing extension data across different genotypes. Graphs were generated with Graphpad Prism and Adobe Illustrator CS5.

3.2.4 Interspecific Courtship:
For inter-species courtship preference assay, each 3-5 day old socially naïve control (Canton S) or dMBDR2-deficient male was paired with one 5-7 day old socially naïve conspecific female (D. mel) and one similarly aged female from a different but related species – D. virilis. Courship was primarily characterized by the number of single wing extensions and copulatory abdominal bendings. Various standard measures of courtship were recorded including – a) latency to courtship or first unilateral wing extension, b) duration of each wing extension, c) total time spent courting each female, d) number of copulatory abdominal bendings, and e) courtship index (C.I.) defined as total time courting both females as a fraction of latency to copulation or total scoring period, in case there’s no successful mating event. These behaviors were scored for a total period of 10
minutes (600 seconds) or up to the point of successful mating event, whichever came earlier.

3.2.5 Statistics:

One-way analysis of variance (ANOVA) with Sidak’s multiple-comparison test was performed in case of three or more comparison groups, and a standard pairwise t-test in case of only two comparisons. If data did not meet key parametric assumptions, non-parametric version of the test or bootstrapping based resampling methods were employed using the Resampling Procedures v1.3 (Howell, 2009). In this case, sample distribution was empirically determined by random sampling of residuals with replacement and F-statistic was computed for each of the 50,000 bootstrapped residuals. The resulting distribution was used to evaluate the likelihood of obtaining an F-statistic greater than the value obtained from the sample means at 95% confidence level (Howell, 2012). In case of more than two comparisons, \( \alpha \)-values were manually adjusted for sequential Holm-Sidak’s correction \( (1- \alpha)^{1/i} \), where \( i=number \ of \ comparisons \). Results were cross validated with permutation tests that involve randomization without replacement. For a 2x2 factorial design to assess if MBDR2-induced variations in social behavior varied across levels of ectopically-induced methylation, an ordinary two-way ANOVA was performed.
3.3 Results

3.3.1 Reduction in dMBD-R2 levels results in decreased conspecific aggression and an increase in male-male courtship

To test the hypothesis that endogenous methyl-binding domain (MBD) proteins in *Drosophila* play a role in male social behavior, we first examined conspecific agonistic interactions in males with reduced dMBD levels. For this purpose, we employed targeted knockdown strategies using the UAS-Gal4 system to selectively manipulate dMBD-levels in OA neurons. dMBD-specific RNAi constructs (*UAS-dMBDR2-RNAi, and UAS-dMBD2/3-RNAi*) were expressed under the control of tyrosine decarboxylase (*Tdc2*) promoter. These lines have previously been demonstrated to reduce dMBD transcript levels in Chapter II (fig 2.4.8).

Pairs of *tdc2-gal4; UAS-dMBD-R2-IR, tdc2-gal; UAS-dMBD-2/3-IR*, or transgenic control males were placed in an aggression chamber and multiple aggression parameters were quantified including latency to the first lunge, total numbers of lunges, and total number of agonistic wing threats. When two males were paired in a standard aggression assay, dMBD-R2-deficient males exhibited a strong reduction in the average number of lunges on each other (a key phenotype in establishment of dominant-subordinate relationships) as compared to the transgenic controls (fig 3.1a). These males also demonstrated a five-fold reduction in the number of agonistic wing threats (fig 3.1b).

In parallel, the onset of aggression (typically marked by the first lunge) was significantly delayed as well (fig 3.1c). In *wt* and transgenic control males, at least 80% of dyadic interactions within the aggression paradigm result in establishing clear dominance hierarchy relationships. However, only 11.76% of social encounters involving dMBD-R2-deficient males engaged in fighting resulting in a significant decrease in formation of social hierarchy in this group (fig 3.1d). One of the possible explanations for such significant reduction in male aggressiveness is a general dampening of the arousal systems, independent of aggression-specific circuitry. However, the observed decrease in aggression in MBDR2-deficient males was not correlated with the waking activity levels. Contrary to that, these males are slightly more active as compared to the transgenic control males (*Chapter II, fig 2.4.8*).
A second explanation for a decrease in aggression may be that males are engaging in an alternative behavior. Within the allotted fight assay time, interactions between wildtype and transgenic control male pairings include high levels of aggression accompanied by a relatively low baseline level of male-to-male courtship. dMBD-R2-deficient males, on the other hand, displayed a substantial three-fold increase in the number of single wing extensions – a key measure of courtship – towards the second male (fig 3.1 e). This increase in male-male courtship potentially at the expense of conspecific aggression is also observed in males that lack OA (Certel et al., 2007). Similar behavioral alterations were observed, albeit to a lesser degree, in males with reduced expression of dMBD2/3 in the OA neurons (fig 3.2). These results demonstrate Drosophila MBD proteins are required for context-dependent male social behavior and identifies a neuronal subpopulation, OA neurons, functionally important for this behavioral plasticity. As the observed behavioral phenotype was more pronounced in tdc2-gal4;UAS-dMBD-R2-IR males, we focused our attention on MBD-R2 for subsequent investigations.
3.3.2 **MBD-R2 knockdown in a small subset of neurons modulates aggression but not courtship**

