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Drk-mediated molecular pathways in learning and Protein Synthesis-Independent Memory (PSIM) in *Drosophila melanogaster*

Conducted in: Dr. Skoulakis lab, Institute for Fundamental Biomedical Research, Biomedical Sciences Research Centre "Alexander Fleming," Vari 16672, Greece

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Παράβαση της ανωτέρω ακαδημαϊκής μου ευθύνης αποτελεί ουσιώδη λόγο για την ανάκληση του διπλώματός μου».

Ημερομηνία

11/09/2023

Ο Δηλών

Αθανάσιος Μανώλης

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Α. ΠΕΡΙΛΗΨΗ

Η κατανόηση των μοριακών μηχανισμών που διέπουν τη μνήμη είναι αναγκαία για την ανάπτυξη μεθόδων προς αντιμετώπιση διαταραχών της μνήμης, συμπεριλαμβανομένων των νευροεκφυλιστικών νοσημάτων και των γνωσιακών παθήσεων που σχετίζονται με την γήρανση. Η μάθηση και η μνήμη μελετώνται σε θηλαστικά και απλούστερους οργανισμούς μοντέλα, όπως η *Drosophila* melanogaster. Ενδιαφέρον προκαλεί το γεγονός ότι οι μύγες μπορούν να σχηματίσουν έναν τύπο μνήμης που, αντίθετα από την ευρέως μελετημένη μακροπρόθεσμη μνήμη, είναι ανεξάρτητος από τη σύνθεση νέων πρωτεϊνών (Protein Synthesis Independent Memory-PSIM). Παρόλο που η PSIM έχει προσδιοριστεί μόνο στη *Drosophila*, είναι απίθανο να μην έχει διατηρηθεί μέσω της εξέλιξης. Πράγματι, ορισμένα χαρακτηριστικά της PSIM έχουν αναγνωριστεί σε άλλα ασπόνδυλα και σπονδυλωτά, όπως ο άνθρωπος. Επειδή οι μηχανισμοί που διέπουν την PSIM είναι σε μεγάλο βαθμό άγνωστοι, χρησιμοποιήθηκε η μέθοδος της εντεταμένης εκπαίδευσης (Massed Conditioning-MC), που οδηγεί αποκλειστικά στο σχηματισμό PSIM, για να αποκαλυφθούν μοριακά στοιχεία αυτής της νέας μνήμης στη *Drosophila*. Προηγούμενες μελέτες του εργαστηρίου έχουν δείξει ότι η πρωτεΐνη Drk, η οποία δρα κάτω από υποδοχείς κινάσης-τυροσίνης, είναι απαραίτητη στα Μισχοειδή Σωμάτια (Mushroom Bodies-MBs), το "κέντρο" της μάθησης και της μνήμης στη μύγα, για την κανονική μνήμη που προκαλείται από την MC. Από πρωτεομική ανάλυση εντός των ενήλικων MBs, εντοπίστηκε ένας αριθμός πρωτεϊνών που πιθανά αλληλεπιδρούν με την Drk, συμπεριλαμβανομένων νέων μορίων που ενέχονται στην PSIM και ίσως συμμετέχουν στα ίδια σηματοδοτικά μονοπάτια με την Drk. Η παρούσα μελέτη παρουσιάζει τη λειτουργική επαλήθευση αυτών των πρωτεϊνών που αλληλεπιδρούν με την Drk, η οποία υποστηρίζει τον ρόλο ορισμένων από αυτές στην μνήμη που προκαλείται από την MC. Η περαιτέρω μελέτη της λειτουργίας και της σηματοδότησής τους θα συμβάλει, ελπίζουμε, στην κατανόηση των μηχανισμών που βρίσκονται στη βάση των μνημών που είναι ανεξάρτητες της σύνθεσης πρωτεϊνών, όχι μόνο στη *Drosophila*, αλλά και στα θηλαστικά.

B. ABSTRACT

Understanding the molecular mechanisms that govern memory is necessary to develop methods to combat memory disorders including dementias and age-dependent cognitive impairments. Learning and memory are being studied in mammalian and simpler model organisms, as *Drosophila melanogaster*. Interestingly, flies are able to form a memory type which unlike the well-studied Long-Term Memory is independent of novel protein synthesis (PSIM). Although PSIM has been identified only in *Drosophila*, it is unlikely that it has not been conserved through evolution. In fact, PSIM- characteristics have been identified in other invertebrates and vertebrates, including humans. Since the mechanisms that govern PSIM are largely unknown, the massed conditioning (MC) training method, which solely yields PSIM, was used to reveal molecular components of this novel memory in *Drosophila*. Previous studies in the lab have revealed that the adaptor protein Drk, which acts downstream of tyrosine-kinase receptors, is essential in the Mushroom Bodies (MBs), -the center of learning and memory in flies- for normal MC-elicited memory. Proteomic analysis within the adult MBs helped identification of probable Drk-interacting proteins, including novel molecules that are engaged in PSIM and might participate in the same signaling pathway as Drk . The present study presents a functional validation of these Drk-interacting proteins, which supports roles for a number of them in MC-elicited memory. Further study of their function and signaling will hopefully aid our understanding of the mechanisms that underlie protein synthesis-independent memories not only in *Drosophila*, but also in mammals.

Acknowledgments

I would first like to thank Dr. Michaelidis for believing in me and giving me the opportunity to test my abilities in a new environment and Dr. Skoulakis for the trust he showed me by welcoming me to his laboratory as well as for the excellent cooperation we had throughout this time. In addition, I would like to sincerely thank Anna Bourouliti, for all the time and energy she devoted to my training, but also for her support in all areas and in every difficulty. Furthermore, big thanks to Alex Charonitakis and Dr. Katerina Papanikolopoulou for helping me with the dissections and the Confocal Microscopy. Finally, a big thank you to all present and past members of the Skoulakis Lab who made this journey even more beautiful.

C. INTRODUCTION

1. Learning and memory

Learning and memory are two of the most magical abilities of our mind. Understanding the mechanisms of these two fundamental components of cognition has been a subject of intense research in the fields of neuroscience and psychology. Learning is the biological process of acquiring new knowledge about the world, and memory is the process of retaining and reconstructing that knowledge over time [1]. The ability to learn and retain information allows organisms to adapt to changing environments, make better decisions based on events from ore-experienced stimuli and is overall essential for survival. Memory and learning are complex processes involving numerous brain regions, cellular mechanisms, and molecular pathways. Recent advances in technology and experimental techniques have enabled researchers to study memory and learning at various levels of analysis, from genes and molecules to circuits and behavior. However, despite decades of research, the precise neural and molecular mechanisms underlying these processes remain elusive [2], [3].

1.1. Definition of memory

Early neuroanatomists in the 19th century observed that memories are not primarily formed through generation of new neurons, since the number of neurons in the brain does not increase significantly after reaching adulthood, but rather through strengthening of the connections between them. The discovery of long-term potentiation in 1966 provided further evidence that memories may be encoded in the strength of synaptic signals between neurons. This marked the beginning of our understanding of memory as a neuro-chemical process. Research of *Aplysia californica* by Eric Kandel, earned him the Nobel prize, and showed that classical conditioning leads to memory storage that can be observed on a molecular level in simple organisms. Today, memory is defined as the faculty of encoding, storing, and retrieving information, with researchers increasingly exploring the chemistry behind memory formation and recall [4].

