The Actin-binding Protein Moesin and Memory Formation in *Drosophila*

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Abstract

Moesin is a cytoskeletal adaptor protein that plays an important role in modification of the actin cytoskeleton and the formation of dendritic spines, which may be crucial to long-term potentiation. Moesin has also been found to be overexpressed in brains affected by Alzheimer's disease. Despite being identified as a potential memory gene and linked to several neurological diseases, its role in memory has not been evaluated. The role of Moesin in the *Drosophila melanogaster* brain was investigated by characterizing the impact of modulating Moesin expression on several aspects of development and behavior. Moesin is involved in both brain and eye development. Knockdown and overexpression of Moesin led to defects in the development of the mushroom body, a brain structure critical for memory formation and recall. Further, knockdown of Moesin throughout development resulted in a significant deficit in long-term memory. Additionally, knockdown of Moesin restricted to adulthood also resulted in a significant deficit in long-term memory, which suggests that Moesin also has a non-developmental role in memory. Further, this requirement for Moesin in long-term memory was traced to the alpha/beta and gamma neurons of the mushroom body. Through the use of a phosphomimetic Moesin mutant that mimics the phosphorylated, activated form of Moesin, the regulation of Moesin in the *Drosophila* brain was analyzed. Expression of this mutant in neurons disrupted photoreceptor development in the *Drosophila* eye and a novel sensorimotor phenotype attributed to its expression in the brain was identified resulting in a defect in stereotypical climbing behavior. These results suggest a critical role for Moesin in general neurological functioning and the molecular pathways involved in its activation require further investigation.

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Abbreviations

BDSC	Bloomington Drosophila Stock Center
CAMKII	Ca2+/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division control protein 42 homolog
CI	Courtship index
CRE	cAMP response element
CREB	cAMP response element binding protein
CPEB	Cytoplasmic polyadenylation element binding protein
ELAV	Embryonic lethal, abnormal vision
ERM	Ezrin, Radixin, Moesin
FasII	Fasciclin II
FERM	Four point one, Ezrin, Radixin, Moesin
GluRIIA	Glutamine receptor IIA
HDAC4	Histone deacetylase 4
IHC	Immunohistochemistry
KD	Knockdown
L1CAM	L1 cell adhesion molecule
LPTC	Lobula plate tangential cells
LRRK2	Leucine-rich repeat kinase-2
LTM	Long-term memory
MARCM	Mosaic analysis with a repressible cell marker
MI	Memory index
MOE	Moesin
NGF	Nerve growth factor
OE	Overexpression
PKA	cAMP-dependent protein kinase A
Rac1	Ras-related C3 botulinum toxin substrate 1

RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
SEM	Scanning electron miscroscopy
STM	Short-term memory
TARGET	Temporal and regional gene expression targeting
UAS	Upstream activating sequence
VDRC	Vienna Drosophila Resource Center
WB	Western Blot

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Chapter 1: Introduction

1.1 The Molecular Basis of Memory Formation

The first cellular theory of memory storage, put forward by Santiago Ramon y Cajal, proposed that memory storage is based upon an anatomical change in the functional connections between neurons [1, 2]. These neuronal connections would later be termed synapses and the modulation of their strength in response to learning would become known as synaptic plasticity [3, 4]. Models were advanced to describe a basic mechanism for synaptic plasticity, however, the evaluation of these models required the development of tractable behavioral systems for the analysis of specific changes in neuronal connections following learning [5, 6]. Several elementary model systems were utilized in the initial attempts to identify the neuronal changes underlying learning and memory including the conditioning of basic behaviors such as the gill-withdrawal reflex of the sea slug *Aplysia* and olfactory conditioning in the common fruit fly *Drosophila.* This reductionist approach revealed that even animals with relatively simple nervous systems—from approximately 20,000 neurons in the central nervous system of *Aplysia* to 300,000 in *Drosophila*—are capable of multiple forms of learning which give rise to short- and long-term memory [7]. These simple forms of learning provided a tractable system for the investigation the molecular basis of learning and memory and identified key components of memory consolidation across species.

The first molecular mechanism of memory to be identified was a change in synaptic strength resulting from the modulation of neurotransmitter release. A single noxious stimulus to the tail in *Aplysia* was found to increase the strength of the synaptic connections between the sensory and motor neurons that control the gill-withdrawal reflex [8]. This sensitizing stimulus activates modulatory neurons to release the neurotransmitter serotonin onto the sensory neuron [9-11]. Serotonin release by these modulatory neurons leads to an increase in the concentration of cyclic adenosine monophosphate (cAMP) in the sensory neuron [8]. cAMP is known as an intracellular "second" messenger as it is activated by "first" messengers, such as hormones, which cannot pass through the cell membrane [12]. In the sensory neuron, elevated levels of cAMP induce the release of the neurotransmitter glutamate at the sensory to motor

neuron synapse, leading to a temporary strengthening of this connection [8]. This elementary system revealed a principle link between the modification of a behavior and biochemical changes at the level of the synapse.

The first clue that the molecular mechanisms of memory may be shared among animals came from the isolation of learning mutants from large-scale mutagenesis screens in *Drosophila*. Mutations in single genes were identified which impair olfactory learning and several of these mutant genes were found to encode components of the cAMP signaling pathway including adenylyl cyclase, cAMP phosphodiesterase, cAMP-dependent protein kinase (PKA), as well as the transcription factor cAMP-response element binding protein (CREB) [13-17].

The continued analysis in these model systems revealed that these simple forms of learning give rise to distinct phases of memory storage with short- and long-term stages. Whereas a single training session produces a short-term memory lasting minutes to hours, repeated training sessions that are temporally spaced give rise to long-term memory that lasts from days to weeks [18-20]. These behavioral changes were soon discovered to have biological parallels in synaptic plasticity, suggesting that short- and long-term memory storage share aspects of a common mechanism [21, 22]. Examination of the sensory to motor neuron synapse responsible for the *Aplysia* gill-withdrawal reflex in dissociated cell culture has revealed how training timing can influence behavioral changes at the cellular and molecular level. Plastic changes in synaptic strength can be reproduced in this cell culture system by substituting a brief application of serotonin in place of the noxious stimulus to the tail used *in vivo* [9, 10, 23]. A single application of serotonin gives rise to a short-term increase in synaptic strength (short-term facilitation), while changes in synaptic strength lasting more than a week (long-term facilitation) require repeated, spaced applications [23]. By highlighting a common mechanism in the establishment of short- and long-term facilitation, these molecular insights indicate a potential pathway for the conversion of short-term memory into long-term memory.

This conversion process, termed consolidation, was found to be initiated by the prolonged elevation of cAMP levels induced by the repeated release of serotonin and resulting in

new protein synthesis [24, 25]. Transient elevation of cAMP levels in sensory neurons resulting from a single noxious stimulus to the tail or serotonin application leads to cAMP-dependent protein kinase (PKA) activation and localized effects at the synaptic terminal that enhance neurotransmitter availability and release for short-term facilitation [26]. However, the repeated release of serotonin results in a sustained increase in cAMP levels in sensory neurons that stimulates PKA to move to the nucleus where it activates gene expression through the phosphorylation of transcription factors [26, 27].

A requirement for new protein synthesis following training for the consolidation of long-term memory has also been observed in vertebrates and the signaling cascade leading to this gene expression is highly conserved [28, 29]. The discovery of the cAMP Response Element (CRE), a conserved DNA sequence in promoter elements that are activated by cAMP as well as a transcription factor that binds this element, the cAMP Response Element Binding protein (CREB), revealed a link from cAMP signaling to targeted gene expression shared between *Aplysia*, *Drosophila* and vertebrates [30]. The connection between CRE-driven transcription, downstream of cAMP signaling, and synaptic plasticity was first described during long-term facilitation in *Aplysia* sensory neurons. PKA was found to activate gene expression through an *Aplysia* cAMP Response Element Binding protein (CREB1) and disruption of CREB1 binding blocked long-term facilitation with no effect on short-term facilitation [31].

As the gene expression required for consolidation takes place in the nucleus, the question arose of how the newly synthesized mRNAs and proteins would be specifically conveyed to the synapses whose activation triggered their expression. A “synaptic tag” model was proposed in which the newly synthesized mRNAs and proteins are transported throughout the cell but only utilized at synapses that have been tagged by previous synaptic activity [32, 33].

In the search for a regulator of protein translation at the synapse, the cytoplasmic polyadenylation element binding protein (CPEB) was identified for its ability to activate dormant mRNA in *Xenopus* oocytes [34]. In *Aplysia*, blocking CPEB at active synapses was found to prevent the maintenance of long-term synaptic facilitation without

impacting its initiation [35, 36]. In *Drosophila*, the CPEB Orb2 has been found to be critical for the formation of long-lasting memory [37, 38]. Orb2 is thought to act as both a synaptic tag and regulator of translation at the synapse through the cooperation of its two distinct isoforms which link the acquisition and consolidation of long-term memory [37, 39-41]. The Orb2A isoform represents the putative synaptic tag, which is localized to active synapses during memory acquisition [39, 41]. Upon reactivation of these synapses Orb2A recruits Orb2B into complex to regulate local translation of CaMKII, a protein critical for the persistence of memory across species [41-43]. The "synaptic capture" or tagging model has since been supported by further studies in *Aplysia* [44, 45] as well as in the rodent hippocampus [46-48] and the recent finding that a rodent CPEB required for memory maintenance shares functional properties with the *Drosophila* and *Aplysia* CPEBs suggests this mechanism may be conserved in higher organisms [49-52].

Taken together, the pathway comprised of cAMP, PKA, CRE, CREB, and CPEB represents a conserved molecular mechanism upon which memory is formed across a broad range of species. The discovery of a conserved molecular biology of memory storage not only highlights the value of the reductionist approach to the study of neuronal plasticity but also provides a framework for the investigation of the downstream effectors that contribute to the structural and functional changes at the synapse during memory consolidation. Notably, the establishment of the synapse as the site of memory storage underscores the need to determine the precise factors that mediate their modification as neurodevelopmental and neurodegenerative disorders, such as Autism Spectrum Disorder and Alzheimer's disease, are commonly characterized by both synaptic deficits and cognitive impairment [53, 54].

1.2 The ERM Family in Neurons

1.2.1 Biology of the ERM Family

The ERM (Ezrin/Radixin/Moesin) family of proteins are adaptor molecules which provide an integral link between the actin cytoskeleton and the plasma membrane. ERM proteins have been implicated in various fundamental physiological processes including the regulation of cell shape, motility and signaling, but the scope of ERM function in neurons is only beginning to emerge [55-59]. The ERM family members interact with integral membrane proteins through an N-terminal FERM (Four point one, Ezrin, Radixin, Moesin) domain and associates with the actin cytoskeleton through a C-terminal actin-binding domain (Figure 1). ERM activity is regulated through head to tail folding in which an intramolecular association between the N- and C-terminal domains results in a "closed", inactive conformation state. Phosphorylation of a conserved threonine residue in the C-terminal actin-binding domain relieves this intermolecular association resulting in an "open", active conformation state and the unmasking of ligand-binding sites (Figure 2).

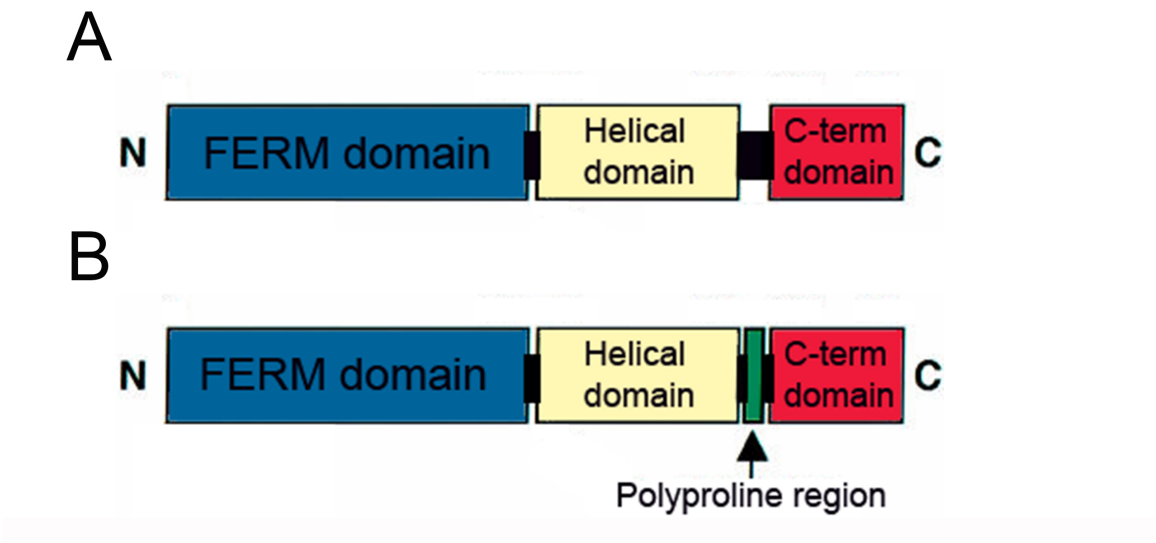


Figure 1: Domain organization of Drosophila Moesin and human ERM proteins.

A. Schematic of the domain organization of human and *Drosophila* Moesin. B. Schematic of the domain organization of human Ezrin and Radixin.

Vertebrates express three ERM genes *Ezrin*, *Radixin*, and *Moesin* while invertebrates have a single ERM gene. The sole ERM homologue in *Drosophila melanogaster* was designated Moesin, as like vertebrate Moesin it lacks the polyproline region present in Ezrin and Radixin (Figure 1A). There is a high level of conservation among ERM genes throughout evolution. Drosophila Moesin shares a 58% amino acid identity with human Moesin and some regions have upwards of 95% amino acid identity [60]. The N-terminal FERM domain (Figure 3A) is composed of three subdomains in a cloverleaf arrangement [61]. The FERM domain allows ERM proteins to act as adaptor molecules linking the actin cytoskeleton to scaffolding proteins or integral plasma membrane proteins [56]. The C-terminal domain (Figure 3B) contains the conserved threonine residue (position 559 in *Drosophila*) that can be phosphorylated to regulate the domain's interaction with actin [61].

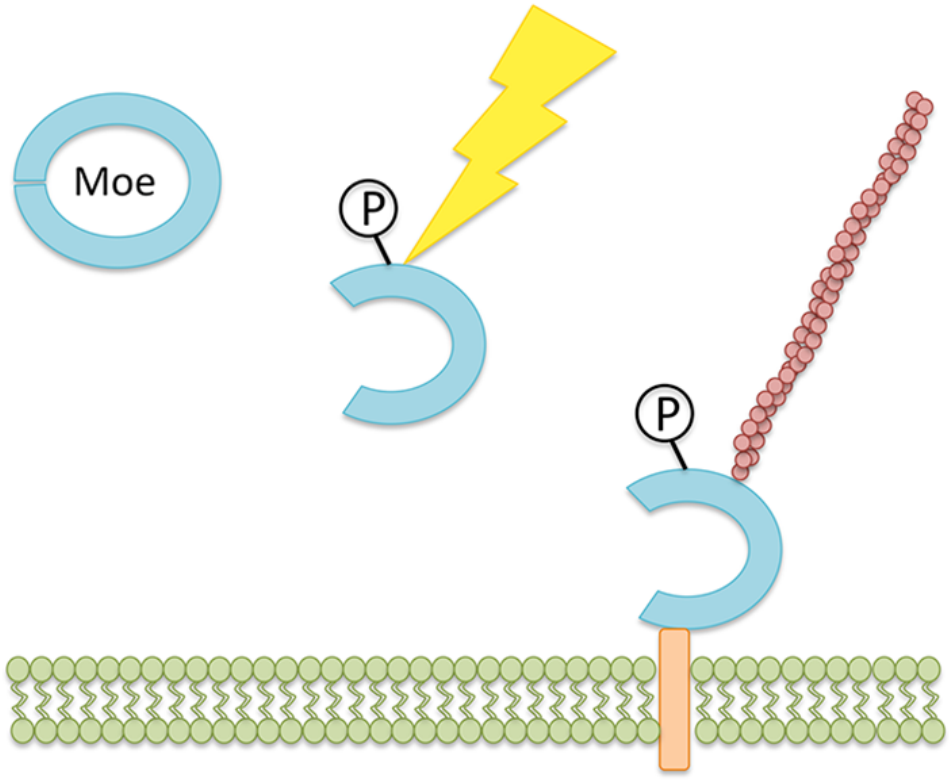


Figure 2: Phosphoregulation of Moesin

Inactive Moesin exists in a closed autoinhibitory conformation. It is activated by phosphorylation within its actin-binding domain which releases this autoinhibition, resulting in an open state allowing the binding of actin and linkage to the plasma membrane

ERM protein expression in the central nervous system of vertebrates peaks during development, and these proteins are found to localize to regions of neuronal growth in the developing brain [62, 63]. Despite our incomplete understanding of ERM function in neurons, specific roles for ERM proteins have been described in axonal guidance, dendritic morphogenesis, and synaptic plasticity.