Under the control of *Tdc2* promoter, around 137 nuclei distributed across the adult brain in discrete clusters are estimated to express the gal4-driven transgenic RNAi construct (Busch et al., 2009; Cole et al., 2005). However, aggression and reproductive behaviors are for the most part mutually-exclusive (Certel et al., 2007; Petrovich et al., 2001). To determine if the dMBD-R2 mediated male aggression and courtship phenotypes can be separated into distinct OA neuronal subpopulations, we further restricted the expression of MBD-R2-RNAi construct to an even smaller subset of neurons. For this purpose, we employed the Gal80-based enhancer-trap system under the control of choline acetyltransferase (*Cha*) promoter to spatially refine the expression of the RNAi construct to a small subset of non-cholinergic *Tdc2* neurons. Adding the *cha-gal80* transgene (*tdc2-gal4;cha-Gal80/UAS-6XGFP*) limits the number of OA neurons with Gal4 activity to neurons within the sub-oesophageal medial cluster (SM), the ventrolateral cluster (OA-VL1 and OA-VL2) (*fig. 3.3a-a’*). A subset of these OA neurons has been shown to play a role in aggression by group-housed males (Zhou et al., 2008). Therefore, we predicted that males with a dMBD-R2 reduction in this OA neuronal subset would exhibit a decrease in aggression only. As anticipated, *tdc2-gal4;cha-Gal80/UAS-dMBD-R2-IR* males did not engage in male-male courtship over and above baseline levels observed in control pairings (*fig. 3.3b*). However, a significant reduction was observed in the number of lunges and wing threats (*fig 3.3 c-d*). This result suggests the male-male courtship quantified in Figure 1 is not a compensatory behavioral artefact of reduced male aggressiveness but may occur as a result of alterations in OA-mediated courtship-specific circuitry. These observations are consistent with previous reports (Certel et al., 2010) suggesting that male aggression and courtship are regulated by distinct, independent subsets of *Tdc2* neurons.

Furthermore, not all aggression parameters are altered in *tdc2-gal4;cha-Gal80/UAS-dMBD-R2-IR* males. The delay in onset to aggression (latency) was not altered significantly (*fig 3.3 d*) and the experimental males were equally likely to form dominance hierarchy relationships as control groups (*fig S1*). In this case, roughly 80% of
dyadic interactions resulted in establishment of dominance hierarchy relationships, which is in striking contrast to the dominance outcomes in males with reduced dMBD-R2 levels in the entire tdc2-Gal4 neuronal population (fig 3.1d). Taken together, the behavior of tdc2-gal4;cha-Gal80/UAS-dMBD-R2-IR males allows us to determine the contribution of a limited number of OA neurons to distinct aggression phenotypes and supports the hypothesis that the male-male courtship observed in the aggression context is regulated, at least to some extent, independent of the circuitry that controls aggression. These observations also lend support to the hypothesis that whether or not an organism will decide to engage in an aggressive encounter and the delay in onset of such encounter is regulated differently and independently of the circuitry that controls the intensity of aggression.
3.3.3 Reducing MBD-R2 levels in adult-specific OA neurons recapitulates male aggression deficits

Previous studies have determined MBD proteins can mediate the plasticity of neuronal gene chromatin during development, signaling, and stress responses (Ballas et al., 2009; Chen et al., 2003; Martinowich et al., 2003; Nuber et al., 2005). Therefore, the deficits in male social behavior we observe may be due to changes in OA neuronal differentiation or connectivity during the course of the development.

To determine if observed alterations in male social behavior were caused by potential alterations in neuronal maturation and/or connectively during early development, we used Gal80-based temperature-sensitive conditional activation system to restrict the expression of MBD-RNAi construct to adult male neurons, and not during early embryonic or larval stages. For this purpose, tdc2-gal4; tub-Gal80ts/UAS-dMBD-R2 -RNAi progeny was raised at non-permissive temperatures (18-19°C), at which Gal80ts represses Gal4 activity, thereby restricting transgenic expression. Figure 3.4 illustrates Gal80ts based suppression of GFP reporter expression in UAS-CD8:GFP/+; Act5c-Gal4/Tub-Gal80 larvae (fig 3.4a) and pupae (fig 3.4b) raised at 19°C. Subsequently, adult males 48 hours post-eclosion were shifted to 30°C for 24-36 hours prior to transference into the fight chamber where the males fought at 25°C (see Materials and Methods). This inducible activation system allowed us to delineate effects due to developmental alterations as opposed to acute modulation of octopaminergic circuit output in adults.

When dMBD-R2 levels were reduced post-eclosion, tdc2-gal4; tub-Gal80ts/UAS-dMBD2 -RNAi males displayed a significant reduction in the number of lunges and delayed onset of aggression as compared to controls (fig. 3.4 c, d). Experimental males did not exhibit an increase in aggressive wing threats (Fig. 3.4 e), however, male-male courtship as measured by the single wing extension remained significantly elevated in dMBD-R2 adult deficient males (fig. 3.4f). These results indicate that dMBD-R2 has a functional role in adult OA neurons.
3.3.4 MBDR2-deficient males display high-levels of interspecies courtship

Our previous work and others have established that males lacking OA and/or the gustatory receptor Gr32a exhibit elevated levels of male-male courtship (Andrews et al., 2014). In addition, Gr32a-expressing neurons have been shown to be important for the inhibition of inter-specific courtship in *Drosophila* (Fan et al., 2013); and OA neurons within the subesophageal zone (SEZ) directly receive Gr32a-neuron chemosensory pheromonal information (Andrews et al., 2014). Since dMBDR2-deficient males displayed impaired inhibition of male-male courtship, we asked if such impairment extended to the regulation of species-specific courtship displays as well.