1.2. Types of memory

It is a fact that classification of memory types is complex and there is an ongoing debate among researchers about the number and distinction of memory types. However, based on the latest scientific advances related to this subject, there is a general classification of memory types (Fig.1).

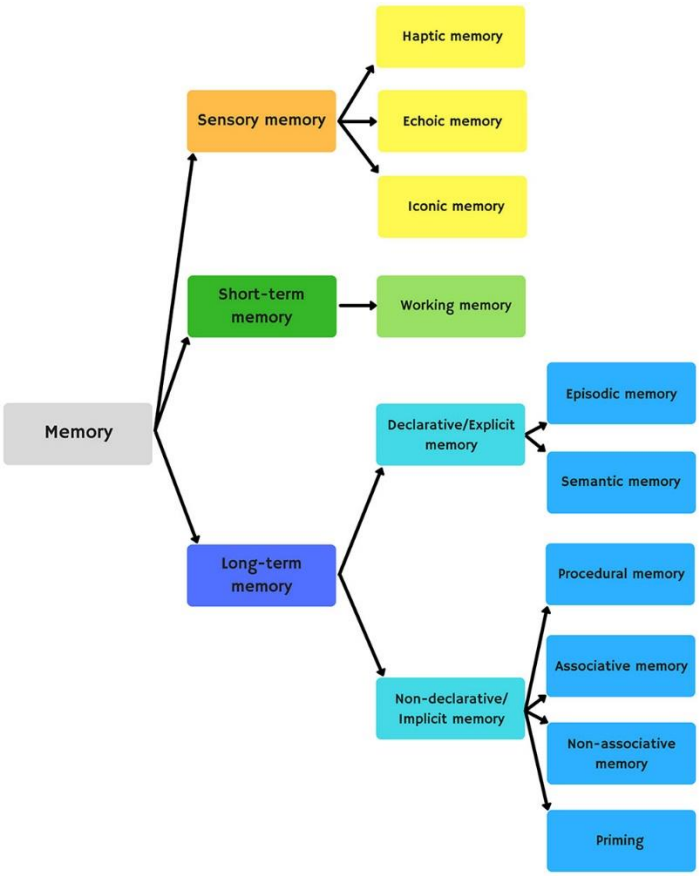


Fig.1 Memory classification [5]

Thus, among them there are three major classifications, which can be stated as follows:

1) Sensory memory

Sensory memory refers to the not consciously controlled ability to temporarily hold a significant amount of information that individuals encounter daily. It encompasses three distinct types: iconic memory, which retains visual information; echoic memory, which retains auditory information from auditory stimuli; and haptic memory, which retains information obtained through touch.

2) Short-term memory

Short-term memory is the ability to maintain information available for a brief period of time. Different models propose the existence of a short-term storehouse with limited capacity enough to transmit information to the structures responsible for long-term memory, generating new deductions and logical reasoning based on existing information. So, it serves as a working memory system that allows for the temporary retention and manipulation of information, facilitating various crucial complex cognitive processes such as language comprehension, learning and reasoning.

3) Long-term memory

Long-term memory is the ability to store and maintain information for extended periods, potentially throughout a person's lifetime. It encompasses two main types:

declarative or explicit memory and non-declarative or implicit memory. Explicit memory refers to information that can be consciously recalled and retrieved. Declarative memory consists of two subtypes: episodic memory, which involves memories of specific events and experiences; and semantic memory, which pertains to general knowledge and facts. On the other hand, implicit memory comprises unconscious memories, including various abilities and skills. There are four main types of implicit memory: procedural memory, which involves remembering how to perform tasks or skills; associative memory, which relates to the formation of connections between stimuli; non-associative memory, which concerns the recognition of changes in stimuli over time; and priming, which involves the facilitation of processing and recognition of information based on previous exposure [4], [5].

1.3. How do we study behavior?

During the effort to distinguish between the disparate approaches to memory storage, it soon became clear that one needed to develop tractable behavioral systems. Such systems would make it more likely to see how specific changes in the neuronal components of a behavior modify that behavior during learning and memory storage. In the mid-twentieth century, the study of learning and memory was revolutionized by the application of reductionist techniques to identify the cellular and molecular basis of these processes. Simple behavioral systems, such as the flexion reflex of cats, the eye-blink response of rabbits, and various forms of reflex learning in invertebrates, emerged as tractable models for studying learning and memory [1], [6]–[8]. Remarkably, even animals with relatively few nerve cells have impressive learning capabilities. For example, *Drosophila*, an invertebrate with approximately 100,000 nerve cells in its central nervous system, is capable of various elementary forms of learning. Each of these forms of learning can give rise to short- or long-term memories, which suggests that implicit memory storage does not depend on specialized neurons that store information. Rather, the capability for storing implicit memory is built into the neural architecture of the reflex pathway itself and depends on its capability for synaptic plasticity[1].

1.4. Associative memory and the Pavlovian assay

Formation of associative memory is achieved through either of two forms of conditioning: classical conditioning and operant conditioning which involve the establishment of associations between stimuli or stimuli and actions, respectively. In both cases a behavior may be altered, like when a behavior is followed by a positive reinforcement or a removal of negative reinforcement, it increases the likelihood of that behavior recurring. [5].
The Pavlovian or Pavlov's dog experiment is a famous example of classical conditioning conducted by Russian physiologist Ivan Pavlov. In Pavlov's studies, dogs associated a neutral stimulus with a natural reflex response. When a dog detects the presence of food, it naturally produces saliva as an unconditioned response. Through repeated pairings of the sound of a bell with the act of giving the dog food, the dog starts associating the bell sound (conditioned stimulus) with the presence of food (unconditioned stimulus). As this pairing is consistently repeated, the dog forms an association between the unconditioned stimulus and the conditioned stimulus, resulting in the dog producing saliva when it hears the bell alone [5], [9].

The Pavlovian assay, which is based on classical conditioning principles, can also be conducted with flies as experimental subjects, since *Drosophila* has been shown to be capable of an array of learning tasks. In this type of experiments a neutral stimulus, such as a particular odor or light, can be paired with a biologically significant stimulus or event, such as an electric shock (punishment) or food (reward). Through repeated pairings, the flies can learn to associate the neutral stimulus with the presence or absence of the biologically significant event. For example, in an odor-based Pavlovian assay, flies may be exposed to a specific odor (conditioned stimulus-CS) while simultaneously receiving a mild electric shock (unconditioned stimulus-US). Over time, the flies can learn to associate the odor with the aversive stimulus, and their behavior may change accordingly when they encounter the odor alone displaying avoidance behaviors [10].

2. Drosophila melanogaster

2.1. *Drosophila* in biomedical research

Drosophila melanogaster
https://bcs.mit.edu/drosophila 1

The intricate neural circuitry and large number of cells in the mammalian brain make it difficult to comprehend behaviors, brain development, and neurological disease. To overcome these challenges, simpler model systems like *Drosophila melanogaster*, *Caenorhabditis elegans*, or the zebrafish (*Danio rerio*) are used as research models to understand neurological processes in a more manageable way. *Drosophila melanogaster*, an invertebrate model, has been extensively used to study human neurological diseases at least for 30 years already. These models offer several advantages over studying rodents, such as simpler neural circuitry, shorter lifespan, and larger brood size, which enable researchers to study the effects of genetic modifications and environmental factors on neurological function and disease development. *Drosophila's* central nervous system shares many similarities with mammals, making it a valuable tool to study behaviors and processes relevant to human neurological diseases such as Parkinson's and Alzheimer's. Furthermore, *Drosophila* shares a significant portion of its genetic code with humans, and more specifically approximately 75% of human disease genes have a counterpart in the fly genome. This gives the chance to researchers to gain insights into the workings and diseases of the human brain [11]–[14].