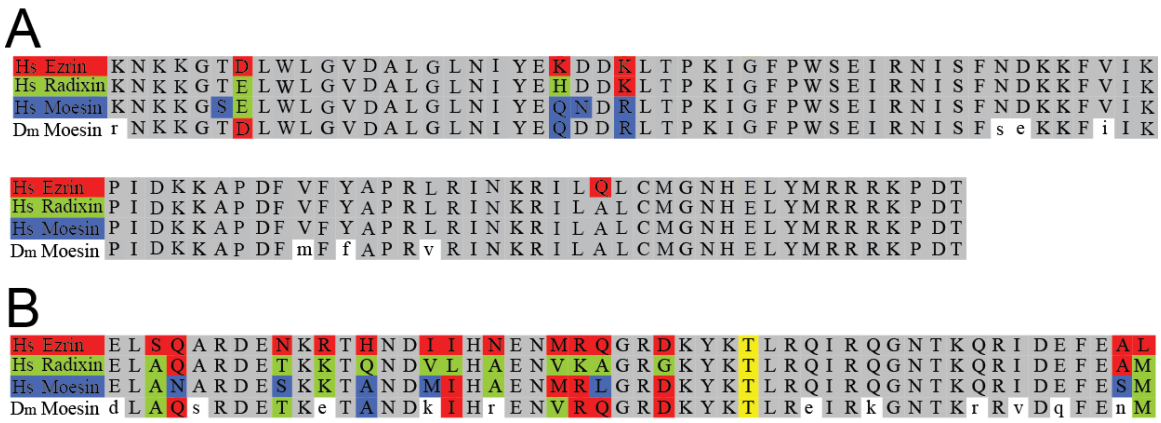


Figure 3: Conservation between Drosophila Moesin and human ERM proteins.

A. Alignment of the FERM F3 lobe amino-acid sequences of *Drosophila* Moesin and human ERM proteins. B. Alignment of the C-terminal amino-acid sequences of *Drosophila* Moesin and human ERM proteins. Conserved threonine residue that is phosphorylated for activation highlighted in yellow.

1.2.2 Axonal Guidance

A key component of neuronal development is the guidance of the growing axons toward synaptic targets [64]. Navigation of these growing axons through developing tissues is directed by a motile growth cone at the tip of the axon (Figure 4) [65]. The dynamic activity of the growth cone is characterized by persistent extension and withdrawal of actin-rich membrane protrusions, which bear membrane receptors that detect extrinsic guidance cues [66, 67]. ERM proteins have been identified as prominent components of axonal growth cones that localize to these membrane protrusions, which undergo the elongation and retraction critical for growth cone motility [62, 66, 68-70].

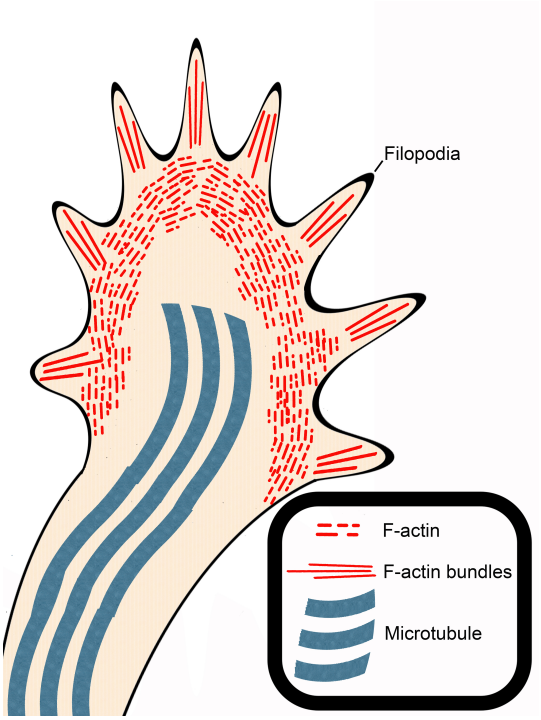


Figure 4: Membrane dynamics at the axonal growth cone

Axon growth is directed by a highly motile growth cone at its tip. The navigation of axons toward their synaptic targets is enabled by the dynamic activity of its finger-like membrane protrusions, termed filopodia. These actin-rich projections bear membrane receptors that detect extrinsic guidance cues, and the persistent extension and withdrawal of these protrusions guides axon growth.

Functional studies have revealed that collapse of the growth cone in chick sensory neurons was concomitant with a loss of radixin and that reformation of the growth cone

in response to nerve growth factor (NGF), results in the relocalization of radixin [70]. The association of radixin with growth cone formation and collapse led to analysis of the biochemical functions of ERM proteins in mammalian neurons which revealed a crucial neurodevelopmental role. Radixin and Moesin were found to be the primary ERM proteins in the growth cones of cultured rat hippocampal pyramidal neurons, and the double suppression of Moesin and radixin expression led to disorganization of F-actin at the growth cone resulting in defects in morphology and motility, while suppression of either Moesin or radixin did not impair axonal development suggesting some degree of functional redundancy [62]. The examination of ERM activation in response to extracellular growth and guidance cues such as NGF has revealed that ERM proteins are critical regulators of the cytoskeletal remodeling required at the growth cone leading margin for axon outgrowth, with an ERM-dependent increase in F-actin content at the growth cone observed in response to NGF stimulation [71, 72]. ERM proteins are locally activated at the leading edge of finger-like projections from the growth cone toward the attractive growth cue where they colocalize with the adhesion molecule L1 [72]. ERM proteins are thought to control two crucial aspects of actin filament dynamics at the leading edge in order to promote axon outgrowth. First, ERM proteins are able to anchor polymerizing actin filaments to the leading edge, acting as a driving force for membrane protrusion [64, 72]. Second, ERM proteins may also link actin polymerization to L1, allowing the growth cone to be pulled forward against L1-substrate adhesions [64, 72, 73].

Additionally, ERM proteins were found to coordinate the growth cone response to secreted guidance cues such as Sema3A. Exposure to this chemorepulsive agent rapidly inactivates ERM proteins leading to a loss of actin bundles from the growth cone filopodia [74]. This depolymerization of actin is accompanied by an ERM-dependent internalization of L1CAM and the Sema3A receptor Nrp-1 [75]. These modifications result in localized filopodial retraction, allowing for growth cone turning in response to the repulsive cue, a critical tool for guiding axon orientation for proper navigation. Taken together, these studies reveal ERM proteins to be key regulators of axonal development and highlight a crucial role in axon guidance.

1.2.3 Dendritic Morphogenesis

In addition to a role in axonal pathfinding, ERM proteins appear to function in dendritic morphogenesis as their suppression leads to altered formation of dendritic spines, the actin-rich membrane protrusions upon which most excitatory synapses are made. These specialized dendritic compartments allow for the modification of individual synapses, an important tenet of synaptic plasticity [76, 77]. ERM knockdown in cultured hippocampal neurons results in decreased density of dendritic filopodia, the precursors to dendritic spines [78]. Interestingly, this decrease in filopodial density is concomitant with a promotion of spine maturation suggesting that ERM proteins not only promote filopodia formation but also restrain filopodia-to-spine conversion. This suppression of spine maturation is dependent upon an interaction with Telencephalin (also known as intercellular adhesion molecule-5), a cell adhesion molecule which promotes filopodia formation but acts as a brake on filopodia-to-spine transition [78-81]. Activated ERM proteins colocalize with Telencephalin at the tips of dendritic filopodia and are thought to anchor Telencephalin at the cell surface of these spine precursors [78].

The transition from filopodia to spines in response to activity is an important aspect of synaptogenesis. While this maturation process has many components, one integral step in this transition is the mobilization of Telencephalin which is present in filopodia but excluded from mature spines [79]. This impediment to spine maturation can be relieved by the Ras-related small GTPase ARF6, a neuronal activity-responsive regulator of spine development that governs the trafficking of Telencephalin from filopodia [82-84]. ARF6 activation triggers Rac1-mediated dephosphorylation of ERM proteins and release from Telencephalin [83, 84]. The loss of ERM-dependent Telencephalin anchorage at the cell surface facilitates Telencephalin internalization and its trafficking from filopodia [83]. Removal of Telencephalin permits the shape changes associated with spine maturation driven by Rac-1-mediated actin remodeling [79, 83].

While ERM proteins appear to retard dendritic spine maturation through an interaction with Telencephalin, recent evidence suggests activated Moesin performs a pro-spine role in response to sex steroids in cortical neurons [85, 86]. These hormones are critical modulators of neuronal morphology and function and have been demonstrated to play a

critical role not only in brain development but also learning and memory [87-91]. Treatment with the sex steroids estradiol or progesterone induces Moesin phosphorylation via a signaling cascade involving the Rho family GTPase RhoA and the Rho-associated kinase, ROCK-2 [85, 86]. Activation of this pathway is associated with increased dendritic spine density and a redistribution of Moesin to membrane sites where spines are formed. Actin remodeling by Moesin appears to be critical for sex steroid-induced spine enrichment, however, it's not clear if the apparent, opposing roles of ERM proteins in spine development are reconciled within individual neurons or whether these functions are segregated to different brain regions. ERM proteins represent an intriguing signaling target involved in dendritic modification and their rapid effects on spine formation suggest they may be involved in the plasticity of synapses.

1.2.4 Synaptic Plasticity

A potential role for ERM proteins in synaptic plasticity was recently suggested following the study of ERM response to the neurotransmitter glumatate. Synaptic plasticity is the process whereby synapses strengthen or weaken due to modulations in their activity, a key component of information processing and storage in the brain [92]. Exposure of hippocampal neurons to glumatate induces ERM phosphorylation and an increase in the number of active synaptic boutons, the pre-synaptic axon terminals that contact dendritic spines to form a synapse [93]. This glutamate-induced enrichment of active synaptic boutons is thought to occur through the conversion of pre-existing silent synapses, a process that requires actin remodelling [94, 95]. While it's not clear if ERM proteins participate in this actin reorganization, the increase in active synaptic boutons is diminished by Moesin knockdown as well as impairment of ERM phosphorylation, indicating that phosphorylated ERM proteins may be involved in the synaptic response to activity [93].

A major factor influencing the plasticity of synapses is the regulation of membrane neurotransmitter receptor density [96]. Intracellular clustering of these receptors provides a reservoir from which membrane receptor density and therefore synaptic strength can be modulated [97-99]. Scaffolding proteins, including the ERM family, are able to anchor diffusing receptors intracellularly or at the synapse and may therefore be critical to the

plasticity of synapses [97-101]. Radixin-mediated extrasynaptic clustering has recently been shown to regulate the synaptic density of the $GABA_A$ receptor, which receives inhibitory synaptic transmission [97, 98]. Activated radixin binds the α5-subunit of the $GABA_A$ receptor facilitating its anchorage at the actin cytoskeleton [97]. The diffusion of $GABA_A$ receptors between their extrasynaptic anchorage and the synapse is regulated by neuronal activity-dependent radixin phosphorylation [97, 98]. This integration of synaptic activity with the control of receptor density reveals one aspect in which ERM proteins may be important to synaptic plasticity. Radixin knockout mice exhibit increased synaptic $GABA_A$ receptor density in hippocampal neurons which is associated with impairments in both short-term and reversal memory [98]. Interestingly, behavioral training was found to reduce radixin phosphorylation in the mouse hippocampus which raises the possibility that radixin regulation may be a critical link in the coupling of training and memory formation through its control of reversible extrasynaptic receptor clustering [98, 99].

1.2.5 Learning and Memory

The evidence that radixin is involved in the regulation of neurotransmitter receptor density at the synapse reveals a mechanism through which ERM proteins may directly impact the potentiation of neurons, and further research will be required to clarify the role of ERM proteins in synaptic plasticity, learning and memory. *Moesin* was first identified as a candidate memory gene through DNA microarray analysis of the transcriptional response to behavioral training in *Drosophila melanogaster*, from which it was identified that expression of *Moesin* was significantly increased both immediately and 24 hours after a training paradigm that leads to long-term memory formation [102]. As this training paradigm leads to the formation of long-term memory lasting days, this transcriptional response suggesting that Moesin may be involved in long-term memory formation [103].

While there has been a dearth of research into Moesin's function in memory, a recent murine study noted that transcription of *Moesin* was also induced by overexpression of nuclear-restricted HDAC4, a histone deacetylase whose overexpression repressed a set of genes essential for synaptic function [104]. HDAC4 has been shown to be a critical

regulator of long-term memory, as overexpression as well as knockdown of HDAC4 in the *Drosophila* brain resulted in impairment of long-term memory but not short-term memory [105]. Further, the overexpression of a catalytically inactive mutant of HDAC4 with no deacetylase activity was also found to impair long-term memory [105]. Since HDAC4's effect on memory is not due to its deacetylase activity, the specific function of HDAC4 in memory formation could be revealed through the study of its potential gene targets such as *Moesin*.

A recent screen for HDAC4-interacting genes in the neurons of the *Drosophila* eye identified a genetic interaction with *Moesin.* Knockdown of *Moesin* was found to enhance a rough eye phenotype caused by overexpression of HDAC4 and characterized by the loss of pigment and disorganization of photoreceptor clusters of the *Drosophila* eye [106]. Although this type of phenotypic screening cannot determine whether there is a direct physical interaction between Moesin and HDAC4 resulting in the enhanced phenotype, it does provide evidence that Moesin is involved in the same pathway that produces this rough eye phenotype. The relationship between Moesin and HDAC4 has yet to be characterized despite its potential associations with neuronal development and long-term memory formation.

1.2.6 Neurodevelopmental and Neurodegenerative Disease

In addition to defined roles in axonal and dendritic morphogenesis and potential roles in neuronal plasticity and memory, Moesin has also been linked with several neurodevelopmental and neurodegenerative disorders. Moesin was found to be significantly reduced in the cortex of fetuses diagnosed with Down syndrome, which presents with abnormal neurogenesis [107, 108]. More recently, it was discovered that a mutation predisposing humans to late-onset Parkinson's disease, an autosomal dominant point mutation in the LRRK2 (leucine-rich repeat kinase-2) gene, enhances LRRK2 activation of Moesin *in vitro* [109]. In order to determine whether Moesin was a physiological substrate of LRRK2, a subsequent experiment investigated the activation of Moesin by this LRRK2 mutant in cultured hippocampal neurons. It was found that the mutant LRRK2 not only increased activation of Moesin but also resulted in abnormal neuronal morphogenesis, which suggests a potential physiological link between Moesin

regulation and the development of late-onset Parkinson's disease [110].

Additionally, a genome-wide association study searching for genes and pathways involved in Autism Spectrum Disorder identified a noncoding RNA that binds the Moesin gene transcript and decreases Moesin protein levels when overexpressed [111]. Moreover, the expression of this transcript was found to be 12.7-fold greater in the postmortem cerebral cortex of individuals with Autism Spectrum Disorder relative to controls, suggesting a possible link between Moesin and a pathway contributing to Autism Spectrum Disorder risk [111]. Moesin was also identified as the highest upregulated protein in a proteomic analysis of Alzheimer's disease hippocampal tissue [112]. As Alzheimer's disease neuropathology is characterized by the loss of cytoskeletal stability at dendritic spines, the regulation of Moesin represents a target for the study of the synaptic deficits and cognitive impairments that are a hallmark of this disorder [53, 54]. The association of Moesin with neurological disease underscores the need to discern the impacts of Moesin expression in brain development and examine the effects of Moesin regulation in the adult brain.