Since *D. virilis* and *D. melanogaster* diverged ~40 million years ago (mya), we began by pairing a single *tdc2-gal4/UAS-dMBD-R2-IR* socially naïve male with one conspecific (*D. melanogaster; Canton S*) female and one *D. virilis* female in a courtship choice assay (see materials and methods). Although, a recent study reported little or no courtship between intact *wildtype* males and *D. virilis* females (Fan et al., 2013); socially naïve control (Canton S) males in our study did exhibit interspecific courtship with *D. virilis* females (fig 3.5 a-d). However, inter-specific courtship by control males was quickly terminated in favor of conspecific pursuits. In contrast, *tdc2-gal4/+; UAS-MBD2-RNAi/+* males displayed significantly high levels of interspecific courtship (fig 3.5 a-d). The number of single wing extensions (SWE) towards *D. virilis* females was increased in MBDR2-deficient males as compared to the control group (fig 3.5 a).

Additionally, the number of copulatory abdominal bendings towards *D. virilis* females was also increased in experimental males (fig 3.5 d). Although, the average duration of conspecific wing extensions remained the same in both control and experimental groups, the duration of interspecific wing extensions towards *D. virilis* females was shortened in the control group, and increased in MBDR2-deficient males (fig 3.5 b). Overall, experimental males spent ~80% of total time courting *D. virilis* females and only ~20% time courting conspecific CS females (fig 3.5 c).

While the latency to initiate courtship (fig 3.5 f) and overall courtship vigor – measured by courtship index (C.I.) (fig 3.5 e) – were not altered, MBDR2-deficient males...
exhibited a significant delay in copulating with conspecific females (*fig 3.5 f*). In terms of reproductive fitness, one of the consequences of observed disinhibition of interspecific courtship in experimental males was a significant reduction in conspecific mating success (*fig 3.5 g*). Together, these results suggest male *Drosophila* require dMBD-R2 function in OA neurons to respond correctly to sex- and species-specific cues.
3.3.5 Selective hypermethylation in OA neurons increases male-male courtship

The function of dMBDR2 as a component of NSL chromatin remodeling machinery has been characterized in recent years (Raja et al., 2010; Lam et al., 2012; Prestel et al., 2010). Not unlike its extensively studied vertebrate homolog – MeCP2, dMBDR2 binds genomic DNA, interacts with histone acetyltransferases (HAT) and is involved in chromatin restructuring and regulation of gene expression (Raja et al., 2010; Lam et al., 2012; Prestel et al., 2010). However, despite the presence of methyl-CpG binding domain (MBD) and structural conservation of DNA binding sites, its ability to interact with methyl-\(^5\)C tags remains elusive (Boffelli et al., 2014).

Due to the relatively sparse distribution of \(^5\)C-methylation in *Drosophila*, we postulated that dMBD-R2 exerts its effects on social behavior through methylation-independent interactions. Therefore, we first sought to characterize the hyper-methylation phenotype in context of social behavior and asked if selective hypermethylation of OA neuron genome alters male aggression and courtship. For this purpose, we expressed the murine *de novo* DNA methyltransferase DNMT3a in OA neurons with the Gal4-UAS system. DMNT3a expression has previously been reported to cause cytosine methylation in *Drosophila* and cause at least three-fold increase in embryonic methylation levels (Lyko et al., 1999; Lyko et al., 2000; Weissmann et al., 2003).

We found that experimentally-induced hypermethylation of OA neurons did not significantly alter male aggressiveness. While the initiation of aggression was delayed in *tdc2-gal4/+UAS-Dnmt3a/+* males (*fig 3.6 c*), no statistically significant changes were observed in the number of lunges or wing threats (*fig 3.6 a-b*). The overall frequency of dominance hierarchy relationships remained comparable to transgenic control males as well (*fig 3.6 d*). However, the experimental males exhibited a significant increase in male-male courtship within the aggression paradigm (*fig 3.6 e*). As the latency to the first lunge was increased in addition to impaired disinhibition of male-male courtship, these results suggest an increased uncertainty in behavioral object choice.
3.3.6 Effects of dMBDR2-knockdown vary across levels of genomic methylation

Experimentally-induced de novo DNA methylation in Drosophila has previously been demonstrated to cause an increase in histone H3K9 methylation and a reduction in histone H3S10 phosphorylation (Weissmann et al., 2003). As H3K9me is associated with the formation of transcriptionally inactive heterochromatin (Peters et al., 2002; Lehner et al., 2003) and H3S10 serves as a marker for transcriptionally-active loci (Nowak and Corces, 2000), the expression of murine DNMT3a in our study is expected to cause DNA compaction and/or suppression of transcriptional activity in OA neurons.

Furthermore, dMBDR2 is a component of non-specific lethal (NSL) multi-subunit complex that also contains the Male absent on first (MOF) histone H4K16 acetyltransferase (HAT) (Raja et al., 2010). This complex is primarily associated with active chromatin states and 66% of all transcriptionally-active gene promoters are bound by dMBDR2 (Lam et al., 2012). However, there is no linear relationship between the presence of dMBD-R2 and transcriptional activity. While dMBDR2-depletion in embryonic cells is associated with a reduced expression of target genes (Prestel et al., 2010), dMBDR2-knockdown in larval salivary glands on the other hand results in differential expression of 3996 genes; some of which are up-regulated while others are down-regulated ((Raja et al., 2010), and figure 6 therein).