2.2. Life cycle

Drosophila melanogaster is advantageous as a model organism due to its short life cycle, which allows the production of large numbers of progeny for genetic crosses. The process of development from a fertilized egg to an adult *Drosophila* lasts for approximately 9-10 days at 25°C, however, temperature can greatly influence the speed of this process, with flies cultured at 18° requiring ~19 days from egg to adult. Embryogenesis taking around 24 hours, followed by three larval stages with molting events between each stage. Larval development is complete five days after fertilization, and the animal metamorphoses within a hard pupal case for 4-5 days. During this time,

most larval tissues break down, and adult structures develop from 19 imaginal discs present in the larvae. The adult flies emerge from the pupal case in a process called eclosion and become sexually mature in about 8-12 hours, allowing the life cycle to repeat itself (Fig.2) [11], [15].

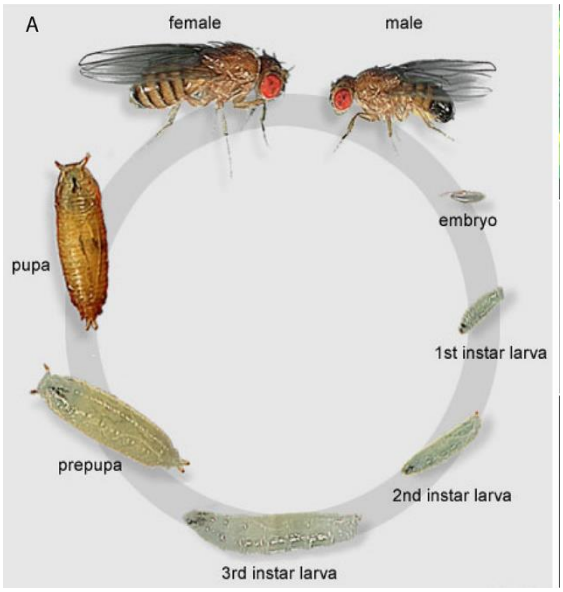


Fig.2 Drosophila melanogaster develops in approximately 10 days, moving through embryo, three larval, pupal, and adult stages. (Modified image by [11]*)*

2.3. Genetic tools and molecular techniques

GAL4-UAS and TARGET systems

In *Drosophila* a wide armamentarium of genetic tools is available. In a reverse genetic approach, a candidate gene is evaluated for its potential functional role. However, until recently, it was necessary to choose between having temporal control or spatial control over the expression of a transgene. Nowadays, targeted gene expression is a useful tool for studying and regulating specific genes, cells, and tissues in an intact organism. One of the most important genetic systems used in reverse genetic approaches is the GAL4/UAS-system (Fig. 3 (A)). This approach involves directing the expression of a transgene to a specific location or time period within the organism using the GAL4-upstream activator sequence (UAS) system, which is adapted from yeast. The system involves using a cloned promoter or enhancer to direct the expression of the GAL4 transcriptional activator in a spatially restricted manner. This activator then drives the expression of a gene of interest downstream of a UAS binding site. The advantage of this system is that the driver and target genes are carried in different parental lines, allowing for a combinatorial approach with different driver and target lines to address various biological questions. Once a line expressing GAL4 in a specific spatial pattern is generated, it can be crossed with any UAS target line, enabling the GAL4 line to be used as a general resource. Similarly, when a UAS target line is generated, the target gene can be transcribed anywhere in the organism by crossing it with the appropriate GAL4 line. In the TARGET System, a modification of the conventional GAL4-UAS system, a temperature-sensitive allele of GAL80 is used to regulate gene expression. At a lower temperature of 19°C, the transcription of a specific gene is suppressed, but this

repression is removed when the temperature is increased to 30°C, allowing for high levels of expression of in a specific tissue. So, this approach provides a general method for achieving combined temporal and regional gene expression targeting (TARGET) with the conventional GAL4-UAS system in *Drosophila* and allows us to express genes of interest in specific phases of flies' development and adulthood (Fig. 3 (B)) [12], [16].

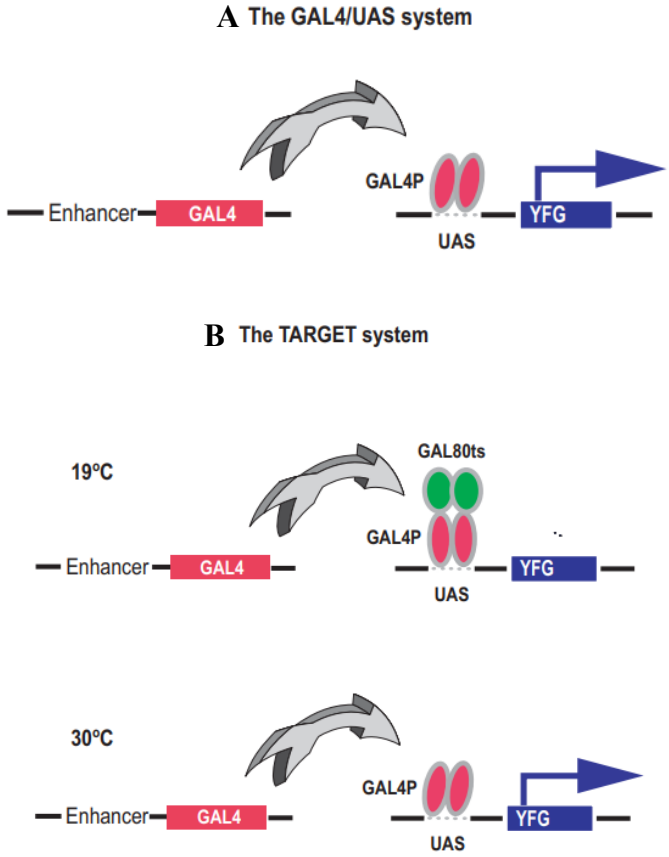


*Fig.3 (**A**) The GAL4-UAS System. This system involves the use of the GAL4 transcriptional activator protein and its cognate UAS binding site to regulate the expression of a gene of interest (YFG) in a specific spatial pattern. The GAL4 protein is directed by a defined promoter or endogenous enhancer, and once bound to the UAS site, it promotes the transcription of YFG in a constitutive manner. (**B**) The TARGET System. This modification of the conventional GAL4-UAS system uses a temperature-sensitive allele of GAL80 to regulate gene expression. At a lower temperature of 19°C, the transcription of YFG is suppressed, but this repression is removed when the temperature is increased to 30°C, allowing for high levels of expression of YFG in a specific tissue* [16]

Balancers

Balancers are indispensable tools in *Drosophila* genetics, employed for the long-term maintenance and tracking of mutations within laboratory strains. These specially designed chromosomes possess multiple inversions, rendering them structurally distinct from wild-type chromosomes. These inversions effectively impede recombination with the corresponding regions on wild-type chromosomes, thereby "balancing out" any unfavorable or lethal mutations present. Balancers are frequently utilized to sustain recessive lethal mutations or mutations that confer a disadvantage to

the organism. By introducing the balancer chromosome carrying the mutation into the genetic background of flies, researchers can perpetuate the mutation within the population without its detrimental effects being expressed in every generation. This facilitates the convenient maintenance of single deleterious alleles and enables the preservation of mutations, transgenes, and/or chromosomal aberrations that are linked together on the same chromosome. One of the most popular second chromosome balancers that is used in this thesis is *CyO* and it is characterized by flies with curly wings [17].