1.3 *Drosophila* as a Memory Model

1.3.1 Genetic Dissection of Behavior

Drosophila melanogaster has become a widely used model organism due its ease of cultivation as well as the sophisticated molecular tools available to *Drosophila* researchers. In addition, *Drosophila* exhibit robust, quantifiable behaviors that can be modified by learning [113]. As such, this model organism has become a valuable component in large-scale mutagenesis screens to identify genes involved in learning and memory [114]. Transgenic flies have long been utilized in gene rescue and structure/function experiments and the development of the GAL4/upstream activating sequence (UAS) system has allowed for unprecedented temporal and spatial control of gene expression in *Drosophila* [115, 116]. The GAL4/UAS expression system utilizes a yeast protein, GAL4, which binds to an upstream activating sequence in order to activate a downstream target gene [117]. Tissue-specific expression of a gene of interest can be

achieved by placing GAL4 under the control of a tissue-specific promoter and cloning the gene of interest downstream of a GAL4 responsive promoter [116]. This targeted expression allows for the investigation of which neurons, circuits, or brain regions are involved in a specific process. Further, temporal control of target gene expression can be achieved with the temporal and regional gene expression targeting (TARGET) system which utilizes a temperature sensitive repressor of GAL4-induced transcription, GAL80ts [118]. At 19°C GAL80ts effectively represses GAL4 transcriptional activity and therefore transcription of the GAL4/UAS target gene, while increasing the temperature to 30°C relieves this repression allowing for temporally controlled expression of the GAL4/UAS target gene [119]. Temporal control of gene expression can be utilized to study the effects of genes crucial to development and to isolate the target gene's effect in the adult. The systematic generation of GAL4 driver lines with diverse tissue- and even neuron-specific expression patterns, as well as the engineering of transgenic stocks for RNA interference of nearly every gene in *Drosophila*, enables the rapid assessment of the gene requirements for defined processes [120]. These valuable resources along with an array of mutant *Drosophila* lines are continually deposited in international stock centers, where they are made available to researchers at nominal cost.

In addition to *Drosophila's* amenability to genetic modification, there are a number of behavioral assays that can be employed to measure learning in *Drosophila*, which make it an apt model organism for the study of memory. The olfactory conditioning assay and the courtship suppression assay represent the two most commonly utilized behavioral assays and these behavioral analyses have led to the identification of many genes and pathways necessary for memory formation [121]. Classical olfactory conditioning involves coupling a conditioned stimulus (an odor) with an unconditioned stimulus (an electric shock) to observe whether there is a learned avoidance of the conditioned stimulus even in the absence of the unconditioned stimulus. To measure this conditioned response, flies are first exposed to an odor (Odor A) while being subjected to a series of electrical footshocks. The subjects are then exposed to a second odor (Odor B), which is not paired with any electric shocks. The flies are subsequently presented with Odors A and B at opposite ends of a T-maze and forced to choose one side of the apparatus. Flies with intact memory will avoid Odor A, while flies with disrupted memory function will show

no preference for either odor [122]. To assess short-term memory, flies are evaluated in the T-maze directly following exposure to each odor. Long-term memory formation, however, requires multiple spaced training sessions with intervals between trainings for consolidation of memory, which is typically tested 12 or more hours later.

Courtship suppression is the conditioned response whereby male flies display decreased courtship efforts following rejection by a female [123]. A mated female will reject courtship advances for several hours following copulation [124]. In the courtship suppression assay, a male trained with a mated, unreceptive female will learn the rejection behavior of a mated female and therefore court less when presented with a mated female in the future as compared to naïve male lacking this training. This experience-associated suppression of courtship can last up to seven days and is indicative of associative learning and memory formation [123, 125].

For training, a female who was mated the previous night and a virgin male are placed together in a mating chamber. The male is allowed to court for seven hours, after which time the female is removed. At the same time, a naïve "sham" male is housed alone. Long-term memory is measured 24 hours later as each male is placed with another freshly mated female and his courtship behavior is scored over a 10 minute period. Short-term memory can be assessed an hour after a one-hour training session.

The genetic dissection of behavior in *Drosophila* has identified many genes crucial to memory formation, most of which are conserved in humans. Several of the first memory genes identified encode components in the cAMP signaling pathway, a critical element of memory formation first identified in the sea slug *Aplysia* but well conserved in vertebrates. The continued study of memory formation in *Drosophila* has moved beyond large-scale mutagenesis screens to include direct functional analysis of specific genes and mutations. *Drosophila*'s amenability to genetic modification allows for the rapid evaluation of genes or mutations that may be involved in memory or neurodegenerative and neurodevelopmental disorders.

1.3.2 The Nervous System of *Drosophila Melanogaster*

Seymour Benzer, the pioneer of the genetic dissection of behavior in *Drosophila*, noted

that with about 100,000 neurons the common fruit fly nervous system represents the rough midpoint on a logarithmic scale between a single neuron and the human brain [126]. Despite its relatively small size, the *Drosophila* central nervous system displays complex organization and functional diversity among its highly ordered structures. Mapping behaviors to regions of the brain has long been a goal of researchers for the generation of models to describe the molecular mechanisms underlying behavior. One central brain structure, the mushroom body, has been identified as critical for memory formation. Genetic and chemical disruption of the mushroom body (Figure 5), produces flies which exhibit impaired learning but otherwise behave normally [127, 128].

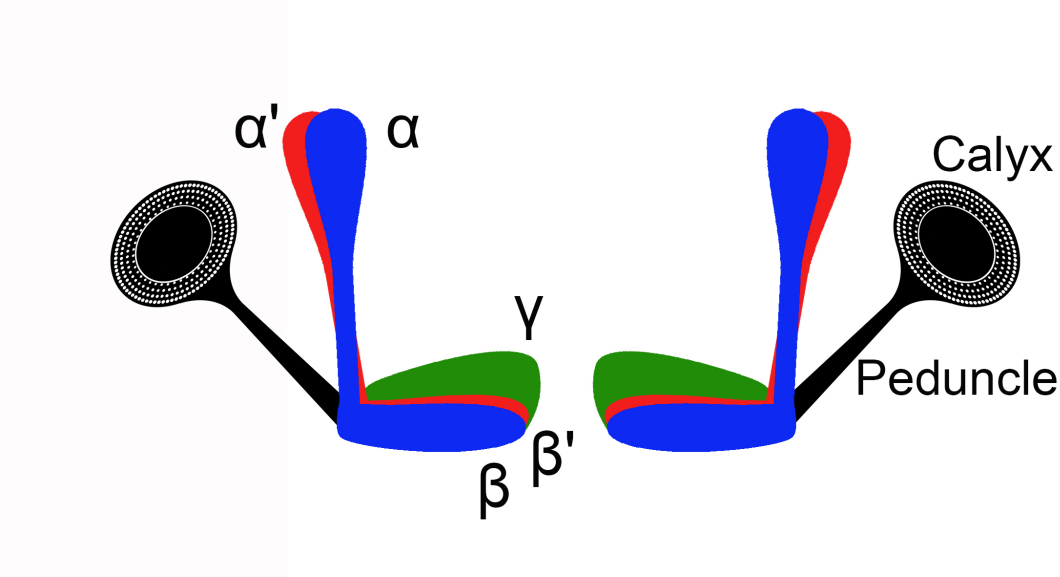


Figure 5: The Drosophila Mushroom Body.

Diagram of the adult *Drosophila* mushroom body. The mushroom body is comprised of three sets of Kenyon cell neurons: alpha/beta (blue), alpha'/beta' (red) and gamma (green). The bundled axons of these neurons travel together in the peduncle before forming the mushroombody lobes. The calyx is the dendritic field of the Kenyon cells which is surrounded by cell bodies (whitedots).

This bilateral structure is composed of approximately 5000 neurons, termed Kenyon cells, arranged into three sets of functional distinct neuronal bundles (alpha/beta, alpha'/beta', and gamma) [129, 130]. Kenyon cell dendrites project anteriorly into the calyx, while the axons fasciculate and project via the peduncle to the heel region (Figure 5). In early larval development, gamma axons branch at the heel to form medial and vertical lobes, which

will be reorganized during metamorphosis to form a single horizontal gamma lobe in the adult mushroom body. During late larval stages, alpha'/beta' neurons project axons through the peduncle to the heel where they too branch to form medial and vertical lobes, which are preserved through metamorphosis. Finally, following metamorphosis, alpha/beta neurons then project axons through the peduncle, which branch at the heel to form medial and vertical lobes. Thus, to form the five mirrored lobes of the adult mushroom body requires close association among distinct axonal layers for their fasciculation, branching, and guidance [131, 132].

The investigation of the axon-axon interactions involved in mushroom body development has revealed that the *Drosophila* L1CAM homolog Neuroglian mediates the adhesive interactions between neuronal bundles required to establish this layered and lobular structure [131, 132]. Interestingly, Moesin was recently identified as the likely binding partner of Neuroglian's FERM–protein interaction domain [132]. This domain was found to be indispensable in both ingrowing and substrate mushroom body neurons for axonal guidance, indicating that the Moesin-Neuroglian interaction may be required in both populations of neurons for proper axonal development. Additionally, the requirement of Moesin in mushroom body development was evaluated through RNA interference, which led to mushroom body defects. This conserved role in axonal navigation highlights a critical neurodevelopmental function for ERM proteins that requires characterization in *Drosophila*.

1.4 Thesis Aims and Objectives

As reviewed in Chapter 1.2, ERM proteins are critical regulators of the actin cytoskeleton within neurons and are integral to several key neuronal processes including axonal guidance and dendritic morphogenesis. Recent studies have also suggested important roles for ERM proteins in neuronal plasticity and identified Moesin as a potential memory gene. Further, Moesin has also been linked to several neurodevelopmental and neurodegenerative disorders, suggesting a more general role for moesin in cognitive function. However, despite evidence of the importance of Moesin in neuronal development and function, there is a lack of comprehensive research into the role of Moesin in brain development and memory formation.

We sought to further examine the effect of moesin expression in brain development and memory in the model organism *Drosophila melanogaster*. As highlighted in Chapter 1.3, *Drosophila* is an apt model organism for the study of candidate memory genes due not only to its amenability to genetic modification, but also the reproducible behavioral assays that can be employed to measure learning and memory.

Drosophila provides a genetically tractable system in which both the expression and activity of its sole ERM protein Moesin can be modulated and the effects on several aspects of development and behavior can be analyzed. My thesis aims to investigate Moesin's role in the development of central structures of the *Drosophila* brain and interrogate the requirement for moesin in memory formation through analysis of courtship behavior.

Specific Aims:

1. To characterize Moesin expression and subcellular localization in the adult Drosophila brain.
2. To examine the effects of modulating Moesin expression and activity on Drosophila brain development.
3. To examine the effect of modulating Moesin expression expression on memory formation.

Chapter 2: Materials & Methods

2.1 *Drosophila* Strains

Transgenic fly lines utilized from the Vienna Drosophila Resource Center and Bloomington Drosophila Stock Center are listed in Table 1. These transgenic lines harbor insertions fused downstream of an upstream activating sequence (UAS) which is a GAL4 responsive promoter. Tissue-specific expression of transgenes was achieved by placing GAL4 expression under the control of a tissue-specific promoter [116]. Temporal control of transgene expression was achieved through the utilization of a temperature sensitive repressor of GAL4-induced transcription, GAL80ts [118]. At 19°C GAL80ts effectively represses GAL4 transcriptional activity and therefore transcription of the GAL4/UAS target gene, while increasing the temperature to 30°C relieves this repression allowing for temporally controlled expression of the GAL4/UAS target gene [119]. Transgenic fly lines driven by ELAV-GAL4 and repressed by GAL80ts were raised at 19°C and switched to 30°C 3 days before dissection. To analyze the effect of Moesin knockdown and overexpression construct on adult viability, homozygotes of each transgenic strain as well as well as a control strain (w1118) were crossed to tubP-GAL4/TM3,Sb, a strain heterozygous for a ubiquitous alpha-tubulin promoter controlled GAL4 driver (tubP-GAL4) and a balancer chromosome (TM3,Sb) carrying the mutant *Sb* ("stubble") gene as a marker, such that half the progeny receive the balancer chromosome with the “stubble” marker (TM3,Sb) while the other half receive the tubP-GAL4 driver and will thus ubiquitously express the UAS-fused construct throughout development [133]. The “stubble” phenotype, severely shortened bristles on the back of the fly, was visualized under dissecting microscope and the total survival percentage was determined by the ratio of the surviving non-Sb adults ubiquitously expressing the transgenic construct to Sb adults which do not express the construct. *Drosophila* strains were cultured on standard fly medium (10g Agar, 40g yeast, 110g ground cornmeal, 130g sugar, 3.3g methyl 4-hydroxybenzoate, 20 mL molasses, and 37 mL of 95% ethanol per liter of water) on a 12 hour light/dark cycle and maintained at a temperature of 25°C unless otherwise indicated.

Name	Description	Genotype	Stock Center	Stock Number
MOE-RNAi1	Inverted hairpin repeat targeted to moesin mRNA	w[1118]; P{w[+mC]=UAS-Moe.IR.528-897}2	VDRC	110654
MOE-RNAi2	Inverted hairpin repeat targeted to moesin mRNA	w[1118]; P{w[+mC]=UAS-Moe.IR.327-775}3	BDSC	8629
MOETD-MYC	Myc-tagged phosphomimetic moesin mutant expression line	w[1118]; P{w[+mC]=UAS-Moe.T559D.MYC}2	BDSC	8630
MOE-MYC	Myc-tagged moesin expression line	w[1118]; P{w[+mC]=UAS-Moe.MYC.K}2	BDSC	8631
tubP-GAL4	Ubiquitous GAL4 driver line	y[1] w[*]; P{w[+mC]=tubP-GAL4}LL7/TM3, Sb[1] Ser[1]	BDSC	5138
ELAV-GAL4	Neuron-specific GAL4 driver line	P{w[+mW.hs]=GawB}elav[C155]	BDSC	458
tubP-GAL80ts	Temperature sensitive regulator of GAL4	w[*]; P{w[+mC]=tubP-GAL80[ts]}10; TM2/TM6B, Tb[1]	BDSC	7108
3A-GAL4	LPTC GAL4 driver line	P{w[+mW.hs]=GawB}3A	BDSC	51629
CD8::GFP	Membrane targeted GFP	y[1] w[*]; P{w[+mC]=UAS-mCD8::GFP.L}LL5, P{UAS-mCD8::GFP.L}2	BDSC	5137
FLAG-HDAC4	FLAG-tagged HDAC4 expression line	w[CS10]; P{3xP3-RFP=attP-86F};UAS-HDAC4	[105]	-
MB247-GAL4	Alpha/beta and gamma mushroom body neuron GAL4 driver	w[*]; P{w[+m*]=Mef2-GAL4.247}3	BDSC	50742

Table 1: *Drosophila* Strains.

2.2 Immunohistochemistry

Whole flies were fixed in PFAT/DMSO (4% paraformaldehyde in 1X phosphate buffered saline+5% dimethyl sulfoxide+0.1% Triton X-100) for one hour then washed in PBT (1X phosphate buffered saline+0.5% Triton X-100). Brains were microdissected in PBT then post fixed in PFAT/DMSO for 20 minutes and stored in methanol at -20°C. Following rehydration in PBT, brains were blocked in immunobuffer (5% normal goat serum in PBT) for >2 hours at room temperature. They were then incubated overnight at room temperature with primary antibody and subsequently incubated overnight at 4°C with secondary antibody (goat anti-mouse Alexa488, goat anti-mouse Alexa555, goat anti-rabbit Alexa488, or goat–anti-rabbit Alexa555, Molecular Probes, 1:200) and mounted with Antifade mounting medium (4% n-propyl gallate in 90% glycerol+10% phosphate buffered saline). The primary antibodies and the concentration utilized in this work are listed in Table 2. For confocal microscopy, optical sections were taken with a Leica TCS SP5 DM6000B Confocal Microscope. Image stacks taken at intervals of 1 μm (whole brain) or 0.5 μm (mushroom body) and were processed with Leica Application Suite Advanced Fluorescence (LAS AF) and ImageJ software.

Name	Concentration	Source	Catalogue Number
α-Drosophila Moesin	IHC 1:1000 WB 1:10,000	D. Keihart, Duke University	-
α-elav	IHC 1:200	DSHB	Elav-9F8A9
α-FasII	IHC 1:200	DSHB	1D4anti-Fasciclin II
α-myc	IHC: 1:50	DSHB	9E10
α-GFP	IHC 1:20,000	Abcam	Ab290
Rabbit α-FLAG	IHC 1:1000	Sigma	F7425
Mouse α-FLAG	IHC 1:5000	Sigma	F1804

Table 2: Primary antibodies.