If the reduction in dMBDR2 levels and ectopically-induced genomic hypermethylation act through completely independent mechanisms on distinct genomic loci, then dMBDR2-knockdown and expression of DNMT3a together in OA neurons should result in an additive effect on measured behavioral outcomes. Since Dnmt3a-induced DNA methylation is likely to occur downstream of dMBD function and given the large number of genomic loci bound by dMBDR2 proteins, a more plausible alternative is that dMBDR2-dependent regulation of transcriptional activity is influenced by methylation-induced alterations in chromatin structure and assembly. However, it remains unknown if dMBDR2 is a critical component in methylation-dependent changes in chromatin compaction and transcriptional activity. If dMBDR2 functions at least partially in the readout of methylated DNA, then reducing dMBD-R2 levels in
conjunction with hypermethylation should rescue or reduce the hypermethylation phenotype.

To test whether the effect of dMBDR2-knockdown on male social behavior varies across different levels of methylation, two-way factorial ANOVA was performed for both, latency to aggression onset and male-male courtship. A significant interaction (dMBDR2 × Dnmt3a) effect was observed between dMBDR2 levels and hypermethylation on both latency to first lunge \((F_{(1,111)} = 25.08, p < 0.0001; V = 0.1459;\) Obs. Power = 1.00, fig. 3.7 a) and male-male courtship \((F_{(1,111)} = 37.89, p < 0.0001; V = 0.246;\) Obs. Power = 1.00, fig. 3.7 b). That is, the effect of dMBDR2 on delay to aggression onset varied across the levels of relative methylation. Simple effects analysis suggests that hypermethylation precludes the expression of dMBDR2-induced effects in context of aggression. At the same time, although both ectopic methylation and reduction in dMBDR2 levels separately increased male-male courtship but when present together, result in a complete rescue of male courtship behavior \((fig. 3.6e, 3.7b).\) As discussed subsequently in section 3.4, these results suggest non-linear multilayered interactions between dMBDR2 and Dnmt3a-induced hypermethylation states in determining the overall behavioral outcome of an organism.
In this chapter, we describe a novel contribution of endogenous methyl-CpG binding proteins in the regulation of male social behavior in *Drosophila*. Across species, methyl binding proteins (MBPs) play a critical role in spatiotemporal regulation of gene expression. This dynamic regulation of transcriptional activity can be achieved in a methylation-dependent or –independent manner by structuring and remodeling of chromatin states through association with various histone modification complexes.

At least two different modes of genomic methylation have recently been confirmed in *Drosophila* (Capuano et al., 2014; Zhang et al., 2015). Although, both of these methylation states have been associated with the regulation of gene expression (Zhang et al., 2015; Takayama et al., 2014), the underlying mechanistic processes that translate these epigenetic marks to appropriate functional states remain obscure.

There are multiple MBD-containing proteins in *Drosophila*, including dSETDB1 (egg), Toutatis (tou), dMBD-R2 and dMBD2/3. Of these, dSETDB1/Egg has been categorized to the histone (lysine) methyltransferase (HMT) family of MBD proteins (Völkel and Angrand, 2007), Toutatis to the histone acetyltransferase (HAT) family of MBD proteins (Vanolst et al., 2005; Emelyanov et al., 2012), and both dMBD-R2 and dMBD2/3 (Hendrich and Tweedie, 2003) rest in the MBD family. While all of these proteins have been implicated for their roles in various chromatin remodeling complexes, only dSETDB1/Egg (Gou et al., 2010) and dMBD2/3 (Roder et al., 2000) (but see Ballestar et al., 2001) have been demonstrated to associate with methylated cytosine residues *in vitro*. Furthermore, none of these genes, to my knowledge, have been studied for their role in context of gross organismal behavior in *Drosophila*. In this study, my colleagues and I tried to fill in that gap by exploring the role of dMBD-R2 in context of highly dynamic species- and sex-specific behavioral interactions. We found that both dMBD-R2 and dMBD2/3 mediate OA neuromodulatory processes in context of aggression and courtship.

We also explored the possibility of an interaction between DNA methylation states and dMBD-R2 function. Polyten chromosome staining by our lab (Chapter II; fig:
2.12) and others (Raja et al., 2010) revealed extensive genome-wide association of
dMBD2. Although, a direct association between dMBD2 and m5C has not been
demonstrated, we asked if dMBD2 function could be altered by differential methylation
states. A direct investigation of this hypothesis by eliminating the endogenous
methylation states is constrained by relatively sparse distribution of methylated cytosines
and lack of a known DNA methyltransferase in Drosophila (Takayama et al., 2014).
Overexpression of a demethylase like dTet (Dunwell et al., 2013; Guo et al., 2011) would
have opened up the possibility of increased levels of oxidated residues including 5-
hydroxymethylcytosine (5hmC) (Guo et al., 2011). As 5hmC has recently been shown to
act as an epigenetic signature in its own right and interact with the human MBD-
containing protein – MeCp2 (Mellén et al., 2012), such an experimental design would
have further confounded our analysis. Therefore, we attempted to address this question
by ectopically inducing a targeted hypermethylation state by expressing murine de novo
DNA methyltransferase (Dnmt3a) selectively in OA neurons. Using a 2 x 2 factorial
design, we found that the effects of dMBD2 on male social behavior varied across
levels of DNA methylation.