3. Mushroom Bodies-Center of olfactory learning and memory in *Drosophila* melanogaster

3.1. Anatomy

The Mushroom Bodies (MBs) are bilateral neuronal clusters located in the dorsal posterior region of the brain in insects (Fig.4). They have complex and visually attractive architecture, initially studied in detail in larger insect species. In adult *Drosophila*, each brain hemisphere contains approximately 2,500 neurons comprising the MBs. The primary neurons, called Kenyon cells, are organized in a highly distinctive manner. Their cell bodies are situated in the dorsal posterior region of the brain, surrounding their main dendritic projections that extend into a neuropil region known as the calyx. The axons of Kenyon cells form a bundle called the peduncle, which projects anteriorly and ventrally. This axon bundle then bifurcates into two major branches: a horizontal branch and a vertical branch. The horizontal branch further divides into three lobes named β, β', and γ, while the vertical branch consists of α and α' lobes. The MB structure encompasses at least three distinct types of Kenyon cells, each with specific axonal projections: one type projecting to α and β lobes, another type projecting to α' and β' lobes, and a third type exclusively projecting to the γ lobe. These subdivisions of axonal projections are not simply arbitrary descriptions but likely reflect developmental and functional differences between the Kenyon cell types [10], [18], [19].

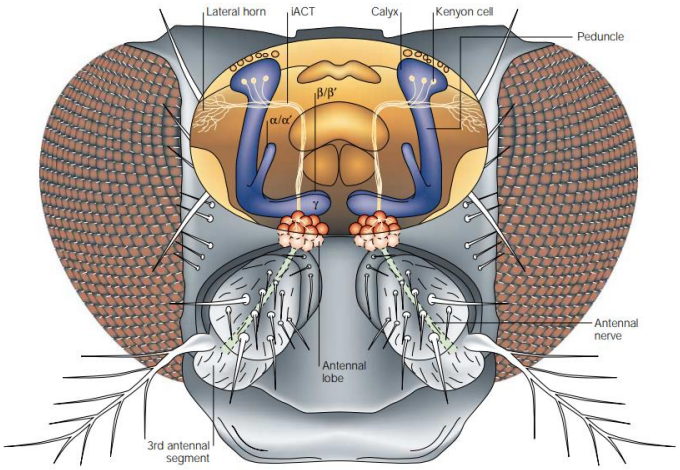


Fig.4 Drosophila brain shown in the head capsule. Mushroom bodies are marked with blue color. [19]

3.2 Function and role in learning and memory

Mushroom bodies, which are conserved brain structures in all insects, receive input from multiple sensory systems. Electrophysiological studies have demonstrated that neurons in the mushroom bodies exhibit responsiveness to various stimuli [20]. This suggests that these structures may serve as sites for sensory integration, a crucial aspect of associative learning. Behavioral experiments have further supported this notion by showing that mushroom bodies are necessary for spatial memory in cockroaches and for olfactory learning in both *Drosophila* and honeybees [20], [21]. As a result, the mushroom bodies have emerged as the initial brain region demonstrated to be involved in insect olfactory learning, leading to extensive research focusing on their structure and function. In line with their role in olfactory associative learning, the Kenyon cells within the mushroom bodies seem to receive input from various sensory modalities, including olfaction, electric shock, and taste. In *Drosophila*, the primary olfactory system provides the most prominent neuronal inputs to the mushroom bodies. Notably, the olfactory system, particularly the mushroom bodies, can be genetically and developmentally manipulated in *Drosophila*, allowing for detailed investigations and experimental manipulations. Such experiments have led to the identification of several genes that play a role in olfactory memory and they show preferential expression in the Kenyon cells of the mushroom bodies (MB). Therefore, the mushroom bodies have now emerged as the central hub for learning and memory in *Drosophila*, as their essential role in these processes has been established [19].

4. Learning and memory in *Drosophila*

As it is already described, *Drosophila* has been shown to be capable of multiple forms of punishment and reward learning and memory tasks. Among these, the most robust is a Pavlovian assay in which the animals learn to associate a conditioned stimulus (CS, odors) with an unconditioned stimulus (US, footshock). (Fig.5) Consequently, the field has focused a great deal of research effort on understanding this brand of behavioral plasticity [3], [10].

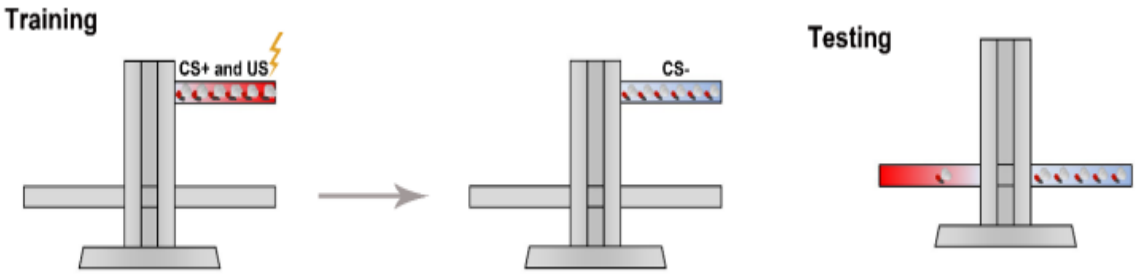


Fig.5 Aversive olfactory association paradigm in Drosophila. Training of flies consists of pairing an odor (CS+) with electric foot shock (US) followed by presentation of another odor (CS-) in the absence of shock. Testing of the performance for the previously learned association involves simultaneous presentation of the CS+ and CS- odors for the flies to choose (Modified figure from [3]*).*

4.1. Memory phases in *Drosophila*

Olfactory learning in *Drosophila* shows many of the behavioral properties generally described for Pavlovian learning in other animals, including acquisition, extinction, CS/US saliency, order dependence, temporal specificity, conditioned excitation, conditioned inhibition and CS/US pre-exposure effects. The same is true for memory formation. Early experiments with behavior-genetic analyses of the "learning" mutants *dunce* and *rutabaga* have revealed that single-gene mutations can disrupt associative learning, without blocking it completely. Instead, analyses of memory formation after olfactory learning in these mutants have suggested disruptions of functionally distinct memory phases [10], [22].

Therefore, the paradigm of classical conditioning in *Drosophila* has become a preferred method for studying and characterizing new learning and memory mutants. The reliability and robustness of this assay have contributed to its widespread use in memory-related research. Through genetic and pharmacological investigations, researchers have further subdivided the memory of conditioned associations in mutants and transgenic flies, aligning them with the phases observed in other invertebrate and vertebrate models.
Here are the commonly recognized memory phases in *Drosophila* (Fig. 6) originated as an inference from the literature [10], [13].