2.3 Western Blot

Flies were collected in tubes and frozen in a dry ice/ethanol bath. The tubes were vortexed to snap the heads from the bodies, and the heads were collected. Cytoplasmic extracts were prepared by homogenizing heads with a disposable mortar and pestle in radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 25 mM Tris, pH 8.0). Following centrifugation at 13,000 g for 2 minutes at 4°C, the supernatant was retained as the cytoplasmic fraction. Protein concentration was then determined with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). 20 µg of each sample was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and resolved at 200V. Protein was transferred onto nitrocellulose and blocked for >2 hours in 5% skim milk powder in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH7.6). The membrane was incubated overnight at 4°C in primary antibody and one hour in secondary antibody. Antibodies used were anti-Moesin (D. Kiehart, Duke University, 1:50,000) and anti-alpha-tubulin (12G10 clone, Developmental Studies Hybridoma Bank, 1:500). Detection was performed with ECL Plus (GE).

2.4 Scanning Electron Microscopy

In order to produce high quality images of the surface of the eye, flies were removed from food vials and placed in a new vial with a disc of filter paper soaked in distilled water the night prior to fixation to avoid any food residue on the eye. For fixation, flies were anesthetised with CO2 and were placed in primary modified Karnovsky's fixative (3% gluteraldehyde 2% formaldehyde in 0.1M phosphate buffer, pH 7.2) with Triton X-100 and vacuum infiltrated until wet. The fixative was replaced and the samples were allowed to fix for at least 8 hours at room temperature. Three washes of 10-15 minutes each, in phosphate buffer (0.1M, pH 7.2) followed by dehydration in graded ethanol series (25%, 50%, 75%, 95%, 100%) for 10-15 minutes each and a final 100% ethanol wash for 1 hour were performed. Samples were critical point dried using liquid CO2 as the CP fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus). Samples were mounted on to aluminium stubs and sputter coated with gold (Baltec SCD 050 sputter coater) and viewed in the FEI Quanta 200 Environmental

Scanning Electron Microscope at an accelerating voltage of 20kV. Microscopy was conducted by technicians at the Manawatu Microscopy and Imaging Centre (MMIC, Massey University, Palmerston North – New Zealand).

2.5 Behavioral Analyses

The repeat training courtship suppression assay [37, 105, 106, 133] was used to assess one-hour and 24-hour memory. In this assay, a male trained with a mated, unreceptive female will learn the rejection behavior of a mated female and therefore court less when presented with a mated female in the future as compared to naïve male lacking this training. All behavioral assays and statistical analyses were performed as described in Fitzsimons and Scott, 2011 [133].

A training session consists of pairing a virgin male with a female who was mated the previous night for 1 to 7 hours. The male is left to court the mated female for the duration of the training session, after which time the female is removed. A one-hour training session was administered for the analysis of short-term memory, while a seven-hour training session was applied in long-term memory assessment. In parallel, a naïve "sham" male is housed alone. Long-term memory was measured 24 hours after training by pairing each male with another freshly mated female and scoring his courtship activity (licking, chasing, or orienting toward the female, wing extension and vibration) over a ten-minute period. Short-term memory was assessed in the same manner one hour after the training session. In order to generate a memory score from this courtship data a memory index is calculated by comparing the percentage of the ten-minute period spent engaging in courtship behavior (courtship index) against the mean of the sham flies of its genotype [125, 134, 135]. 16 sham males and 16 trained males were analyzed for each genotype. Memory was measured on a scale of 0 to 1, with 0 being perfect memory, and a score of 1 indicating no memory [105, 106, 133].

Chapter 3: Results

3.1 Moesin is expressed throughout the fly brain.

The expression pattern of Moesin has not been described in the *Drosophila* brain, therefore, immunohistochemical staining of whole-mount fly brains was carried out in order to examine the expression pattern throughout the brain, as well as the subcellular localization of Moesin within neurons. No commercial antibody for *Drosophila* Moesin exists, however a *Drosophila* Moesin antibody was kindly gifted by an academic researcher (Professor Dan Kiehart, Duke University) and has been validated for its specificity to Moesin [136-138]. In order to visualize the subcellular localization of Moesin, brains were counter-stained with anti-ELAV antibody. ELAV is an RNA binding protein that predominantly localizes to the nucleus and is commonly used as a marker of neuronal nuclei [139, 140]. Anterior and posterior confocal projections though the brain are shown. Endogenous Moesin expression in the *Drosophila* brain was observed to be widespread throughout all regions of the brain (Figure 6C, J). Moesin did not localize with ELAV (Figure 6D, E) and visualization of a single optical plane though the anterior portion of the central brain (F-I) revealed that Moesin was primarily cytoplasmic as observed by the Moesin-positive cytoplasmic haloes surrounding the ELAV-positive nuclei (Figure 6I, O). Significant expression was not observed in the axons that comprise the lobes of the mushroom body, a region of the brain critical for memory formation and recall [141, 142], while magnification of the cell bodies of the mushroom body neurons (termed Kenyon cells) revealed cytoplasmic localization of Moesin (Figure 6M-P).

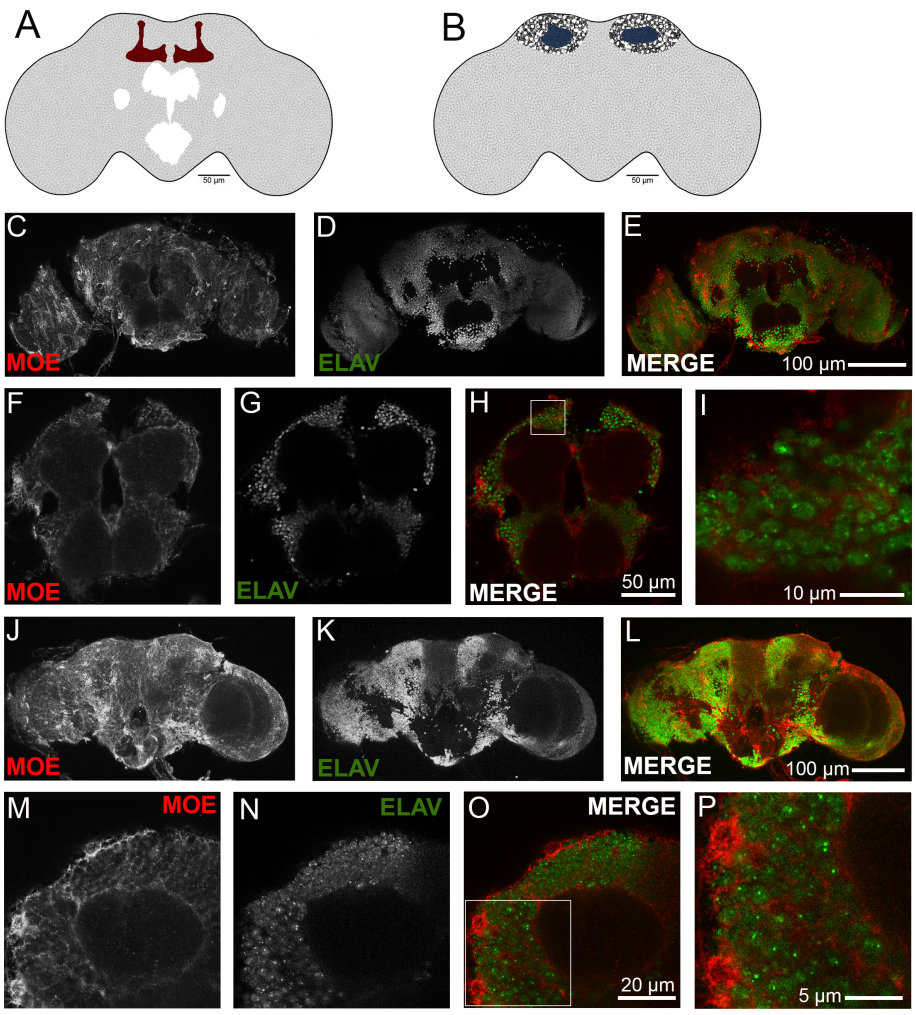


Figure 6: Endogenous Moesin expression in the fly brain.

A. Diagram of the anterior view of the Drosophila brain illustrating the mushroom body (red) and the pattern of neuronal nuclei (grey) stained by anti-elav antibodies. B. Diagram of the posterior view of the Drosophila brain illustrating the calyx (blue) encircled by the Kenyon cells (black and white pattern) and the pattern of neuronal nuclei (grey) stained by anti-elav antibodies. C-P. Immunohistochemistry with anti-Moesin (red) and anti-ELAV (green) antibodies on whole mount brains. C. Frontal confocal projection through the brain illustrating widespread Moesin expression. D-E. ELAV staining reveals neuronal nuclei, scale bar = 100 µm. F-I. 1 µm optical slice through the central lobes of the brain illustrating non-nuclear Moesin expression, appearing as a cytoplasmic halo around the elav stained nuclei, scale bar = 50 µm. G. Magnification of area surrounded by the white square in F, scale bar = 10 µm. H-J. Posterior confocal projection through the brain. K-M. 1 µm optical slice through the calyx illustrating non-nuclear Moesin expression in Kenyon cells, scale bare = 20 µm. N. Magnification of area surrounded by the white square in M, scale bar = 5 µm.

3.2 Constructs for the genetic manipulation of Moesin

Given that Moesin is expressed highly throughout the brain, including the mushroom body, an investigation into the role of Moesin in neurological function was performed by genetic manipulation of Moesin via overexpression and knockdown, followed by analysis of phenotypic assessment parameters including viability, brain development, learning and memory. The GAL4/UAS system was utilized to drive tissue-specific expression of UAS-MOE knockdown and overexpression constructs. In this system a tissue-specific driver expresses the protein GAL4 which binds an upstream activating sequence (UAS) to activate expression of the UAS-fused construct (see Section 1.3.1 for more detail). Each of the constructs utilized in this study is fused to an upstream activating sequence and by crossing these strains to a GAL4 driver line we can induce tissue-specific expression of these transgenes. In order to achieve specific knockdown of Moesin, transgenic flies harboring RNA interference constructs designed to target Moesin were obtained from two stock centers (Bloomington Drosophila Stock Center [137], and Vienna Drosophila Resource Center, [120]). Each transgenic construct harbors an inverted hairpin repeat targeted against a different region of Moesin mRNA to compare the efficacy of Moesin knockdown. In addition to assessing the impact of reducing Moesin expression, two constructs designed to overexpress Moesin fused with a Myc tag for high-affinity detection were also used. Full length wild-type Moesin was overexpressed (termed MOE-MYC), as was full length Moesin harbouring a T559D mutation (termed MOETD-MYC), which mimics phosphorylation of threonine 559 and thus acts as a constitutively active form of Moesin [143]. Phosphorylation at this site relieves Moesin's autoinhibitory head-to-tail binding, leading to activation by revealing the previously obscured the actin the binding site (Figure 2)

3.3 Validation and characterization of UAS-MOE constructs.

The UAS-MOE knockdown and overexpression constructs were first assessed via western blotting to confirm the appropriate expression and knockdown of Moesin, respectively. Each of the UAS-MOE constructs was crossed to a line harboring the neuron-specific driver ELAV-GAL4 in order to induce expression throughout the brain. Whole-cell lysates of *Drosophila* heads were prepared and analyzed via western blot for

Moesin expression (Figure 7). In the control, a specific band of approximately 75 kDa was observed, which is the estimated molecular weight of *Drosophila* Moesin. A clear reduction of Moesin levels in the head resulted from expression of each knockdown construct, confirming that each targeted Moesin. In the UAS-MOE overexpression strains a higher molecular weight band was detected in addition to endogenous Moesin, which represents the MOE-MYC and MOETD-MYC products In the two strains co-expressing MOE-MYC and a MOE-RNAi construct the levels of both endogenous Moesin and MOE-MYC are reduced, which is expected as both endogenous Moesin and the MOE-MYC constructs contain the mRNA sequences targeted by RNAi.

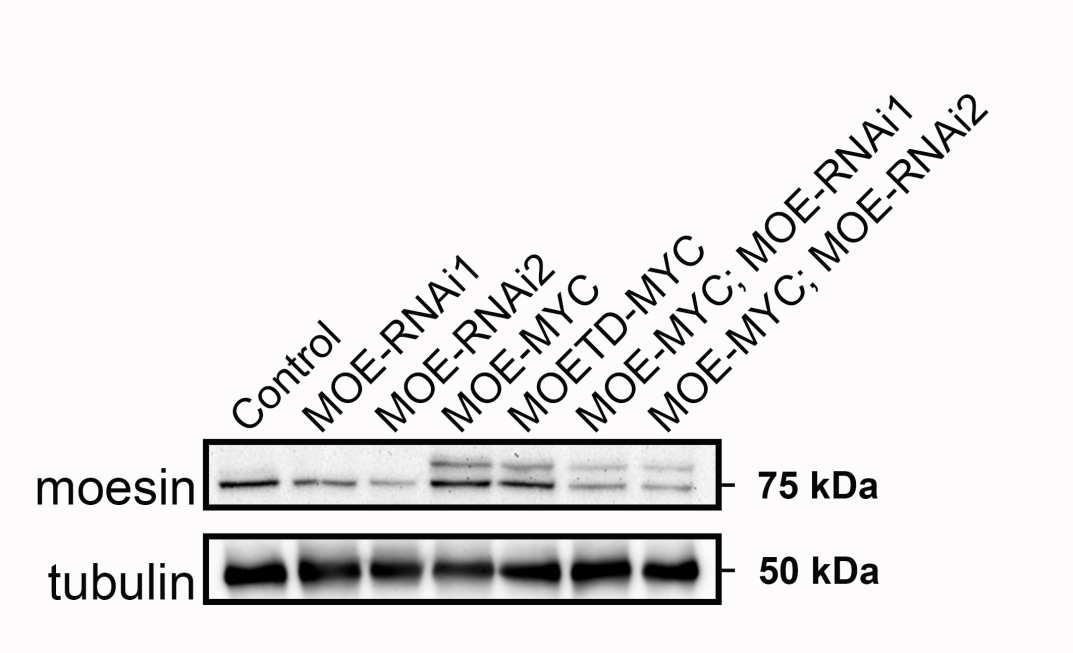
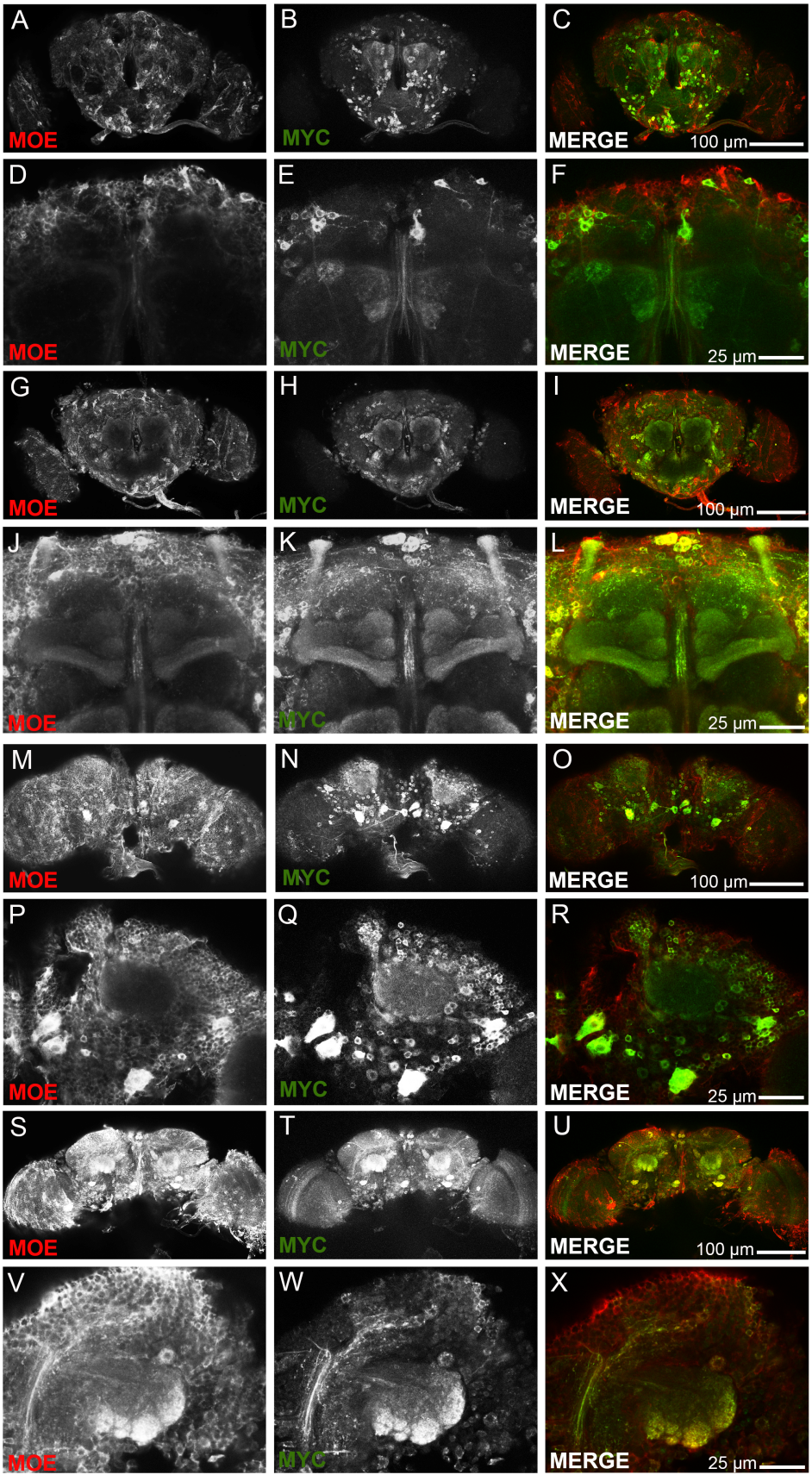


Figure 7: Characterization of ELAV-GAL4-mediated expression of UAS-MOE constructs.