While a concurrent dMBD2-knockdown completely rescued the
hypermethylation-induced homosexual courtship phenotype in our study (fig 3.7b), one
must tread the water cautiously with respect to proposing a direct functional association
between genomic methylation and dMBD2 proteins. In addition to the lack of direct
evidence for methylation-dependence of dMBD2-function, there are a number of
different factors that may further confound our interpretation of these results. In addition
to genomic hypermethylation, Dnmt3a expression in Drosophila can cause an increase in
H3K9 methylation – a hallmark of chromatin silencing and heterochromatin formation
(Weissmann et al., 2003). Since – a) dSETDB1 is the only essential H3K9
methyltransferase in Drosophila (Koch et al., 2009), b) SETDB1 has been shown to
interact with Dnmt3a in mammalian context (Li et al., 2006), and c) Dnmt3a can itself
repress transcription through ATRX-like PHD domains and direct association with
histone deacetylase HDAC1, independent of its CpG methylation activity (Bachman et
al., 2001). It is plausible, therefore, that the alterations in latency to aggression (fig 3.6c)
and inter-male courtship (fig 3.6e) in Dnmt3a-expressing males are caused by direct
alterations in chromatin structure and transcriptional activity through Dnmt3a-dSETDB1 or HDAC1 interactions, and not by genomic hypermethylation \textit{per se}. A further concern that dSETDB1 itself binds methylated cytosines in the $^5$CpA dinucleotide context (Gou et al., 2010) is mitigated by CpG selective hypermethylation activity of Dnmt3a (Oka et al., 2006). As a result, an alternative interpretation of these results may suggest that dMBDR2 rescues Dnmt3a/dSETDB1-mediated alterations in male social behavior. For what it’s worth, Dnmt3a also displays extensive co-localization with MBD1 and MeCP2 in mouse somatic cells, ES cells and NIH 3T3 cells (Bachman et al., 2001; Lewis et al., 1992; Hendrich and Bird, 1998).

At the same time, a low level ubiquitous expression of mouse Dnmt3a has been reported to greatly increase the proportion of methylated $^5$CpG-residues to 4% – a very significant increase from the 0% $^5$mCpG levels detected by the same assay in comparison lines expressing maintenance methyltransferase Dnmt1 (see (Lyko et al., 1999); Table 1 from the article has been reproduced here as Table 3.1). Furthermore, depletion of MBD-R2 impairs the development of salivary glands and results in a reduced gland size (Raja et al., 2010). Coincidentally, or perhaps not, a significant reduction in salivary gland size was also reported in hypermethylated flies by a separate group (Weissmann et al., 2003). Because of a very significant increase in methylation levels and shared phenotypic alterations, we cannot completely exclude the possibility that hypermethylation plays a role in observed behavioral shifts in aggression and courtship in Dnmt3a lines in our study, in favor of the alternative hypothesis outlined above (fig 3.6 c, e). At this point, our results suggest that dMBDR2-function varies across levels of genomic methylation in \textit{Drosophila}.

The observation that \textit{Drosophila} MBD-containing proteins play a significant role in the regulation of social behavior is consistent with the role of MBD-family proteins in other organisms. In both mice and humans, the MBD-containing protein – MeCP2 – is critical for normal functioning of genes associated with the regulation of social behavior (Huppke et al., 2006; Tantra et al., 2014; Moretti et al., 2005). Multiple accounts of socio-behavioral effects of the mammalian methyl CpG binding protein 2 (MeCP2) have associated this key MBD-family protein with the modulation of territoriality and
aggression in mammals. In mice, conditional knockout of MeCP2 in serotonergic neurons, and separately in a subset of hypothalamic neurons, results in a significant increase in aggressive attacks towards unfamiliar cage mates in a resident-intruder assay (Fyffe et al., 2008; Samaco et al., 2009). Alterations in MeCP2 expression have also been associated with poor impulse control and social aggression in schizophrenia cohorts as well as monogenic disorders such as rett syndrome and MeCP2-duplication syndrome in humans (Huppke et al., 2006; Tantra et al., 2014; Ramocki et al., 2009). The direction of MeCP2-induced alterations in social behavior varies significantly with the genetic background. That is, depending on the specific genetic context, an increase or decrease in MeCP2 levels may modulate aggressive phenotypes in either direction. For instance, both Rett syndrome patients, in which there’s a loss of MeCP2 function, and patients with MeCP2 duplication syndrome display bouts of hostility and/or uncontrolled aggression (Huppke et al., 2006; Ramocki et al., 2009). Such context-dependence and non-linear association between MBD proteins and the direction of behavioral change may explain why both reduction of dMBDR2 and increase in genomic methylation separately alter the delay to aggression onset (compare fig 3.1c and fig 3.6c) and male-male courtship (compare fig 3.1e and fig 3.6e) in the same direction. In support of this hypothesis, as mentioned previously, both reduction in dMBDR2 levels and hypermethylation have separately been reported to alter the size of the salivary glands in the same direction (Raja et al., 2010; Weissmann et al., 2003).