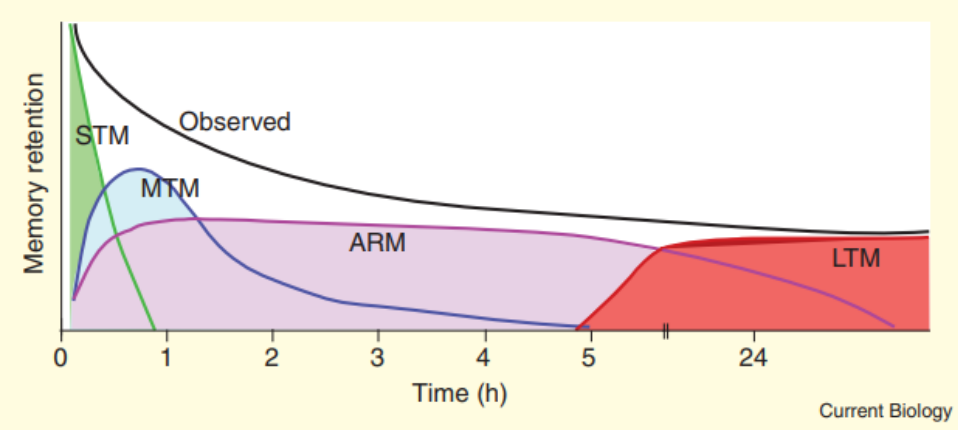


Fig.6 Dissection of memory phases [10]

- **Short-term memory (STM):** Short-term memory refers to the initial phase of memory formation and is assumed to correspond to high learning levels immediately after training. It decays in less than an hour and is independent of transcription and translation and three types of consolidated memories. A typical training session for *Drosophila* involves a single cycle, where the conditioned stimulus (CS) odor is presented for approximately 1 minute along with 12 electric shock pulses. This training protocol efficiently induces conditioned behavior in flies, encompassing both short-term memory (STM) and middle-term memory (MTM). [10], [13], [23]

- **Middle-term memory (MTM):** The existence of middle-term memory (MTM), lasting from 1 to 4 hours and dependent on new protein synthesis from pre-existing messages, was supported by experiments conducted on amnesiac mutants. The first evidence came from comparing memory retention curves of

normal flies and amnesiac mutants. It was observed that amnesiac mutant flies displayed near-normal memory retention immediately after a single training session and again around seven hours later. However, during the intermediate time period, their memory retention was significantly lower than that of normal flies. Further evidence for MTM came from "reversal retention" experiments. Previous studies had shown that normal flies were capable of "reversal learning," where they could learn to associate a previously irrelevant stimulus with a different outcome. By applying this reversal learning task at various time points after initial training, a window of sensitivity for reversal learning was identified. This reversal-sensitive phase occurred after short-term memory (STM) but before anesthesia-resistant memory (ARM), suggesting that MTM was specifically affected. Importantly, the retention curves for reversal learning in both normal flies and amnesiac mutants were identical, indicating that the amnesiac mutation and reversal learning disrupted MTM selectively. This genetic analysis demonstrated that early memory could be divided into distinct STM and MTM phases with different functional properties.[10], [13]

- Long-term memory (LTM): Long-term memory (LTM) is a stable and enduring form of memory that can persist for days or even weeks. Its establishment relies on the synthesis of new proteins and gene expression, leading to lasting changes in neuronal circuits and synaptic morphology. Normal protein synthesis during training and the involvement of the transcription factor CREB are crucial for LTM formation. Its persistence, as well as its absolute dependence on new protein synthesis suggest that LTM is energetically costly. According to the consolidation theory, it takes hours to convert labile memory into LTM. Thus, in aversive olfactory conditioning in *Drosophila*, LTM does not develop from a single training trial, massed training trials, or backward-spaced training. It only emerges after spaced conditioning (SC), requiring repeated efforts for acquisition and it becomes apparent no sooner than 6 h post training [3], [23], [24]

- Anesthesia Resistant Memory (ARM): In addition to the protein synthesis-dependent memory phases mentioned above, *Drosophila* also exhibits a form of memory that can be formed rapidly, since it is detectable as early as 2 h after training. It does not rely on new protein synthesis as it is not sensitive to the protein synthesis inhibitor cycloheximide. It is produced by massed conditioning (MC), which involves 10 consecutive training sessions, resulted in stronger memory retention lasting about three days. Even though Anesthesia Resistant Memory has been only described in *Drosophila*, it seems unlikely that there are no traces of it in other organisms [22].

4.2. Anesthesia Resistant Memory (ARM)

Memory consolidation is a highly intricate and time-dependent cognitive process that takes place over prolonged durations in diverse species, with the requirement of novel protein synthesis serving as a key component. However, in the case of *Drosophila* a fascinating phenomenon emerges as an exception to the conventional understanding of memory consolidation. Specifically, *Drosophila* exhibits a distinct form of memory

termed anesthesia-resistant memory (ARM), which displays an accelerated consolidation rate and a remarkable resistance to disruption by cold anesthesia administered shortly after the learning event. Intriguingly, the consolidation of ARM does not rely on the synthesis of new proteins as a fundamental requirement. This was proven with a series of experiments using the protein synthesis inhibitor Cycloheximide (CXM), where CXM-feeding to flies affected 1 day retention after Spaced Training but Not Massed Training (Fig. 7).

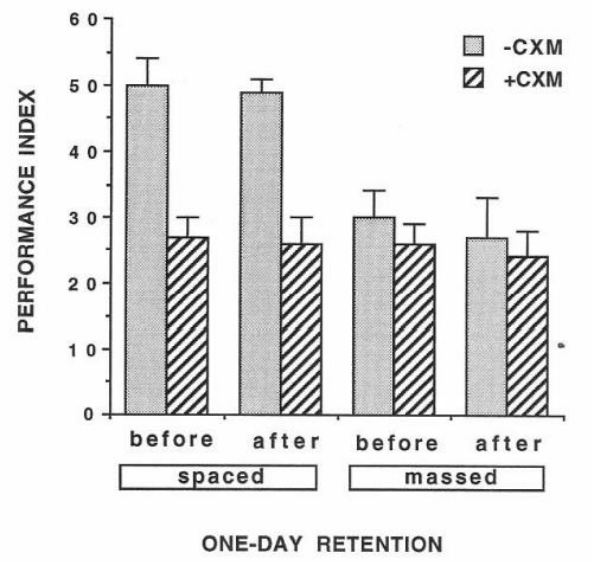


Fig. 7. CXM feeding affects 1 Day retention after Spaced Training but not Massed Training [25]

To explore the complex dynamics of memory consolidation in *Drosophila*, researchers have utilized a sophisticated experimental strategy involving the induction of temporary immobilization through acute exposure to cold temperatures, colloquially known as cold shock (4°C). By subjecting the fruit flies to cold shock immediately following a session of conditioning involving the pairing of specific odors with aversive footshocks, memories that have not yet undergone complete consolidation enter a labile state, rendering them susceptible to disruption. This implies that the cold shock intervention interferes with the ongoing consolidation process, thereby compromising the stability of the memories. Notably, already consolidated ARM displays an extraordinary resilience, remaining impervious to the disruptive effects of cold shock, thereby suggesting a relatively swift consolidation timeframe for this particular memory type. In contrast, the disrupted memory, termed anesthesia-sensitive memory (ASM), represents a labile phase of the association, vulnerable to impairment induced by the cold shock. The distinctive responses of ARM and ASM to the cold shock intervention (Fig. 8) provide compelling evidence that they represent separate and distinct memories of the same odor-shock association, each characterized by distinct underlying consolidation mechanisms and exhibiting unique consolidation kinetics. The unveiling of a mutant variant within *Drosophila* known as *radish*, which selectively eliminates ARM while leaving ASM intact, constitutes a crucial discovery that further supports the notion of ARM and ASM representing discrete memories in various stages of consolidation [3], [25]–[28].