Western blot shows the expression of Moesin in fly head lysates of UAS-MOE strains crossed to the driver ELAV-GAL4. The wild-type strain w1118 was also crossed to ELAV-GAL4 as a control genotype. Blots were probed with anti-Moesin to detect Moesin as well as MOE-MYC and MOETD-MYC. Anti-α-tubulin antibody was used as a loading control.

In order to characterize the expression pattern of the UAS-MOE constructs in the brain, and to examine whether activation of Moesin altered its subcellular localization within neurons, the expression pattern of MOE-MYC was compared to endogenous Moesin, and

to that of the phosphomimetic mutant MOETD-MYC (Figure 8). As overexpression of Moesin could potentially result in alterations in neuronal development, for the purposes of this experiment, the overexpression was restricted to adulthood with the TARGET system which utilizes a temperature-sensitive repressor of GAL4 transcription, GAL80ts. (see Section 1.3.1 for more detail). Flies were raised at a GAL4 repressive temperature (19°C) throughout larval and pupal development, and following eclosion UAS-MOE expression was induced at a GAL4 permissive temperature (30°C) for 48 hours in adults. The expression pattern of MOE-MYC is very similar to that of endogenous Moesin, suggesting that they are similarly regulated (Figure 8A). Higher magnification of the mushroom body showed that both endogenous Moesin and MOE-MYC were concentrated in the cytoplasm and excluded from the nucleus (Figure 8B). In contrast to endogenous Moesin, MOE-MYC could be detected at low level in the mushroom body lobes, which are the axonal projections of the Kenyon cells (Figure 8D-F). Expression of MOETD-MYC also resulted in robust expression throughout the brain (Figure 8G-I), however clear differences in subcellular localization were observed, with the phosphomimetic mutant localizing to actin-rich regions. This was consistent with previous studies indicating a marked difference in the subcellular localization of wild-type Moesin and constitutively active Moesin in photoreceptors and oocytes, typified by the close association of the phosphomimic with the actin cytoskeleton [137, 144]. In order to further analyze the localization of wild-type and phosphomimetic Moesin, confocal sections through the mushroom body region were taken which reveal high Moesin staining in the mushroom body lobes when MOETD-MYC was expressed (Figure 8J-L). Confocal projections through the posterior of the brain reveals strong MOE-MYC and MOETD-MYC staining of the mushroom body calyx (8N,T). Confocal sections through the mushroom body calyx reveal that while MOE-MYC and MOETD-MYC each label the dendritic field, the phosphomimic also strongly the axons entering the peduncle (Figure 8P-R, V-X).


A
MOE
B
MYC
C
MERGE
100 µm
D
MOE
E
MYC
F
MERGE
25 µm
G
MOE
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T
MYC
U
MERGE
100 µm
V
MOE
W
MYC
X
MERGE
25 µm

Figure 8: Overexpression of MOE-MYC and MOETD-MYC in the fly brain.

A-H. Immunohistochemistry with anti-Moesin (red) and anti-Myc (green) antibodies on whole mount brains. A-C. Frontal confocal projection through the brain of a fly expressing MOE-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 100 µm. D-F. Confocal sections through the mushroom body region of the brain of a fly expressing MOE-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 25 µm. G-I. Frontal confocal projection through the brain of a fly expressing MOETD-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 100 µm. J-L. Confocal sections through the mushroom body region of the brain of a fly expressing MOETD-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 25 µm. M-O. Posterior confocal projection through the brain of a fly expressing MOE-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 100. P-R. . Confocal sections through the mushroom body calyx region of the brain of a fly expressing MOE-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 25 µm. S-U. Posterior confocal projection through the brain of a fly expressing MOETD-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 100. V-X. . Confocal sections through the mushroom body calyx region of the brain of a fly expressing MOETD-MYC with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system, scale bar = 25 µm.

3.4 Alteration of wild-type levels of Moesin impairs viability.

The viability of flies expressing UAS-MOE constructs was evaluated by crossing homozygotes of each strain to a heterozygous driver of GAL4 expression under control of the alpha tubulin promoter balanced over a chromosome carrying the mutant *Sb* ("stubble") gene as a marker, tubP-GAL4/TM3,Sb [133]. In this manner, half the progeny will receive the balancer chromosome with the “stubble” marker (TM3,Sb) while the other half receive the tubP-GAL4 driver and will thus ubiquitously express the UAS-fused construct throughout development. As the “stubble” phenotype is easily visualized by the severely shortened bristles on the back of the fly, the lethality of the UAS-fused construct can be determined by comparing the number of surviving progeny with either wild-type or “stubble” bristles. As Moesin null mutants are non-viable [145], this experiment provided a means to evaluate the viability of two MOE-RNAi strains which each harbor an inverted hairpin repeat targeted to a distinct region of Moesin mRNA. No flies that carried both MOE-RNAi and tubP-GAL4 survived to adulthood, verifying the efficacy of each knockdown construct (Tables 3 & 4). Additionally, the viability of the

two UAS-MOE overexpression constructs was analyzed. Ubiquitous expression of myc-tagged wild-type Moesin (MOE-MYC) also impacted viability, resulting in a survival rate of 9.1% when raised at 18°C and 50.3% at 25°C. Expression of MOETD-MYC resulted in 100% lethality at both 18°C and 25°C.

In order to evaluate whether co-expression of MOE-MYC and MOE-RNAi could rescue viability, the MOE-MYC overexpression strain was recombined with each of the MOE-RNAi strains. However, no flies survived, indicating that the net level of Moesin resulting from their co-expression does not meet requirements for viable development. As MOE-MYC expression resulted in partial viability, this suggests that in combination, the level of Moesin is still lower than wild-type. This is in agreement with the western blotting data (Figure 7), in which MOE-MYC expression was lower than that of endogenous Moesin, and combination of MOE-MYC and each Moesin RNAi appeared to together result in a total amount of Moesin that was lower than wild-type.

Strain	Sb males	WT males	Sb females	WT females	Total survival of WT (%)
w1118	184	192	205	135	84.1
MOE-RNAi1	317	0	314	0	0
MOE-RNAi2	229	0	244	0	0
MOE-MYC	223	1	217	39	9.1
MOETD-MYC	202	0	209	0	0
MOE-MYC; MOE-RNAi1	210	0	283	0	0
MOE-MYC; MOE-RNAI2	310	0	363	0	0

Table 3: Survival of flies at 18℃.

Strain	Sb males	WT males	Sb females	WT females	Total survival of WT (%)
w1118	284	282	304	308	100.3
MOE-RNAi1	225	0	250	0	0
MOE-RNAi2	255	0	243	0	0
MOE-MYC	293	31	295	264	50.2
MOETD-MYC	214	0	215	0	0
MOE-MYC; MOE-RNAi1	273	0	271	0	0
MOE-MYC; MOE-RNAI2	220	0	251	0	0

Table 4: Survival of flies at 25℃.

3.5 Phosphomimetic Moesin expression impairs motor function.

In order to investigate MOE's role in neurological development, the UAS-MOE constructs were expressed pan-neuronally throughout development with the ELAV-GAL4 driver. A visual inspection of flies in their vials did not reveal any gross physical or behavioral changes in flies expressing MOE-MYC or MOE-RNAi. However, expression of phosphomimetic Moesin produced flies lacking stereotypical climbing behavior. Flies tapped to the bottom of a vial rapidly climb to the top, and this behavior has previously been exploited to measure cognitive deficits in *Drosophila* models of neurodegenerative disorders [146]. Flies expressing MOETD-MYC in neurons displayed severely reduced climbing behavior and congregated at the bottom of vials (Figure 9A). This sensorimotor phenotype highlights the importance of Moesin phosphoregulation in neurological function.

3.6 Phosphomimetic Moesin expression disrupts photoreceptor development.

Microscopic examination of MOETD-MYC flies also revealed a "rough eye" phenotype, which is indicative of malformed or missing photoreceptor clusters, producing a rough,

glassy look to the eye [106]. As *elav* is expressed in cells of neuronal progenitor origin including the photoreceptors of the eye, the ELAV-GAL4 driver induces target gene expression in the eye as well as the brain [139]. The discovery of this eye development phenotype led to the examination of each of our transgenic Moesin expression lines by scanning electron microscopy (SEM) to identify if altered Moesin expression resulted in visible disruption of photoreceptor development in the adult. SEM analysis revealed that ELAV-GAL4 driven expression of phosphomimetic Moesin resulted in severe disorganization of bristles and ommatidia (the clusters of photoreceptors which make up the *Drosophila* eye) (Figure 9F). While MOETD-MYC expression resulted in a clear disruption of eye development, no disruption of photoreceptor development was observed in the other UAS-MOE lines tested (Figure 9).

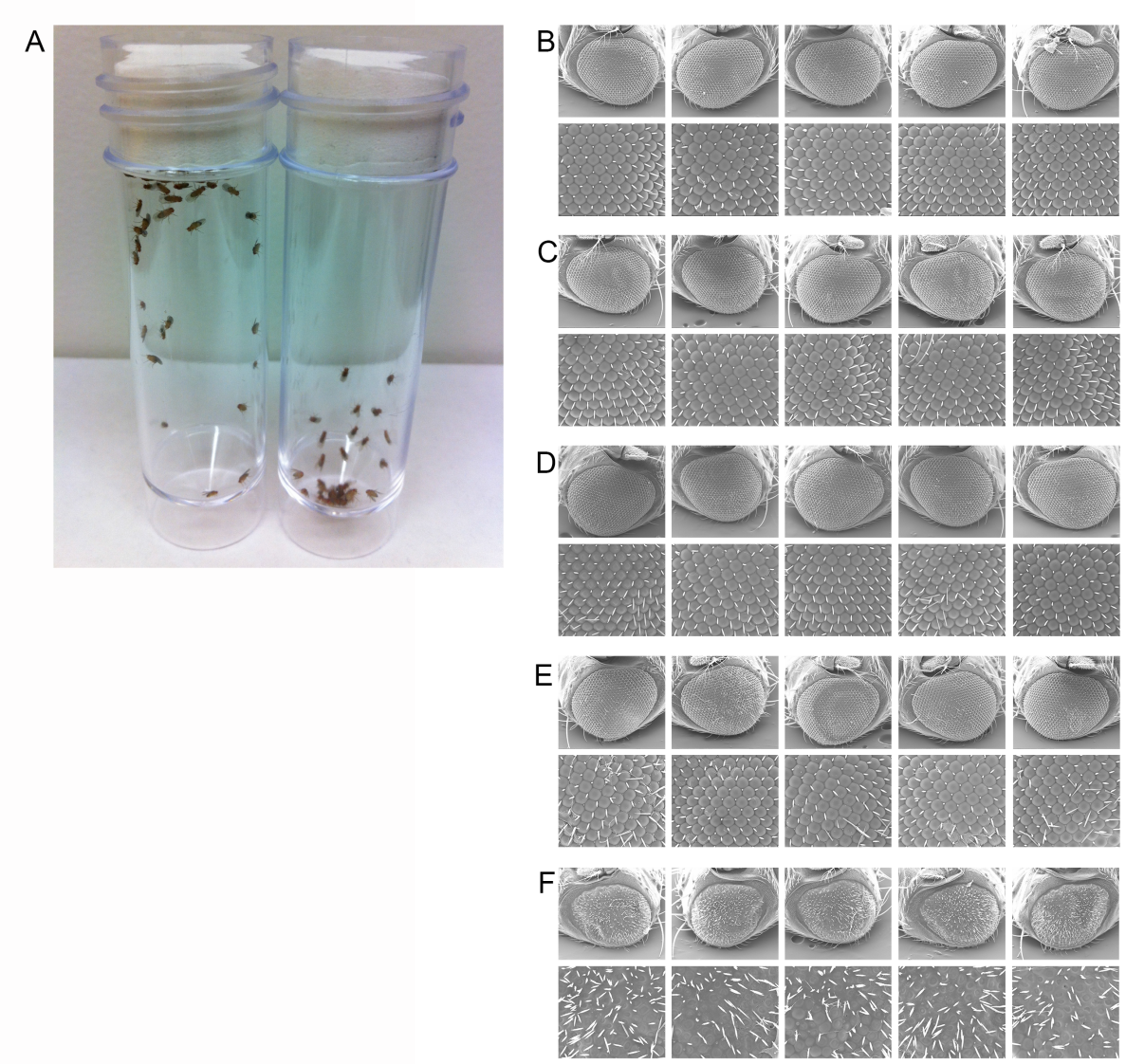


Figure 9: Phosphomimetic Moesin expression disrupts photoreceptor development.

A. An example of the sensorimotor defect that results from pan-neuronal expression of phosphomimetic Moesin. Wild-type flies in the left vial with MOETD-MYC flies in the right. B-E. Upper panel eye magnification: 250x. Lower panel eye magnification: 1000x. B. SEM of wild-type flies crossed to the ELAV-GAL4 driver line. C. SEM of flies expressing MOE-RNAi1 driven by ELAV-GAL4. D. SEM of flies expressing MOE-RNAi2 driven by ELAV-GAL4. E. SEM of flies expressing MOE-MYC driven by ELAV-GAL4. F. SEM of flies expressing MOETD-MYC driven by ELAV-GAL4.

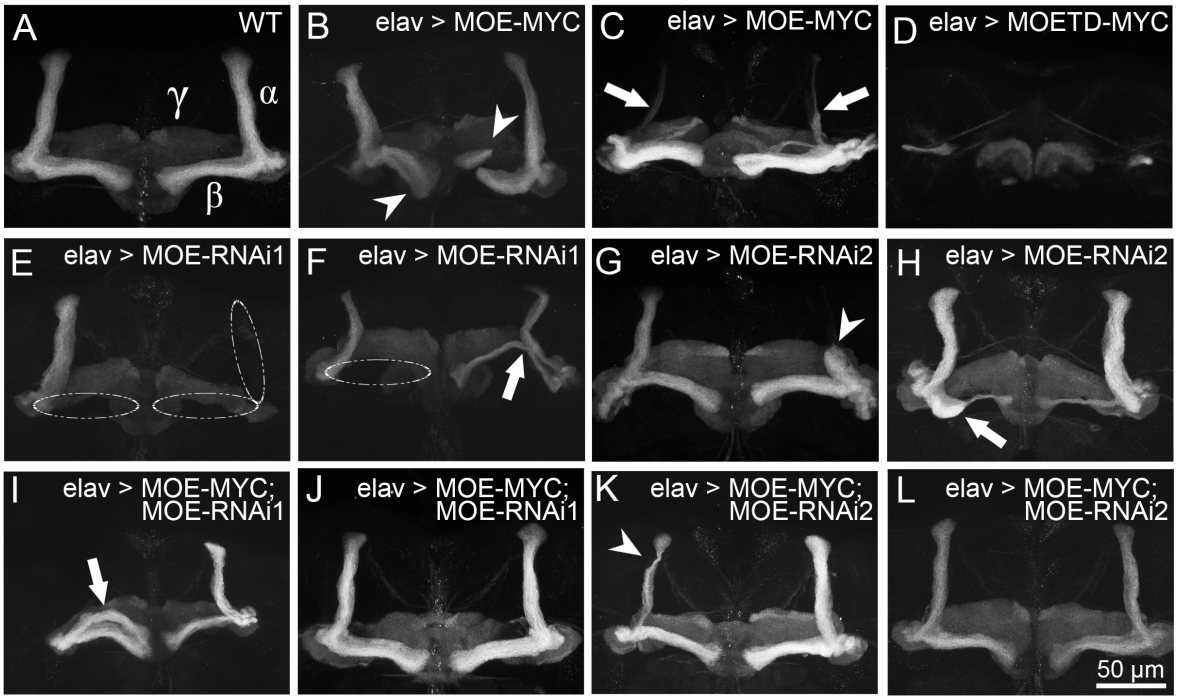
3.7 Altered Moesin expression disrupts mushroom body development.