Additional results in our study pertain to the role of dMBDR2 proteins in the regulation of inter-species courtship. We demonstrate that dMBDR2-deficient males enthusiastically, much more so than controls, court females of a distantly-related species (fig 3.5 b-e). Wildtype D. melanogaster males have previously been reported to interact sexually with other, distantly related, sympatric drosophilid species (Dawson and McRobert, 2011; Dukas, 2004). However, such interspecific courtship interactions are reproductively futile and energetically inefficient as very few species are able to copulate and hybridize with D. melanogaster (David et al., 1974; Tsacas and BäChli, 1981). In a few cases where copulation does occur, hybrid incompatibility and sterility has been well documented (Sturtevant, 1920; Barbash, 2010). In many cases, however, Drosophila males adopt pre-mating behavioral strategies for reproductive isolation by restricting courtship displays towards con-specific
females (Spieth, 1974; Spieth and Ringo, 1983). These reports are consistent with recent evidence pointing towards existence of chemosensory and neurobiological filters for species-identification and inhibition of interspecific courtship (Fan et al., 2013; Dukas, 2004). Our group recently demonstrated that OA-neurons act as second-order transducers in Gr3a-mediated chemosensory-information pathway (Andrews et al., 2014). The shorter duration of interspecific wing extensions by control males towards *D. virilis* females (fig 3.5 b; *p=0.0434*) in our study may reflect the ability to reliably process and respond to species-specific identification cues resulting in termination of singing and courtship sequence, or lack thereof in case of dMBD-R2 deficient males (Agrawal et al., 2014). At this point, we do not know if the observed defects in responding to sex- and species-specific cues are due to a requirement for dMBD-R2 in the subset of OA neurons that promote male courtship, or a separate requirement for dMBD-R2 in a set of OA neurons that modulate the inhibition of male-male or interspecies courtship. It has also been suggested that male-female courtship specificity and avoidance of male-male courtship is a learned phenomenon where males learn to refrain from male-male courtship after experiencing antiaphrodisiac pheromones and rejection from other males (Spieth, 1974; Anaka et al., 2008; Hirsch and Tompkins, 1994). Context-inappropriate behaviors such as homosexual courtship or reduced sex or species specificity in courtship attempts may, therefore, suggest learning deficits as well as difficulties in gender recognition. A number of mutants with learning-deficits also display male-male courtship (Anaka et al., 2008; McRobert et al., 2003; Savvateeva et al., 2000). As OA is involved in the formation of courtship memory (Zhou et al., 2012; Chartove et al., 2015), it may therefore also facilitate specification of context-appropriate behaviors through learning and memory of previous social experiences in addition to its role in species and sex recognition. However, it is clear dMBD-R2 plays an important role in the molecular basis of species and sex discrimination in addition to, or in exclusion of, learning and memory of courtship rejection cues in *Drosophila* and contributes to our understanding of pre-mating behavioral strategies for reproductive isolation.
Figure 3.1: dMBDR2 knockdown in OA neurons reduces conspecific aggression and increases male-male courtship.

(A–D) Dyadic agonistic interactions between pairs of males with RNAi-based reduction in dMBDR2 levels in OA neurons (Tdc2-Gal4/+; UAS-MBDR2IR/+; n=20) and individual transgenic controls, UAS-MBDR2IR/+ (n=21) or Tdc2-Gal4 (n=18). (A) Number of lunges (represented by each dot) in a 30 min scoring period after the first lunge by either male in a fighting pair. dMBDR2-deficient males exhibited a significant reduction as compared to controls (****P adj<0.0001). (B) Number of wing threats in the same 30 min scoring period. A significant reduction is observed in average number of wing-threats in dMBDR2-deficient males compared to transgenic controls (****P adj<0.0001). (C) The latency to first lunge or delay to onset of aggression was significantly higher in Tdc2-Gal4/+; UAS-MBDR2IR/+ males as compared to controls.
Percent of encounters that result in fighting and formation of dominance hierarchies in control and experimental groups. Dominance was characterized by 3 consecutive lunges followed by chase behavior. This criterion was relaxed for the experimental group because of extremely low number of lunges in each fight and essentially represents % of encounters that resulted in fighting. Male-male courtship measured by the number of unilateral wing extensions within the aggression paradigm was significantly increased in MBDR2-deficient males as compared to both transgenic controls (****$P_{adj}$<0.0001). Unless noted otherwise one-way ANOVA with Sidak’s multiple comparison test was used in all cases. Data is represented as Mean ± 95% confidence interval (C.I.) of mean. Each $p$-value was adjusted ($P_{adj}$) to account for multiple comparisons at family-wise $\alpha = 0.05$. Only the most conservative value was reported for each family-wise comparison.
Figure 3.2: dMBD2/3 knockdown in OA neurons reduces conspecific aggression and increases male-male courtship

(A–D) Dyadic agonistic interactions between pairs of males with RNAi-based reduction in dMBD2/3 levels in OA neurons (Tdc2-Gal4/+; UAS-MBD2/3IR/+; n=18) and individual transgenic controls, UAS-MBD2/3IR/+ (n=23) or Tdc2-Gal4 (n=18). (A) Number of lunges (represented by each dot) in a 30 min scoring period after the first lunge by either male in a fighting pair. dMBDR2-deficient males exhibited a significant reduction as compared to controls (**P_{adj} = 0.0087). (B) No change was observed in the average number of wing-threats in dMBDR2-deficient males compared to transgenic controls (nsP_{adj} = 0.5106). (C) The latency to first lunge or delay to onset of aggression was significantly higher in Tdc2-Gal4/+; UAS-MBD23IR/+ males as compared to controls (**P_{adj} = 0.0022). (D) Percent of encounters that result in fighting and formation of dominance hierarchies showed a modest decrease in experimental groups. Dominance was characterized by 3 consecutive lunges followed by chase behavior. (E) Male-male courtship measured by the number of unilateral wing extensions within the aggression
paradigm was significantly increased in MBD2/3-deficient males as compared to both transgenic controls (**P_{adj}<0.0001). One-way ANOVA with Sidak’s multiple comparison test was used in all cases. Data is represented as Mean ± S.E.M (standard error of mean). Each p-value was adjusted (P_{adj}) to account for multiple comparisons at family-wise α = 0.05. Only the most conservative value was reported for each family-wise comparison.
Figure 3.3: dMBDR2-knockdown in small subset of OA neurons modulates aggression not courtship.