Fig.8 Calculation of the performance index results in a representation of memory retention. In the case of cold shock treatment two hours post training, MTM loses its ASM counterpart and consists only of ARM (Modified figure from [3]*).*

4.3. ARM vs LTM

It is a fact that consolidated memory after olfactory learning in *Drosophila* consists of two components which mainly differ in the dependence of new protein synthesis. Apart from this distinction, they appear to involve different molecular pathways with those that underlie ARM remaining largely unknown. Indeed, even though they partially overlap in time, they differ in their timing of emergence, duration, and their functional characteristics. These distinctions are analyzed in detail below and summarized in Figure 10 [3].

1) While both Long-Term Memory (LTM) and Anesthesia Resistant Memory (ARM) are triggered by repeated training sessions, they follow distinct protocols. Stable LTM, which relies on protein synthesis, can be achieved by undergoing 5-10 cycles of negatively reinforced olfactory conditioning. Each cycle consists of 12 pairings of an unconditioned stimulus (US) and a conditioned stimulus (CS), with a 15-minute rest period between cycles [Spaced Conditioning (SC)]. In contrast, ARM formation can be induced through two different aversive olfactory conditioning protocols. One protocol mimics the original experiment that produced ARM and involves exposure to cold shock. The alternative protocol does not involve cold shock. It requires 5 to 10 consecutive training cycles, like those used for LTM formation, but without the inclusion of the 15-minute rest interval between cycles [Massed Conditioning (MC)]. Therefore, the main distinction between the two multiple-cycle training protocols, massed and spaced, lies in the absence of a 15-minute resting interval between cycles (Fig. 9) [3], [28].

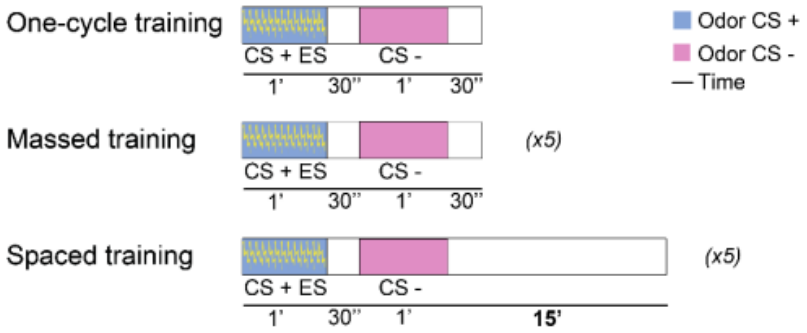


Fig. 9 Training protocols for aversive olfactory association conditioning in Drosophila [3]

2) The formation of Anesthesia Resistant Memory (ARM) begins approximately 30 minutes after training and can be detected as early as 2 hours after training, whereas Long-Term Memory (LTM) is not yet evident at this stage. LTM consolidation occurs more gradually than ARM, with its presence becoming apparent no earlier than 6 hours after training. By this time, memories induced by Massed Conditioning (MC) overlap with LTM in time and can last for 24-48 hours, while MC-elicited memories appear to decay soon after 24 h [3].

3) In addition to the protein synthesis independence, evidence for engagement of distinct molecular mechanisms emerged from observation that mutations in the *radish* (*rad*) gene differentially affect ARM and MC memories, but not LTM. Thus, the Radish protein functions acutely in the adult fly to engender ARM, because its expression 1 h before training is sufficient to rescue memory in mutant *radish* flies [3], [13], [29], [30]. On the other hand, using an inducible transgene that expresses a dominant negative member of the fly CAMP-responsive element-binding proteins (CREBs) family, LTM was specifically and completely blocked only after induction, while ARM and learning were unaffected [13], [25].

4) In addition, different inhibitors seem to affect these distinct types of memory. In the case where flies were treated with the Drok inhibitor Fasudil, a highly significant 24-h ARM deficit was observed whereas spaced conditioning-induced LTM was not affected by the same treatment [18]. Also, as it was mentioned above, formation of ARM is insensitive to the protein synthesis inhibitor cycloheximide (CXM) but in contrast, LTM's formation is cycloheximide sensitive [25]. Finally, administration of p-chlorophenylalanine (pCPA), an inhibitor of serotonin synthesis, specifically impaired 24-h memory after massed training that produced only ARM [31].

5) The long-lasting nature of Long-Term Memory (LTM), coupled with its dependence on protein synthesis, indicates that LTM is energetically demanding. In fact, the absence of food following Spaced Conditioning (SC) leads to fatality shortly after the completion of training. On the other hand, the viability is not significantly impacted by the lack of nourishment after Massed Conditioning (MC) [3], [18].

6) While it is widely recognized that memory tends to decline with age, Long-Term Memory (LTM) is differentially affected compared to memories formed through Massed Conditioning (MC). In this case, memories formed through MC tend to remain relatively intact despite the effects of advanced age [32].

7) Moreover, the influence of social interactions within the group of trained and tested flies appears to have differential effects on Long-Term Memory (LTM) and memories formed through Massed Conditioning (MC). When SC-trained flies are tested the following day, they can recall the learned information whether they are tested individually or in groups. However, flies that underwent MC tend to exhibit better recall when tested in groups rather than individually. This suggests that the retrieval of MC-elicited memories relies on social interactions or the context of the group in which the training took place. Therefore, MC memories may be more dependent on the specific social context, indicating a higher degree of context-dependency compared to LTM [3], [33].

		ARM/PSIM	LTM
1)	training protocol	cold shock or massed training	spaced training
2)	maintenance	<2 days	weeks
3)	genes involved	e.g., *radish*	e.g., *CREB*
4)	affected by inhibitors	pCPA, Fasudil	CXM
5)	excess energy demand	no	yes
6)	affected by aging	no	yes
7)	affected by social context	yes	no

Fig. 10 Main differences between ARM/PSIM and LTM. (Modified figure from [3]*)*

4.4. Distinction between ARM and MC-Elicited Memory

MC-yielded memory was thought to be anesthesia resistant, but that was questioned when Bourouliti and Skoulakis [3], [28] demonstrated that this form of protein-synthesis-independent aversive memory actually consists of two distinct memories with different characteristics [2]. The first point of evidence is that MC-induced memory, like Anesthesia Resistant Memory (ARM), does not require protein synthesis and is unaffected by the protein synthesis inhibitor Cycloheximide (CXM), which disrupts Long-Term Memory (LTM) formed through Spaced Conditioning (SC). Therefore, if MC memory and the memory that survives cold shock 2 hours after training were equivalent, then delivering a cold shock at least two hours after MC should not affect the 24-hour memory of the training. However, they found that a cold shock delivered 2 hours after MC disrupted the memory, suggesting that MC yields an additional memory type that consolidates more slowly than ARM and is susceptible to cold shock. As a result, this MC-elicited memory was referred to as Protein Synthesis-Independent Memory (PSIM) to distinguish it from the genuine ARM that emerges after cold shock. The nature of PSIM, whether it represents a slower consolidation component of ARM induced by multiple training rounds or a distinct type of memory, is currently being investigated. Nevertheless, it is important to avoid assuming that cold shock-resistant memory (ARM) and MC-elicited memories are identical, or that they require the activity of common genes or engage common neuronal circuitry, unless they have been explicitly tested in both types of memory assays. There is a possibility that molecules affecting PSIM may be identified in the future, including some known to play a role in cold-shock-persistent ARM, but which have not been cross-tested for MC-elicited memories. Therefore, the term "MC-memory" is used to refer to PSIM and the ARM-like memory induced by massed conditioning, in order to semantically differentiate it from memory induced by a single round of conditioning that is resistant to cold shock, which is still referred to as ARM.[3].