Additionally, Moesin has been shown to be involved in photoreceptor axon targeting in *Drosophila*. Despite evidence that ERM proteins are involved in axonogenesis in mammalian cells, Moesin's developmental role in the brain has not been studied in detail. However, a recent work describing the role of the L1CAM Neuroglian in the formation of the mushroom body has revealed that Moesin is indeed required for mushroom body development and is the likely binding partner of Neuroglian's ERM–protein interaction domain [132]. This domain was found to be indispensable in both ingrowing and substrate neurons for mushroom body assembly, indicating that MOE-Neuroglian interaction may be required in both populations of neurons for proper axonogenesis. In order to assess this requirement for Moesin in mushroom body development we examined the effects resulting from pan-neuronal knockdown or overexpression of Moesin driven by ELAV-GAL4.

Immunohistochemical staining for the neural marker FasII strongly labels the alpha and beta lobes of the MB and weakly labels the gamma lobe enabling the visualization of mushroom body lobes, and therefore characterization of altered mushroom body development phenotypes in comparison to wild-type [129].

In the wild-type brain, the axons of the alpha and beta neurons each project from the cell bodies in a bundled fiber termed the peduncle, and then bifurcate to form the vertical and horizontal alpha and beta lobes (Figure 4 and Figure 10A). Both overexpression of Moesin and RNAi-mediated knockdown of Moesin resulted in clear disruption of mushroom body development, with defects ranging from misdirected or malformed lobes (Figure 10B) to the complete absence of alpha/beta lobes (Figure 10E), as well as axon arrest/stalling [147], in which the projection of α/β neurons from the peduncle is halted resulting in partially formed lobes (Figure 10G, arrowhead). Additional defects in α/β lobe morphology included lobes that were thin or diminished (Figure 10K, arrowhead), misdirected (Figure 10I), misoriented (Figure 10B) and those with defects in branching (Figure 10F). Alpha/beta axon phenotypes were quantified by hemisphere, as the observed defects were often asymmetrical (Figure 10M). Brain hemispheres were scored as "axon stalling" when one or more partially formed alpha/beta lobes were present. The

complete absence of one or more alpha/beta lobes in a hemisphere was scored as “lobes missing”. Brain hemispheres presenting any other defects of lobe morphology were scored as “abnormal morphology”, while hemispheres with no detected defect were scored as “normal morphology”. Quantification revealed that missing lobes was the primary defect in the two MOE-RNAi lines tested (67% and 70% respectively). While MOE-MYC expression also led to missing lobe defects (25%) and a higher proportion of axon stalling than Moesin knockdown lines (21%), the majority of MOE-MYC mushroom bodies displayed normal morphology (52%). Interestingly, alpha/beta defects were observed in in all MOETD-MYC hemispheres analyzed, with 89% displaying missing lobes. Finally, co-expression of MOE-MYC with either of the MOE-RNAi constructs resulted in rescue of mushroom body development in the majority of brain hemispheres analyzed (79% and 76% respectively).



M

	MOE-MYC	MOETD-MYC	MOE-RNAi1	MOE-RNAi2	MOE-MYC; MOE-RNAi1	MOE-MYC; MOE-RNAi2
n	*104*	*70*	*84*	*88*	*84*	*74*
axon stalling	21	4	10	5	12	11
lobe missing	25	89	67	70	5	5
abnormal morphology	2	7	10	11	5	8
normal morphology	52	0	14	16	79	76

Figure 10: Altered Moesin expression disrupts mushroom body development

A-H. Immunohistochemistry with anti-FasII antibody on whole mount brains reveals mushroom body defects resulting from ELAV-GAL4 driven expression of UAS-MOE constructs. FasII is expressed in the alpha (α), beta (β) and gamma (γ) lobes of the mushroom body and is used as a marker to examine mushroom body structure. All images are frontal confocal projections through the mushroom body region of the brain. Scale bar = 50 μm. A. Wild-type mushroom body. Alpha (α), beta (β) and gamma (γ) lobes of the mushroom body are labeled in white. B. Misoriented beta lobes (arrowheads) in a fly expressing MOE-MYC. C. Thin, reduced alpha lobe projections in a fly expressing MOE-RNAi2. D.Complete disruption of mushroom body development in a fly expressing phosphomimetic Moesin.E. Missing alpha and beta lobes (dashed lines) in a fly expressing MOE-RNAi1. F. Missing beta lobe (dashed line) and alpha/beta branching defect (arrow) in a fly expressing MOE-RNAi1. G. Axon stalling defect characterized by a partially formed alpha lobe (arrowhead) in a fly expressing MOE-RNAi2. H. Beta lobe outgrowth defect (arrow) in a fly expressing MOE-RNAi2. I. Misdirected alpha lobe (arrow) in a fly co-expressing MOE-MYC and MOE-RNAi2.J. Rescue of mushroom body development through coexpression of MOE-MYC with MOE-RNAi1. K. Thin alpha lobe (arrowhead) in a fly co-expressing MOE-MYC and MOE-RNAi1. L. Rescue of mushroom body development through coexpression of MOE-MYC with MOE-RNAi2. M. Summary of mushroom body defects resulting from ELAV-GAL4 driven expression of UAS-MOE constructs. The percentage of each alpha/beta lobe phenotype was calculated from the total number of brain hemispheres analyzed for each genotype (*n)*. Brain hemispheres were scored as “axon stalling” when one or more partially formed alpha/beta lobes were present. The complete absence of one or more alpha/beta lobes in a hemisphere was scored as “lobes missing”. Brain hemispheres presenting any other defects of lobe morphology were scored as “abnormal morphology”, while hemispheres with no detected defect were scored as “normal morphology”.

3.8 Altered Moesin expression disrupts dendritic arborization

We next sought to interrogate Moesin's role in dendritic morphogenesis in the adult central nervous system. The vertical system (VS) of lobula plate tangential cells (LPTCs), a group of visual system interneurons in the optic lobe, represent a model system particularly suited to the study of dendritic development as these neurons display complex but stereotypical dendritic arborization and a GAL4 driver line (3A-GAL4) is available to aid in expression and visualization of these neuronal processes [148]. In addition, dendrites in LPTCs have been shown to bear vertebrate-like spines that are actin-enriched, and therefore this system could be utilized to examine dendritic spine formation and turnover [149]. In rat cortical neurons, actin remodeling by Moesin has been shown to be crucial to dendritic spine growth and development [85, 86]. To visualize the dendritic morphology of these visual system interneurons the 3A-GAL4 driver was used to express CD8::GFP, a plasma membrane targeted GFP, which labels the axons, dendrites, and cell bodies of LPTCs. The characteristic arborization pattern of the six neurons which form the vertical system (VS) of the LPTCs is not altered by 3A-GAL4-driven expression of CD8::GFP (Figure 11A). However, co-expression of MOE-RNAi with CD8::GFP by 3A-GAL4 reveals severely reduced dendritic projections (Figure 11B). Strikingly, when Moesin is overexpressed with 3A-GAL4, the organization of the dendritic arbor is lost (Figure 11C). The strong and contrasting phenotypes resulting from overexpression and knockdown of Moesin in this system reveal that correct dendritic development is reliant on wild-type levels of Moesin.

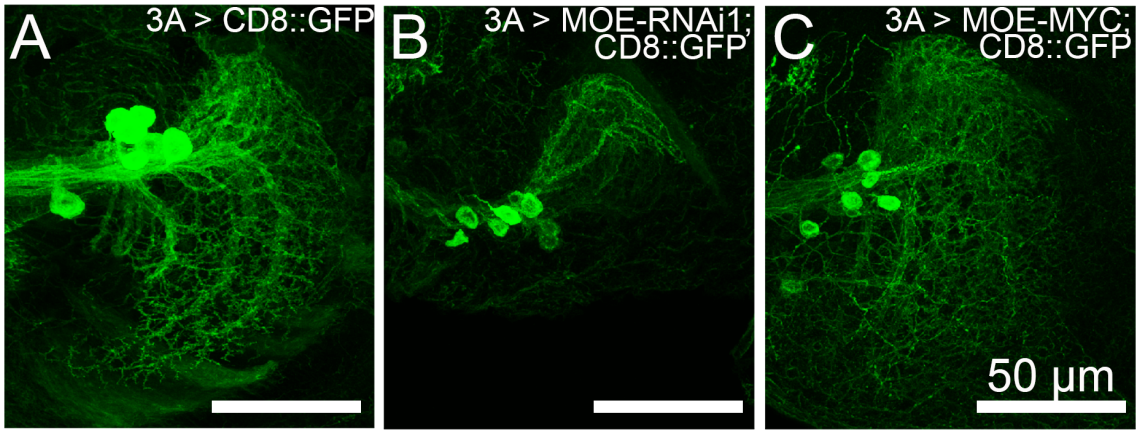


Figure 11: Altered Moesin expression disrupts dendritic arborization.

A-C Immunohistochemistry with anti-GFP (green) antibody on whole mount brains. All images are confocal projections through the optic lobe of the brain. A. The wild-type strain w1118 was crossed to 3A-GAL4 driver line expressing CD8::GFP to visualize the dendritic arbor of the six neurons comprising the vertical system (VS) of lobula plate tangential cells (LPTCs). B. The MOE-RNAi1 strain was crossed to 3A-GAL4 driver line expressing CD8::GFP to examine Moesin knockdown in the dendritic development of LPTCs. The dendritic arbor of the VS is severely reduced with Moesin knockdown. C. The MOE-MYC strain was crossed to 3A-GAL4 driver line expressing CD8::GFP to examine Moesin overexpression in the dendritic development of LPTCs. When Moesin is overexpressed the organization of the VS dendritic arbor is lost.

3.9 Assessing the contribution of Moesin to learning and memory.

3.9.1 Knockdown of Moesin impairs LTM

The repeat training courtship suppression assay was used to assess 24 hour long-term memory through behavioral analysis. In this assay a male trained with a mated, unreceptive female will learn the rejection behavior of a mated female and therefore court less when presented with another mated female as compared to a male lacking this training. In order to assess whether the developmental defects resulting from Moesin knockdown through development impacts long-term memory, we used ELAV-GAL4 to drive pan-neuronal RNAi-mediated knockdown of Moesin. Males with pan-neuronal Moesin knockdown as well as controls from each parental genotype were subjected to a seven hour training session with a mated female. 24 hours later these trained males as well as naïve "sham" males of each genotype were placed with freshly mated females and each male's courtship activity was scored over a ten-minute period. A courtship index,

calculated as the proportion of time spent in courtship activity was generated for the trained and sham groups of each genotype (Figure 12A). A suppression of courtship was observed in the trained group of each parental control genotype, while the Moesin knockdown trained group courted at nearly the same level as sham males lacking training.

In order to generate a memory score from these courtship indices, a memory index was calculated by comparing the courtship index of each trained fly against the mean of the sham flies of its genotype (Figure 12B). A memory index of 0 represents perfect memory, while a score of 1 indicates that memory is the same as that of sham males (no memory). A significant defect in 24-hour long-term memory was detected in Moesin knockdown males while each parental genotype exhibited normal long-term memory.

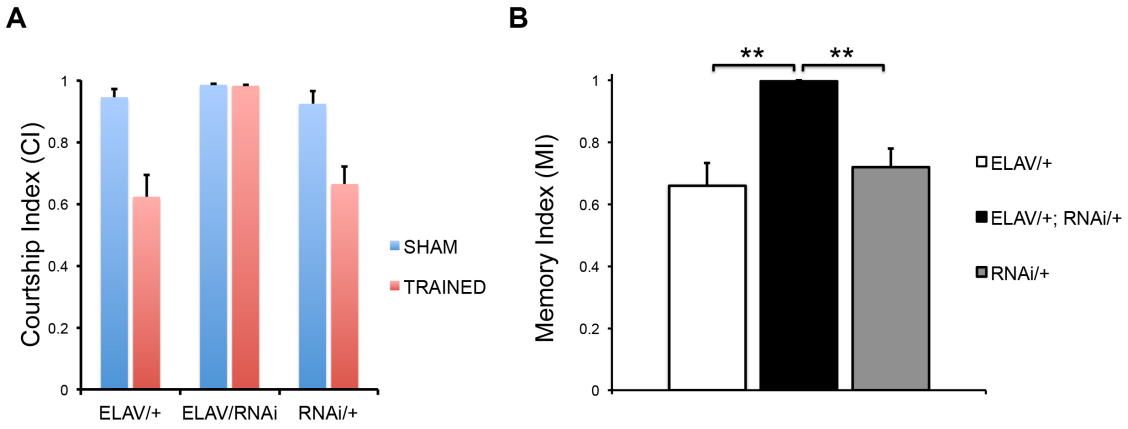


Figure 12: Knockdown of Moesin throughout development impairs LTM.

A. Courtship indices for males trained for 7 hours with a mated female and naïve "sham" males of each genotype. The courtship index represents the average proportion of time spent in courtship activity over a ten-minute period for the trained and sham groups of each genotype. B. 24-hour LTM was significantly impaired by ELAV-GAL4 mediated knockdown of Moesin (ANOVA, post-hoc Tukey's HSD, **p<0.01).

3.9.2 KD of Moesin restricted to adulthood impairs LTM but not STM

Given that Moesin knockdown through development results in morphological defects of the mushroom body, a structure critical for courtship memory, the memory deficits observed are likely due to developmental roles of Moesin, and cannot provide

information about whether Moesin plays a specific role in memory itself. In order to establish whether Moesin also plays a non-developmental role in long-term memory, i.e. whether reduction or overexpression of Moesin specifically in adulthood alters memory in a brain that has developed normally, the temporal and regional gene expression targeting (TARGET) system was utilized to restrict pan-neural Moesin knockdown to the adult brain, allowing the flies to develop normally [118].

In this system, temporal control of transgene expression is achieved through the exploitation of a temperature sensitive repressor of GAL4-induced transcription, GAL80ts [118]. At 19°C GAL80ts effectively represses GAL4 transcriptional activity and therefore transcription of the GAL4/UAS target gene, while increasing the temperature to 30°C relieves this repression allowing for temporally controlled expression of the GAL4/UAS target gene [119].

With the TARGET system flies were raised to adulthood at 19°C (GAL4 repressed) then switched to 30°C (GAL4 active) 3 days before testing to allow induction of MOE-RNAi1 expression. Assessment of 24-hour memory revealed that pan-neuronal Moesin knockdown in adulthood results in significant long-term memory impairment, signifying that Moesin plays a non-developmental role in memory (Figure 13A).

We were interested if this non-developmental role in memory was specific to long-term memory or may impact an earlier phase of memory; therefore we assessed short-term memory one hour after a one-hour training session. We found that adult Moesin knockdown had no impact on short-term memory as all genotypes displayed normal one-hour memory (Figure 13B).

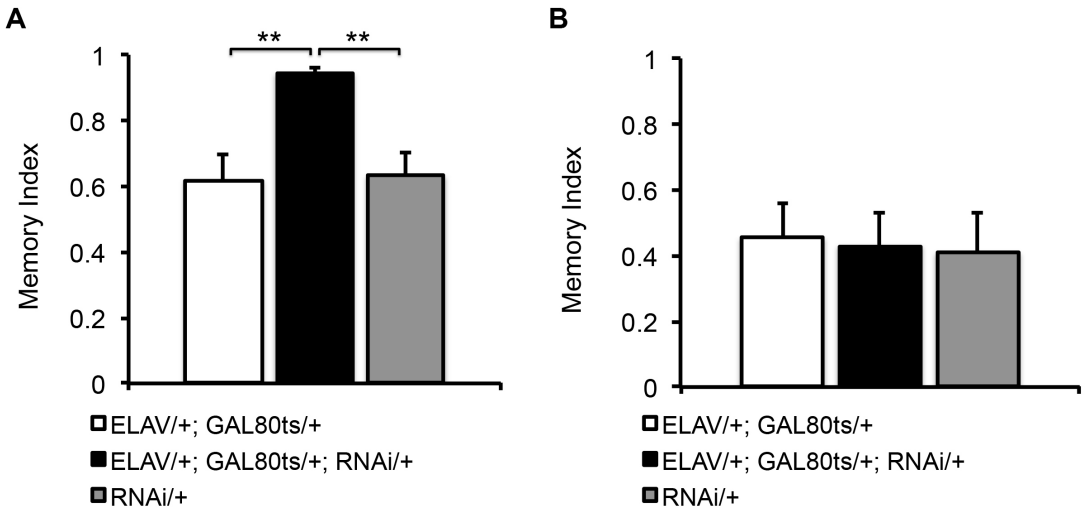


Figure 13: Knockdown of Moesin restricted to adulthood impairs LTM but not STM.