(A–A’’) Subset of OA neurons in adult brain of tdc2-gal4/UASmCD8:gfpl/UAS-Cha-Gal80 male (nc82 labels neuropil regions - blue; anti-GFP - green; mAb | Gray channel panels are shown for enhanced contrast). (B-D) Dyadic agonistic interactions between pairs of males with RNAi-based reduction in dMBDR2 levels in a subset of OA neurons (Tdc2-Gal4/+; UAS-MBDR2IR/Cha-Gal80; n=18) and individual transgenic controls, UAS-MBDR2IR/+ (n=23) or Tdc2-Gal4/+; Cha-Gal80/+ (n=14). (B) Experimental males exhibited low baseline levels of male-male courtship measured by the number of unilateral wing extensions within the aggression paradigm and were not statistically different from one of the transgenic controls (ns\textit{ }P_{adj}=0.0587). (C) Number of lunges (represented by each dot) in a 30 min scoring period after the first lunge by either male in a fighting pair. Experimental males exhibited a significant reduction as compared to controls (\textit{ }**P_{adj}=0.0020). (D) Males with reduced levels of dMBDR2 in Tdc2-Gal4/Cha-Gal80 neurons exhibited a significant reduction in the average number of wing-threats compared to transgenic controls (\textit{ }**P_{adj}=0.0031). (E) The latency to first lunge or delay to onset of aggression was not altered in experimental males as compared to transgenic controls (ns\textit{ }P_{adj}=0.7178). One-way ANOVA with Sidak’s multiple comparison test was used in all cases. Data is represented as Mean ± S.E.M (standard error of mean). Each \textit{p-value} was adjusted (\textit{P}_{adj}) to account for multiple comparisons at family-wise \(\alpha = 0.05\). Only the most conservative value was reported for each family-wise comparison.
Figure 3.4: Reducing MBD-R2 levels in adult OA neurons recapitulates male aggression deficits

(AA’–BB’) Side-by-side comparison of 3rd instar larvae (A-A’), and pupae (B-B’) raised at 18-19°C expressing GFP under the control of actin promoter (Act5c-Gal4) in the presence or absence of temperature-sensitive Tub-Gal80ts repressor. (A-B) represents pseudo-colored heat-maps representing intensity of GFP signal which is quantified in panels (A’-B’) corresponding to the green horizontal lines cutting across the images. UAS-20XmCD8:gfp/+; Act5c-gal4/Tub-Gal80ts larva and pupa raised at 18-19°C display a clear absence of GFP signal in comparison to UAS-20XmCD8:gfp/+; Act5c-gal4/+ larva and pupa also raised at 18-19°C. (C-E) Dyadic agonistic interactions between pairs of males with adult-specific RNAi-based reduction in dMBDR2 levels in OA neurons (Tdc2-Gal4/+; UAS-MBD2IR/Tub-Gal80ts; n=15) and transgenic control, Tdc2-Gal4/+; Tub-Gal80ts/+ (n=11). (C) Number of lunges (represented by each dot) in a 30 min scoring period after the first lunge by either male in a fighting pair. Experimental
males exhibited a significant reduction in lunges as compared to controls (**P = 0.0085). No statistical evidence was obtained for a significant difference in the (D) latency to first lunge or delay to onset of aggression (\(^{\text{ns}}\)P = 0.1357). (E) or number of wing-threats (\(^{\text{ns}}\)P = 0.4792) between experimental and transgenic control males. (F) Adult-specific reduction in MBDR2 in OA neurons increased male-male courtship measured by the number of unilateral wing extensions within the aggression paradigm (**P = 0.0010). Unpaired t-test with Welch’s correction for was used in all cases. Data is represented as Mean ± S.E.M (standard error of mean).
Figure 3.5: MBDR2-deficient males display high-levels of interspecies courtship and reduced conspecific mating