5. Molecular pathways involved in learning and memory

5.1. RAS/RAF/MAPK pathway

There is growing evidence highlighting the fundamental role of the RAS/RAF/MAPK pathway in learning and memory in both vertebrates and invertebrates. However, the

specific mechanisms by which this pathway is activated and regulated in neurons are not yet well understood. Neuronal RAS activation can occur through various means, including receptor-tyrosine kinases (RTKs), G-protein-coupled or NMDA-glutamate receptors, voltage-gated calcium channels, and cell adhesion molecules. Adapter proteins like GRB2/DRK facilitate the connection between RAS and receptors, contributing to signaling selectivity and specificity. Typically, activated RAS triggers the activation of a MEK-kinase, such as RAF, at the plasma membrane. RAF, in turn, activates another kinase called MEK, which further activates MAPK. MAPK regulates the activity of transcription factors in the nucleus and targets proteins in the cytoplasm and membrane. To investigate RAS/RAF/MAPK signaling in *Drosophila* learning and memory, researchers have focused on identifying cascade members present in the mushroom bodies (MBs), which play a significant role in these processes. This study specifically reports on Drk, a protein with a prominent distribution in the MBs, which is crucial for transmitting RTK signals to the RAS/RAF/MAPK pathway [13], [34], [35].

6. The protein Drk/GRB2

The *drk* gene encodes a protein (Drk: downstream of receptor kinase) composed of 211 amino acids, which is the ortholog of the vertebrate GRB2. Its primary structure consists entirely of a central SRC-Homology domain 2 (SH2) which binds to receptor-tyrosine kinases (RTKs). This SH2 domain is flanked by two SH3 domains. The protein interacts with the guanine exchange factor SOS through its N-terminal SH3 domain, forming a complex that is crucial for activating the RAS protein. Furthermore, the C-terminal SH3 domain of Drk binds to DISABLED, a protein that potentially serves as a link between signaling pathways mediated by SRC-like tyrosine kinases and the RAS/RAF/MAPK cascade (Fig. 11). As a result, Drk plays a critical role in initiating multiple signaling cascades that have the potential to lead to the activation of MAPK [34], [36], [37].

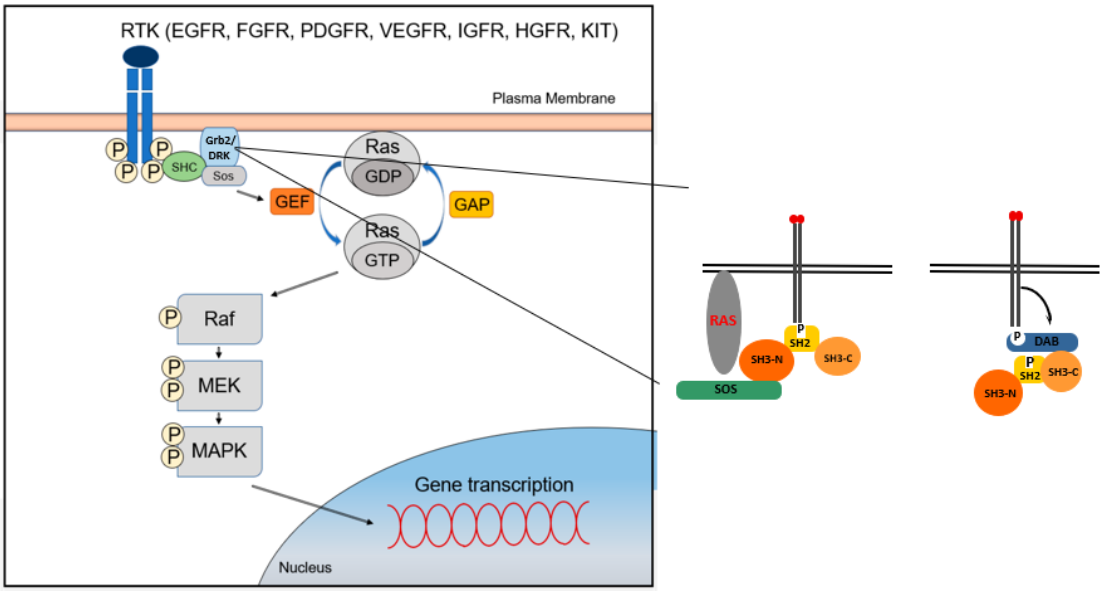


Fig. 11 The RAS-RAF-MAPK signaling pathway and the structure and function of the protein DRK (GRB2) [38].

The investigation of the involvement of Drk in olfactory learning and memory began with the observation that it is predominantly accumulated in Kenyon cells. Specifically, Drk is found in various structures of the adult brain, such as the antennal lobe (AL), ellipsoid body, and notably the α, β, and γ lobes of the mushroom bodies (MBs), while it is not detected in the α'β' lobes (Fig. 12). Like Drk, GRB2 is found to accumulate in the hippocampus and amygdala, which are important regions associated with learning in vertebrates [18], [34].

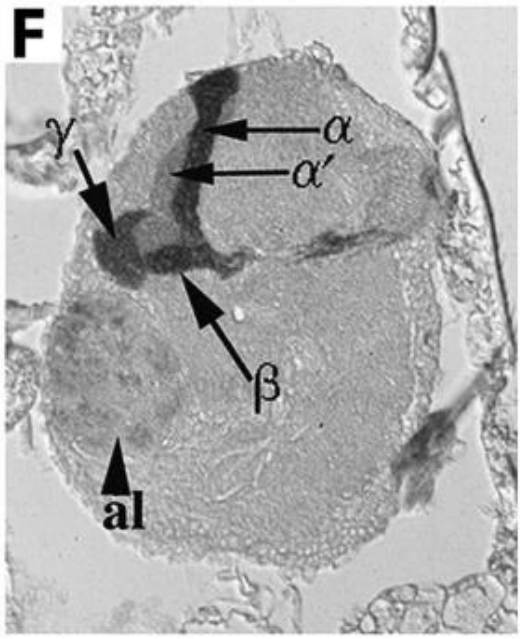


Fig. 12. Distribution of Drk in the Drosophila's adult brain. differential distribution of Drk within the pedunculus, α,β and γ lobes. (al, antennal lobe)

6.1. Role in learning and memory

The distinct distribution pattern of Drk within the mushroom bodies (MBs) led to the hypothesis that it might play a crucial role in olfactory learning and memory, like other proteins highly enriched in these neurons. However, since homozygotes for *drk* mutations do not survive beyond the larval or pupal stages, an alternative approach was taken to investigate the impact of reduced Drk levels on behavioral neuroplasticity. Deletion heterozygotes, which are expected to harbor 50% of the normal protein dosage, were subjected to olfactory associative conditioning.
The findings of the study revealed that the decrease in learning performance observed when animals were trained with 6 or less pairings is a bona fide impairment in learning and can be attributed to the reduction in Drk levels. Interestingly, the data also demonstrated that *drk* mutant heterozygotes and animals with suppressed Drk specifically in the MBs exhibited reduced learning efficiency. However, through extensive overtraining, these animals eventually reached the same level of learning as the control group. In other words, the reduction in Drk impacted the efficiency of learning, but not the actual ability to learn per se (Fig. 13, A).