A. MOE knockdown driven by ELAV-GAL4 in adulthood with the TARGET system led to a significant impairment in 24-hour LTM (ANOVA, post-hoc Tukey's HSD, **p<0.01). B. STM tested one hour following a one-hour training session was not significantly different between parental control genotypes and MOE knockdown males.

3.9.3 MB specific Moesin KD in adulthood impairs LTM but not STM

As long-term courtship memory is mushroom body-dependent [142], we chose to examine the specific requirement for Moesin in these neurons for long-term memory, by restricting Moesin knockdown to the alpha/beta and gamma neurons of the mushroom body using the MB247-GAL4 driver. Mushroom body specific Moesin knockdown restricted to adulthood with the TARGET system impaired 24-hour memory (Figure 14A), indicating that Moesin is required in the mushroom body for long-term courtship memory. To confirm that this memory defect is specific to long-term memory, as was observed with pan-neuronal Moesin knockdown, short-term memory was assessed following mushroom body-specific Moesin knockdown. Like pan-neuronal Moesin knockdown, MB247-GAL4-mediated knockdown in adulthood had no impact on one-hour memory (Figure 14B).

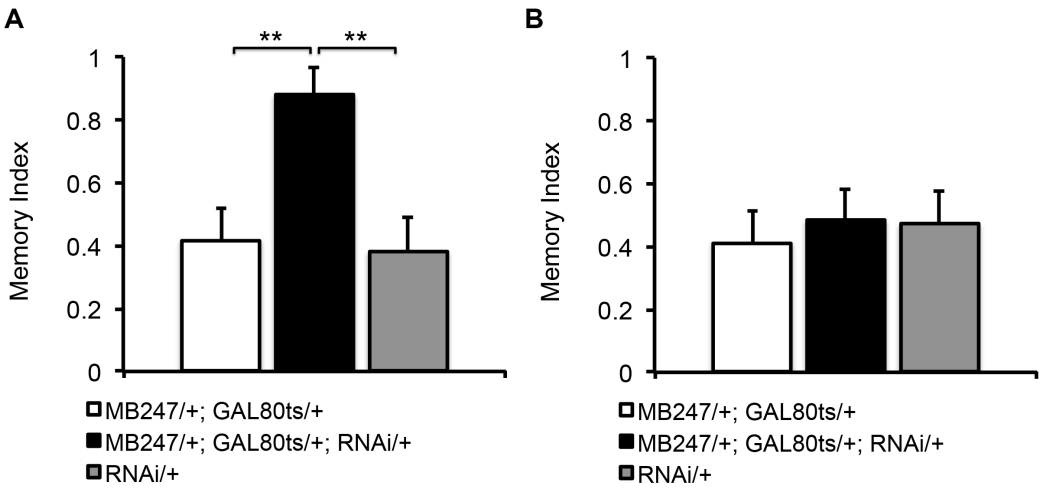


Figure 14: Mushroom body specific Moesin knockdown restricted to adulthood impairs LTM but not STM.

A. Moesin knockdown in the mushroom body driven by MB247-GAL4 in adulthood with the TARGET system led to significant impairment in 24-hour LTM (ANOVA, post-hoc Tukey's HSD, **p<0.01) B. One hour STM was not affected by knockdown of Moesin in the adult MB.

3.9.4 Overexpression of MOE-MYC has no impact on LTM

The requirement for Moesin in the mushroom body for long-term courtship memory led us to investigate the effect of elevated Moesin levels in mushroom body neurons on LTM. The MB247-GAL4 driver was used with the TARGET system to express MOE-MYC in the alpha/beta and gamma neurons of the mushroom body specifically in adulthood. Assessment of 24-hour memory revealed that overexpression of Moesin in the mushroom body had no significant effect on long-term courtship memory (Figure 15).

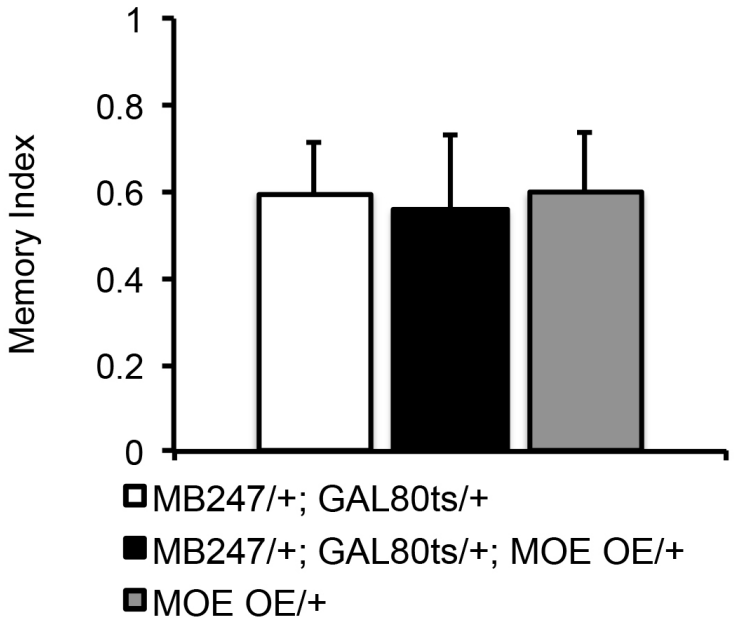


Figure 15: Mushroom body specific Moesin overexpression in adulthood has no impact on LTM.

No significant impairment in 24-hour LTM resulted from MB247-GAL4-mediated MOE-MYC expression in the MB with the TARGET system

3.9.5 Co-expression of MOE-MYC and Moesin RNAi restores LTM

Lastly, we examined whether expression of MOE-MYC could rescue the long-term memory defect caused by Moesin knockdown in the mushroom body. Co-expression of MOE-MYC and MOE-RNAi1 in the mushroom body was driven by MB247-GAL4 and restricted to adulthood with the TARGET system. On assessment of 24-hour courtship memory we found that flies co-expressing Moesin overexpression and knockdown constructs exhibited 24-hour memory indiscernible from parental controls, indicating that sufficient restoration of Moesin levels had been achieved to establish long-term courtship memory (Figure 16).

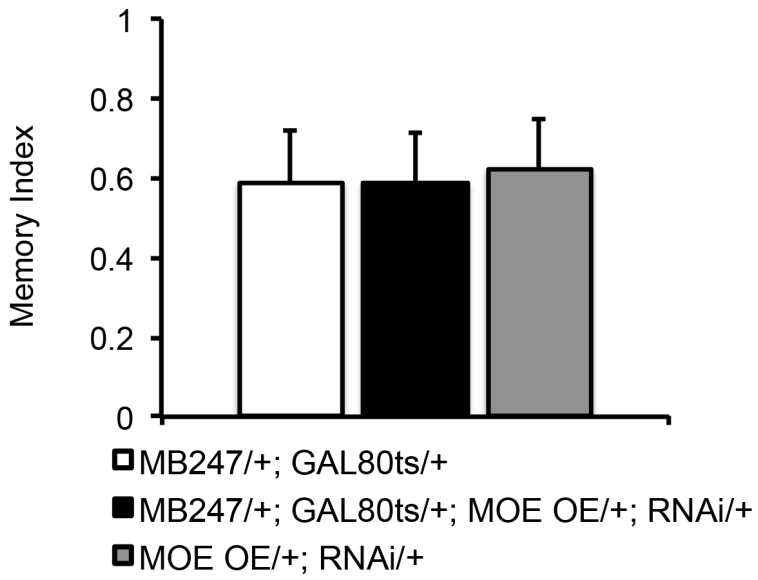


Figure 16: Rescue of Moesin knockdown LTM deficit by Moesin overexpression.

MB247-GAL4-mediated co-expression of MOE-MYC and MOE-RNAi1 with the TARGET system resulted in LTM indistinguishable from parental genotype controls

3.9.6 Characterization of Moesin expression in an HDAC4OE background

HDAC4 has been identified as a master regulator of memory formation in *Drosophila* [105] and overexpression of nuclear-restricted HDAC4 in mice has been shown to upregulate Moesin transcription while repressing a set of genes essential for synaptic function [104]. Further, Moesin knockdown in *Drosophila* was found to enhance an HDAC4 overexpression-induced defect in eye development [106]. To investigate whether there may be a physical interaction between HDAC4 and Moesin may be we examined their expression in the fly brain. As there are currently no *Drosophila* HDAC4 antibodies available, we analyzed the localization of a FLAG-tagged HDAC4 construct expressed throughout the adult brain using the pan-neuronal driver ELAV-GAL4 in the TARGET system. Anti-Moesin staining in the HDAC4OE flies does not appear to differ significantly from the endogenous expression pattern (Figure 17A,D). Additionally, there was no appreciable increase in Moesin expression. FLAG-HDAC4 predominantly localized to the mushroom body, with the highest levels in the alpha and beta lobes. FLAG-HDAC4 was also observed in the calyx, the dendritic field of the Kenyon cells (Figure 17H), and in a subset of Kenyon cell nuclei. Moesin is strongly localized to the

cytoplasm of the Kenyon Cells and largely absent from the mushroom body calyx. These observations do not support a possible physical interaction between HDAC4 and Moesin in the *Drosophila* brain.

As we previously observed the translocation of phosphomimetic Moesin to the mushroom body lobes in the adult fly brain, we next investigated whether activated Moesin may physically interact with HDAC4 by examining the co-localization of MOETD-MYC and FLAG-HDAC4. Myc staining in flies co-expressing MOETD-MYC and FLAG-HDAC4 revealed stereotypical MOETD-MYC localization, with its highest levels in the antennal lobe glomeruli and MB lobes (Figure 18A). Additionally, FLAG-HDAC4 localization did not appear to be affected by MOETD-MYC expression. Merging of these staining patterns reveals that MOETD-MYC is largely absent from the FLAG-HDAC4 stained beta lobes, while the gamma lobes are heavily stained for MOETD-MYC with low levels of FLAG-HDAC4 detected (Figure 18C). Magnification of the mushroom body region highlights a strong co-localization of FLAG-HDAC4 and MOETD-MYC in the tips of the alpha lobes, while FLAG-HDAC4 is largely absent from the MOETD-MYC labeled tips of the alpha' lobes (Figure 18D-F). While the expression pattern of endogenous Moesin and FLAG-HDAC4 is predominantly non-overlapping, the proximal localization between MOETD-MYC and FLAG-HDAC4 suggests that a physical interaction between Moesin and HDAC4 cannot be precluded.

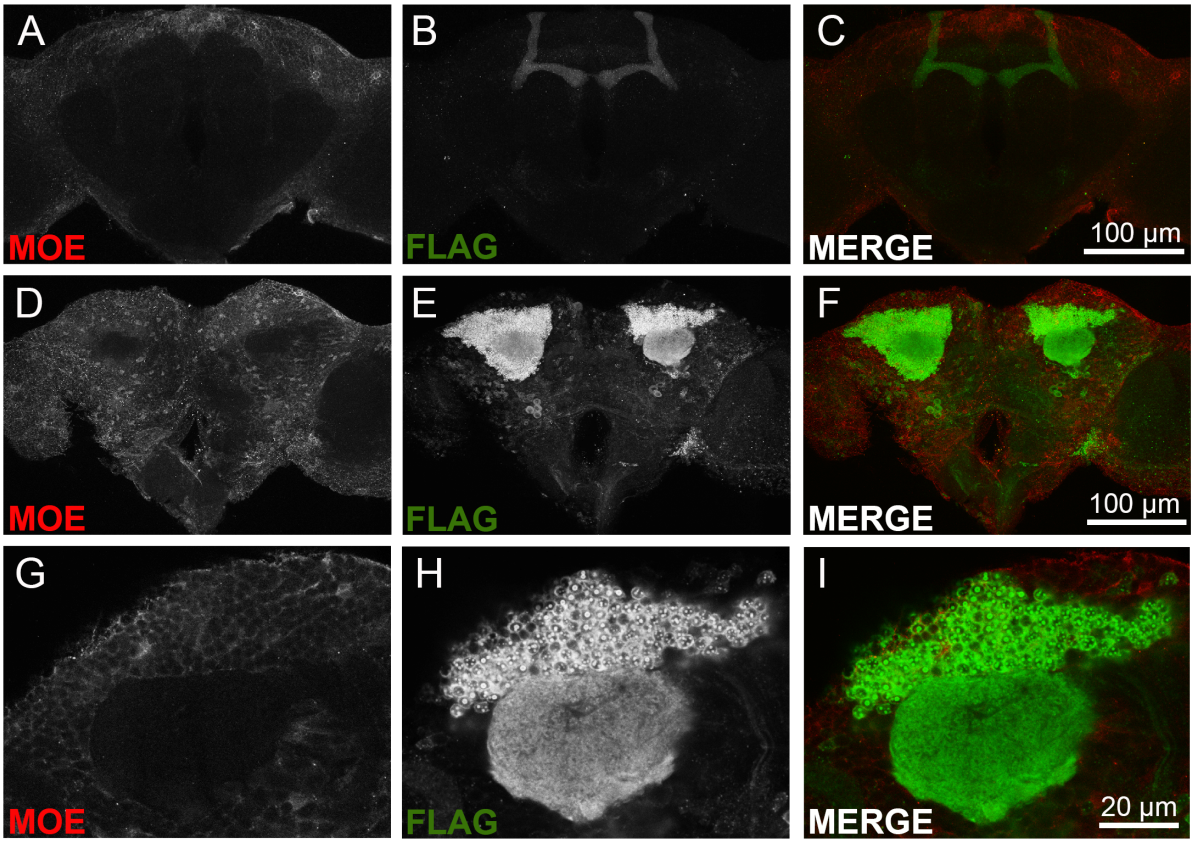


Figure 17: Overexpression of FLAG-tagged HDAC4 in the fly brain.

A-I. Immunohistochemistry with anti-Moesin (red) and anti-FLAG (green) antibodies on whole mount brains of flies expressing FLAG-HDAC4 with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system. A-C. Frontal confocal projection through the brain, scale bar = 100 µm. D-F. Posterior confocal projection through the brain, scale bar = 100 µm. G-I. 1 µm optical slice through the calyx, scale bare = 20 µm.

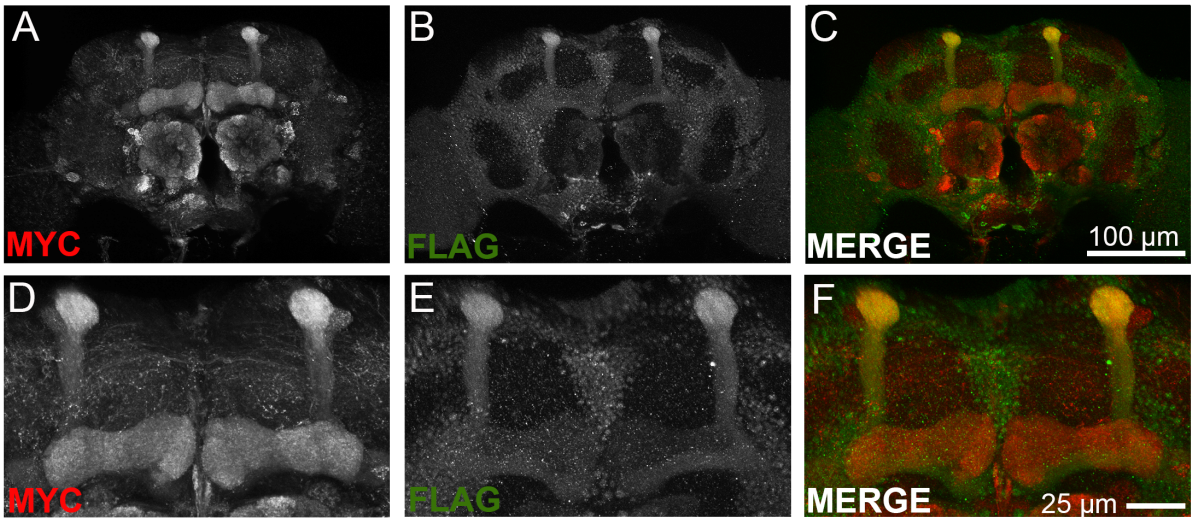


Figure 18: Co-expression of phosphomimetic Moesin and HDAC4 in the fly brain.