(A-D) Courtship behaviors of MBDR2-deficient (*D. mel, Tdc2-Gal4/++; UAS-MBDR2IR/++; n=18) and control (*D. mel, Canton S; n=16) males towards conspecific (*D. mel; labeled CS) and interspecific (*D. virilis; labeled DV) females in a courtship-choice/preference assay. (A) Number of unilateral/single wing extensions (singing; SWE) towards conspecific and interspecific females. Interspecific wing extensions as a fraction of total wing extensions towards either female were calculated as: SWE_DV/(SWE:CS+SWE:DV). MBDR2-deficient males disproportionately courted interspecific female over conspecific female (**p<0.0001). (B) Average length of each unilateral wing extension was estimated. Experimental males exhibited an increase in duration of interspecific wing extensions (**p = 0.0006). Duration of conspecific wing extensions was comparable to the controls (ns P = 0.7142). Control males exhibited shorter wing extensions towards *virilis females as compared to conspecific females (*P = 0.0434). (C) Males with reduced levels of dMBDR2 in Tdc2-Gal4 neurons spent majority of their time courting *virilis females as compared to transgenic controls (**p< 0.0001). (D) Number of interspecific attempted matings or copulatory abdominal bendings in an attempt to mount the female were increased in experimental males (**p=0.0002). (E) Courtship index (C.I.) was calculated as total time spent courting any female as a fraction of total scoring period (600sec). In case of conspecific copulation within the scoring period, time to copulation was used as a denominator. Average C.I. of experimental males was similar to that of control males (nsP=0.6883) (F) The latency to first single wing extension
(courtship) to either female and delay to successful conspecific copulation were measured in control and experimental males. As compared to controls, latency to courtship was not altered (\( ^{*} \)P =0.1637) while conspecific copulation was delayed significantly in \( Tdc2\)\( -\)Gal4/+; UAS-MBDR2IR/+ males (\( ^{*} \)P =0.0153). \( (G) \) Percent of assays that resulted in a successful conspecific mating event was significantly decreased in MBDR2-deficient males (50% mating success rate) as compared to the control groups (81.25% mating success). Mann-Whitney test was used in all cases, unless otherwise specified. Data is represented as Mean ± S.E.M (standard error of mean).
Figure 3.6: Selective hypermethylation of OA neurons increases male-male courtship

(A-D) Aggressive behaviors between pairs of males with selectively-induced genomic \((m^5CpG)\) hypermethylation in OA neurons by expressing mouse DNA methyltransferase Dnmt3a \((Tdc2-Gal4/++; UAS-Dnmt3a/++; n=20)\) and individual transgenic controls, \(UAS-Dnmt3a/+(n=21)\) or \(Tdc2-Gal4/+ (n=18)\). No difference was observed in the (A) number of lunges in a 30 min scoring period \(\text{One-way ANOVA: } ^*p_{adj}=0.1357 \mid \text{Bootstrap: } F_{C1-EXP}=12.046, ^**p=0.001, d=0.571; \text{ and } F_{c2-EXP}=3.032, ^ns p=0.089, d=0.279 \) and (B) number of wing-threats \(^ns p_{adj}=0.2354 \) between experimental and control males. (C) Males with selective hypermethylation in OA neurons exhibited a significant delay in onset of aggression or the latency to first lunge compared to transgenic controls \(\text{One-way ANOVA: } ^**p_{adj}=0.0057 \mid \text{Bootstrap: } F_{C1-EXP}=9.098, ^**p=0.004, d=0.496; \text{ and } F_{c2-EXP}=5.430, ^*p=0.025, d=0.373 \) (D) Percent of fights that resulted in clear-establishment of dominant-subordinate relationship exhibited only a marginal decrease in experimental groups. Dominance was characterized by 3...
consecutive lunges followed by chase behavior. (E) Tdc2-Gal4/+; UAS-Dnmt3a/+ males exhibited an increase in male-male courtship measured by the number of unilateral wing extensions within the aggression paradigm as compared to the transgenic control pairs (One-way ANOVA: *P_{adj} = 0.0178 | Bootstrap: F_{C1-EXP} = 8.428, **p = 0.003, \textit{d} = 0.478; and F_{C2-EXP} = 5.146, p = 0.026, \textit{d} = 0.363; \textit{d} = \text{effect size}; \textit{C1} and \textit{C2} represent respective transgenic control groups \textit{tdc2-gal4/+} and \textit{UAS-Dnmt3a/+}). One-way ANOVA with Sidak’s multiple comparison test was used in all cases. In case of panels C and E where few-extreme values skewed the distribution, instead of data transformations or outlier removal, original data was cross-validated by non-parametric bootstrapping-based resampling methods (see materials and methods) as these data form critical components for subsequent analysis and interpretations with regard to dMBDR2 function. Penal A was also cross-checked with bootstrapping methods to avoid selection bias. In all 3 instances, bootstrapping methods confirmed the validity of parametric ANOVA results. Data is represented as Mean ± S.E.M (standard error of mean). Each \textit{p-value} was adjusted (P_{\text{adj}}) to account for multiple comparisons at family-wise \textit{\alpha} = 0.05. In most cases, only the most conservative value was reported for each family-wise comparison.
Figure 3.7: Effects of dMBDR2-knockdown vary across levels of genomic methylation

(A-B) Two-way (2 x 2) Factorial ANOVA illustrating an interaction effect between dMBDR2-knockdown and selectively-induced genomic (m^5CpG) hypermethylation in OA neurons by expressing mouse DNA methyltransferase Dnmt3a (A) Effect of dMBDR2 on the latency to lunge varies significantly across methylation states (Interaction dMBDR2 x Dnmt3a: $F_{(1, 111)} = 25.08, p < 0.0001; V = 0.1459; \text{Obs. Power} = 1.00$), and (B) Effect of dMBDR2-knockdown on the number of male-male courtship events measured by counting unilateral wing extensions between pairs of males also varies across levels of Dnmt3a-induced methylation states (Interaction dMBDR2 x Dnmt3a: $F_{(1, 111)} = 37.89, p < 0.0001; V = 0.246; \text{Obs. Power} = 1.00$). Additionally, a concurrent dMBD-R2 knockdown rescues Dnmt3a-induced increase in male-male courtship ($F = 9.055, **p=0.003, d=0.503; \text{Bootstrapped ANOVA. d= effect size, see materials and methods}$).
Table 3.1: Indicating UAS-Dmnt3a-induced increase in genomic m⁵CpG levels.

Reproduced from (Lyko et al., 1999)
3.6 REFERENCES


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