A-F. Immunohistochemistry with anti-myc (red) and anti-FLAG (green) antibodies on whole mount brains of flies co-expressing MOETD-MYC and FLAG-HDAC4 with ELAV-GAL4-mediated expression restricted to adulthood with the TARGET system. A-C. Frontal confocal projection through the brain, scale bar = 100 µm. D-F. Confocal sections through the mushroom body region of the brain, scale bar = 25 µm.

Chapter 4: Discussion

Here, we characterize the impact of modulating Moesin expression on several aspects of development and behavior. We describe an essential role for Moesin in neurological function throughout development in addition to a distinct adult role. We present evidence that Moesin is involved in the neurogenesis of the central nervous system and identify an independent role in long-term memory in the adult brain. Additionally, we analyze the role of phosphoregulation of Moesin activity through expression of a phosphomimetic Moesin mutant in the *Drosophila* brain.

4.1 Moesin in involved in brain development

Through its regulation of the actin cytoskeleton the adaptor protein Moesin has been shown to critical to neuronal development in primary cultured neurons [62]. In this study we describe a crucial role for Moesin in the formation of a central brain structure, the mushroom body, as well as a function in establishing complex dendritic arbors critical to the formation of neural circuits.

The examination of axon growth and guidance in mushroom body assembly indicates an integral role for Moesin in axon projection, targeting, and branching. We found that that Moesin knockdown as well as overexpression impaired mushroom body development. In comparison, Moesin overexpression generally resulted in less severe developmental phenotypes, however, the expression of phosphomimetic Moesin resulted in complete disruption of mushroom body assembly, highlighting a critical role for phosphoregulation of Moesin in neurogenesis. Moesin was recently found to interact with the cell adhesion molecule neuroglian in mushroom body assembly [132]. Mutational analysis of Neuroglian, the sole *Drosophila* L1CAM homolog, identified a requirement for the ERM-interaction domain, to which Moesin binds, in the establishment of the mushroom body's highly organized architecture [131]. Neuroglian mutants display severe mushroom body phenotypes including growth and guidance errors, missing lobes and branching defects [131, 132], similar to those herein that result from the modulation of Moesin expression. The deletion of the ERM interaction domain of Neuroglian, however, results in a phenotype in which aberrant axonal projections form a ball-like structure from

continuous circular growth in the posterior of the brain [132]. This phenotype has been described previously as axon stalling, however, it has now been characterized as a guidance error following the discovery that the defect results from a failure of axons to enter the peduncle [131, 132].

In contrast, our data reveal an axon stalling phenotype in which axon growth is arrested subsequent to branching from the peduncle and is observed in both Moesin knockdown and overexpression brains. While this axon stalling defect was not typified by ball-like axonal accumulations, MARCM analysis (mosaic analysis with a repressible cell marker) [150], in which single mushroom body neurons can be labeled, will be required to determine whether this phenotype results from growth cone arrest or a similar guidance error. Additionally, the lack of any aberrant axonal accumulations in the posterior of brain hemispheres with missing mushroom body lobes indicates that these defects are likely the result of branching errors as axons either fail to bifurcate or subsequently segregate into vertical and medial lobes. The prevalence of this lobe formation defect in neuroglian knockdown mushroom bodies and the importance of the ERM protein interaction domain to L1CAM-mediated axon branching in vertebrates suggests that reduction of Moesin expression may impair branching in part due to an interaction with neuroglian [132, 151]. The range of mushroom body defects resulting from the modulation of Moesin expression and activation highlights a central role for Moesin in axon growth and guidance. A screen for genetic interaction with neuroglian in mushroom body assembly identified the guidance cue Sema1a. This transmembrane guidance molecule of the Semaphorin family not only suppressed mutant neuroglian mushroom body phenotypes but is also found to regulate Moesin in photoreceptor axon targeting.

Sema1a has been shown to interact genetically and physically with Moesin in photoreceptor axons to promote its phosphorylation and activation [152]. In turn, Moesin was found to increase Fas2-mediated axon–axon attraction by downregulating the activity of the Rho family GTPase Rho1 in axon terminals. The mediation of axon-axon attraction is critical for a process called fasciculation in which one axon can grow along the surface of another axon or be assembled into bundles through adhesive interactions [153]. This process is particularly critical in mushroom body assembly as each lobe is

comprised of bundled axons, highlighting the need to clarify if Sema1a also regulates Moesin activity during mushroom body development. Our data reveal the importance of phosphoregulation of Moesin in photoreceptor and mushroom body development, as evidenced by the severe defects that result from the neuronal expression of phosphomimetic Moesin. While the modulation of Moesin expression also led to serious defects in mushroom body assembly, no defect was observed in photoreceptors in which Moesin was knocked down or overexpressed. This finding is consistent with previous reports in which reduction of Moesin dosage by up to half did not impair photoreceptor development and only overexpression of phosphomimetic Moesin led to guidance defects [143].

In addition to a role in axon growth and guidance, we present a critical role for Moesin in dendritic morphology. The stereotypical dendritic arborization of the vertical system (VS) of lobula plate tangential cells was severely disrupted by the modulation of Moesin expression. Knockdown of Moesin resulted in a severely reduced dendritic field with little or no dendritic projections from multiple VS neurons, while Moesin overexpression led to a dendritic tree with typical field coverage but lacking its characteristic branching pattern. The complex phenotypes emerging from the modulation of Moesin suggest that it may be involved in multiple aspects of dendritic arborization. Previously characterized regulators of dendritic arborization in *Drosophila* include the Rho GTPases Rac1 and Rho1 which appear to have opposing effects on the growth and complexity of dendrites. Rac1 promotes dendritic branching and extension while Rho1 restricts both branching and branch length [154-157]. As Moesin has been shown to negatively regulate Rho1 activity in *Drosophila* neurons [152], some of the developmental effects resulting from the modulation of Moesin may be mediated by Rho1. Given Rho1's role in restricting dendritic outgrowth, the lack of dendritic projections in some Moesin-knockdown VS neurons may be the result of Rho1 hyperactivity. Further, downregulation of Rho1 activity may account for the overextension of dendrites in VS neurons in which Moesin is overexpressed.

As both the precise navigation of axons to their synaptic targets and the appropriate arborization of dendrites to a receptive field are each critical for proper circuit formation,

the integral role Moesin plays in neuronal development may be the source of the memory impairment observed when Moesin is knocked down during development.

4.2 Moesin is required for long-term memory

Long-term memory is thought to reflect structural and functional changes at specific neuronal synapses [158, 159]. These plastic changes at the synapse are highly contingent upon actin remodeling within particular compartments to enable these dynamic structural modification in neuronal morphology [160-162]. Here, we provide evidence that the actin-binding protein Moesin is necessary for long-term memory processes in *Drosophila*.

The pan-neuronal knockdown of Moesin during development severely impaired 24-hour long-term memory and was associated with defects in assembly of the mushroom body, an important center for associative learning. As mushroom body ablation has been shown to impair courtship memory, the severe defects in development resulting from Moesin knockdown are at least in part responsible for the long-term memory deficit [142]. This finding highlights a critical role for Moesin in the development of brain structures that parallels vertebrate neurodevelopmental disorders [107, 108]. To investigate the post-developmental role of Moesin we utilized a temperature-sensitive expression system for the conditional knockdown of Moesin in the adult brain. Interestingly, restriction of Moesin knockdown to adult brains led to similar defects in long-term memory, while one-hour short-term memory was unaffected. This argues strongly for a post-developmental role for Moesin in memory, as short-term memory, which requires mushroom body function was unimpaired [142]. By targeting Moesin knockdown in the adult specifically to the neurons which comprise the mushroom body, the requirement for Moesin in long-term memory was traced to the alpha/beta and gamma neurons of the mushroom body.

While the precise molecular mechanisms behind courtship learning are still largely unresolved, several steps in the acquisition and consolidation of memory have been elucidated and the synapses of mushroom body gamma neurons have emerged as the

likely site of the plastic modifications that underpin long-term courtship memory in *Drosophila*. Courtship learning represents an increased behavioral response to a male pheromone deposited onto females during mating, thus distinguishing mated females from virgins [39, 135]. This increased sensitivity to the male pheromone cis-vaccenyl acetate is thought to be mediated by a dopamine reinforcement signal and a class of dopaminergic neuron (aSP13) that innervate the gamma lobe of the mushroom body convey this signal for short-term memory [39]. Interestingly, reactivation of the dopaminergic neurons required for the acquisition of short-term memory is both necessary and sufficient for the consolidation of long-term courtship memory [41]. Accordingly, the dopamine receptor DopR1, which is activated in the gamma lobe by these dopaminergic neurons, is required not only during the acquisition phase for short-term memory but also later for the consolidation of long-term courtship memory [39, 41]. One protein found to act downstream of DopR1 in gamma neurons in courtship learning is the CPEB (cytoplasmic polyadenylation element–binding protein) Orb2. This translational regulator has two isoforms which each perform distinct roles during courtship learning. Orb2A is localized to synapses in gamma neurons during memory acquisition and thought to mark activated synapses for consolidation upon subsequent stimulation [40, 41]. Upon post-acquisition dopamine stimulation through DopR1, Orb2A recruits Orb2B into complex at the synapse to regulate local translation of CaMKII [41], a protein critical for long-lasting memory formation [42, 43]. The structural and functional modifications at the synapse resulting from the neuronal activity-dependent synthesis of CaMKII and other potential memory genes rely upon the modification of the actin cytoskeleton. As a key regulator of the actin cytoskeleton in neurons, Moesin may impact the formation or strengthening of synapses in several ways during long-term memory consolidation, however, two potential mechanisms stand out: the anchorage of mRNA at the synapse for localized translation and the development of dendritic spines which mature into synapses.

First, the localized translation of mRNA depends not only on transport to the dendrites but also anchorage at the synapses following translocation and actin-binding proteins have been proposed to fulfill this role [163, 164]. Moesin is required for mRNA anchorage and the proper localization of the RNA-binding protein Staufen in oocytes

[144, 165]. Staufen, which is involved in mRNA translocation and translation in neurons as well as oocytes [166-171], has been found to be required during long-term olfactory memory formation [102]. Additionally, Staufen controls the accumulation of the glutamate receptor IIA subunit (GluRIIA) at synapses through regulation of coracle mRNA localization and translation [172]. Coracle anchors GluRIIA to the actin cytoskeleton and knockdown of Staufen not only reduces Coracle and GlurIIA accumulation at the synapse but also decreases bouton number [172, 173].

Interestingly, Staufen and Moesin are both upregulated following training that leads to long-term memory [102] and may, therefore, participate in a reciprocal interaction between actin dynamics and dendritic protein synthesis that contributes to consolidation [174]. In this model, a feedback loop is generated whereby newly synthesized proteins that regulate the actin cytoskeleton and facilitate mRNA translocation and translation, like Moesin and Staufen, promote further local protein synthesis. During long-term olfactory memory formation, local translation of Staufen and CaMKII mRNA is enabled by a synaptic activity-dependent pathway and it will be of interest to determine if Moesin synthesis is similarly regulated or whether Moesin upregulation may result from secondary CaMKII signaling [42].

Next, as CaMKII signaling regulates the plastic modification of dendrites through cytoskeletal reorganization in *Drosophila*, including the formation of spines, the synthesis of CaMKII during consolidation may initiate the structural modification of dendrites [175]. The formation of dendritic spines is thought to be critical to memory formation and maintenance and the Rho GTPases Rac1, RhoA/Rho1, and Cdc42 have been shown to mediate spine development through the regulation of ERM proteins in vertebrate neurons [83-86]. In hippocampal neurons, ERM proteins promote the formation of filopodia, the precursors to dendritic spines, while also engaging as a molecular brake on their conversion to spines [78-81]. Rac-1 mediated dephosphorylation of ERM proteins is required to overcome this impediment to filopodia-to-spine transition [79, 83, 84]. In cortical neurons, however, Moesin is activated by the vertebrate homolog of Rho1 (RhoA) and its associated kinase (ROCK) to promote sex-steroid induced spine development formation [85, 86].

Vertebrate-like dendritic spines have been identified in *Drosophila* lobula plate tangential cells that are enriched in actin, bear cholinergic receptors, and are sites of synaptic connection [149]. To date, Rac1 is the only protein identified to promote the development of dendritic spines in *Drosophila* [149]. In addition, the Rho GTPase Cdc42 has been identified as the only negative regulator of spine density in *Drosophila* [176]. While Moesin's critical role in dendritic development obscures observation of spine formation in lobula plate tangential cells, the finding that Rho GTPases govern spine formation in *Drosophila* suggests that the pathways observed in vertebrate spine development may be conserved in *Drosophila*. Further, it has recently been revealed that the Kenyon cell neurons which comprise the mushroom body also bear vertebrate-like dendritic spines along their dendritic branches and within their claw-like endings which each enwrap a projection neuron bouton [177-179]. As these projection neurons transmit the cis-vaccenyl acetate signal to the mushroom body where this stimulus is processed [135], these synapses may be the sites of the plastic changes that represent the enhanced sensitivity to the male pheromone and a behavioral adaptation in courtship .

Chapter 5: Conclusion and Future Directions

We have established that the actin-binding protein Moesin plays an essential role in neurological function in *Drosophila*, both during neuronal development and in the adult brain, with a distinct contribution to memory independent of its developmental role. Both Moesin knockdown and overexpression disrupt axonal as well as dendritic development. We describe an axonal stalling phenotype during mushroom body assembly which resembles growth cone arrest, however, analysis of individual Kenyon cells by single cell labeling through the MARCM (mosaic analysis with a repressible cell marker) technique will be required to distinguish this defect from comparable guidance errors [150]. In addition, we found that expression of phosphomimetic Moesin, which mimics activated Moesin, severely impaired mushroom body assembly indicating that the phosphoregulation of Moesin is critical during development. This finding highlights the need to investigate the regulators of Moesin activity during neurogenesis, especially the guidance cue Sema1A, which not only activates Moesin but also interacts with Neuroglian in mushroom body assembly [131, 152].

Further, we describe a requirement for Moesin in long-term courtship memory, independent of any developmental effects. This requirement was traced to the alpha/beta and gamma neurons of the mushroom body in the adult. While this finding is consistent with the emergence of the gamma mushroom body neurons as the likely site of the plastic modifications that underpin long-term courtship memory, the requirement for Moesin in each of the three sets of mushroom body neurons (alpha/beta, alpha'/beta', and gamma) should be compared. We propose that Moesin contributes to long-term memory consolidation through two mechanisms: the promotion of synaptic protein synthesis and the formation of dendritic spines. A primary objective in the investigation of Moesin's role in synaptic protein synthesis will be to determine whether Moesin is required for mRNA anchorage and the proper localization of the RNA-binding protein Staufen in neurons as in oocytes (Jankovics et al., 2002; Polesello et al., 2002). Additionally, as Staufen has been found to control glutamate receptor IIA subunit (GluRIIA) accumulation and bouton number (Gardiol and St. Johnston, 2014), it will be of interest to examine these qualities in Moesin knockdown neurons. Also, while severe

developmental defects impaired the examination of dendritic spine density in Moesin knockdown and overexpression lobula plate tangential cells, a temperature sensitive system to restrict transgene expression to adulthood can be implemented in order to analyze Moesin's role in dendritic modification in the adult brain [118]. One thread linking Moesin's involvement in axon growth and guidance, dendritic arborization, and dendritic spine formation is the activity of the Rho GTPases Rac1, RhoA/Rho1, and Cdc42 which each play a role in these neuronal processes [154-157, 176, 180](Haas et al., 2007). In vertebrates, these Rho GTPases regulate the activation of Moesin in neurons and it will be crucial to identify whether they also govern Moesin activity in *Drosophila*.

Next, we found that the overexpression of Moesin in the adult mushroom body had no impact on long-term memory, which suggests that phosphoregulation of Moesin may be critical to its function in long-term memory. To that end, the effect of phosphomimetic Moesin expression in mushroom body neurons on long-term courtship memory should be examined. Finally, in order to whether determine whether Moesin's role in long-term memory is conserved in higher organisms, memory should next be analyzed in Moesin knockout mice.

Chapter 6: References

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