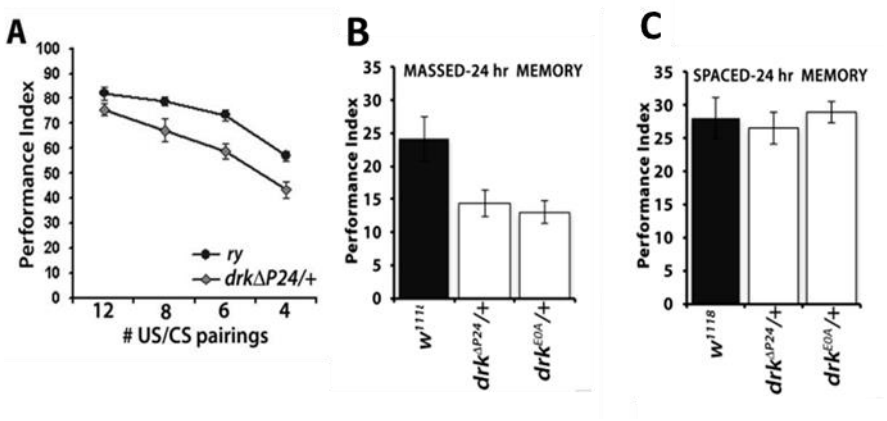


Fig. 13 Learning deficits of drk mutant heterozygotes. (A) Performance immediately after conditioned odor avoidance after training with the indicated number of pairings. The performance of control flies was significantly different from that of drk heterozygotes mutants after 6 and 8 pairings, but not after 12 pairings. (B) PSIM induced after 5× massed training and assessed 24 h later was significantly different in mutants than controls. (C) In contrast, 24-h LTM induced by 5× spaced training was not affected. ry,W1118 control flies. drkΔP24, deletion allele. drkE0A, the two-point mutations within the SH2 domain (amino acid 106 H to Y), (Modified figure from [34]*)*

To investigate the impact of Drk reduction on the RAS/RAF/MAPK pathway and its association with learning and memory deficits, researchers explored whether the deficits observed in *drk* mutant heterozygotes could be reversed or improved through the controlled expression of constitutively active RAS and RAF specifically within the mushroom bodies (MBs). The study demonstrated that the learning deficits in *drk* mutant heterozygotes could be rescued by conditionally expressing Ras and Raf in the MBs. However, the 90-minute memory deficit was not fully restored by this manipulation. Furthermore, the levels of MAPK activation in the brains of adult flies were measured at specific time points after associative training. It was observed that a 50% reduction of Drk did not significantly affect MAPK activation immediately after training but had a significant impact on sustained activation of the kinase, particularly evident at the 90-minute time point. This suggests that the inability to sustain phosphorylated MAPK (pMAPK) levels likely underlies the 90-minute memory deficit. Together with the behavioral rescue of learning, but not the 90-minute memory deficit, through the expression of *raf* transgenes in the MBs, these findings support the idea that RAF activity is involved in the acute elevation of pMAPK levels after training but is not necessary for the sustained activation of MAPK during memory consolidation.
Overall, these results indicate that Drk is likely involved in engaging distinct signaling molecules and cascades that are required for both learning and memory. It is hypothesized that Drk interacts with different molecules through each of its SH3 domains, potentially explaining its dual role in these processes. One dependent on RAF activation for learning and another independent role in memory, which relies on sustaining pMAPK levels, possibly by antagonizing RAF activity [34].

6.2. Drk Reduction Selectively Affects PSIM

Knowing the involvement of *drk* in memory formation, the next step was to determine the specific memory type affected in *drk* mutants. It was found that *drk* heterozygotes

exhibited deficits in protein synthesis-independent memory (PSIM) when subjected to massed conditioning, which involved five consecutive cycles of 12 US/CS pairings. However, when the same mutants underwent conditioned with 5 spaced training cycles that induce protein synthesis-dependent long-term memory (LTM), their performance was comparable to that of the control group. These findings provide robust evidence that Drk is specifically required for the formation of normal PSIM. [34] (Fig. 13 B,C).

In addition, the analysis of *drk* mutant flies revealed a significant decrease in filamentous actin (F-actin) levels compared to control flies, specifically within the calyces. Based on these findings, it is strongly suggested that PSIM formation involves activity-dependent localized changes in the neuronal cytoskeleton, particularly the actin cytoskeleton. These changes in cytoskeletal structure and function are hypothesized to modify synaptic strength or properties, and they appear to persist for at least 24 hours, indicating a stable alteration in memory formation. In summary, the results implicate actin cytoskeleton dynamics as a molecular hallmark of PSIM formation, highlighting the importance of localized structural and functional changes in the neuronal cytoskeleton in the process of long-lasting PSIM. [18]

D. AIM-IMPORTANCE OF PRESENT STUDY

The aim of this study is to investigate underlying molecular mechanisms involved in the formation of protein synthesis independent memory (PSIM) in the fruit fly, *Drosophila melanogaster.*

Memory formation is a fundamental process that allows organisms to learn from past experiences and adapt their behaviors accordingly. While protein synthesis-dependent forms of memory have been extensively studied, recent evidence suggests the existence of protein synthesis-independent mechanisms that contribute to long-lasting memory storage. *Drosophila* provides an excellent model system to investigate these mechanisms due to its well-characterized memory paradigms and genetic tractability. The primary objective of this study is to identify and characterize the molecular components of Drk-mediated pathway regulating Protein Synthesis-Independent Memory (PSIM) in *Drosophila*. This will be achieved through a combination of behavioral assays, genetic manipulations, and molecular analyses.

Specifically, the study aims to:

1. Analyze the effects of genetic manipulations into mushroom bodies of adult flies (RNAi expressing lines) on memory performance in the classical conditioning paradigm, focusing on the impact on PSIM.
2. Examine the molecular and cellular changes associated with PSIM including alterations in signaling pathways and synaptic plasticity.

By elucidating the molecular components underlying PSIM in *Drosophila*, this research aims to contribute to our understanding of the broader mechanisms of memory formation and storage. The findings may have implications for deciphering similar processes in other organisms, including humans, and potentially provide insights into the treatment and management of memory-related disorders.

E. EXPERIMENTAL PROCEDURES

1. *Drosophila* cultures and strains

Drosophila were raised and crosses set up in standard wheat flour–sugar food as previously described [18] and raised in a 12 h night/dark cycle, at 25ºC or 18ºC and 50% humidity.
For crosses male flies carrying UAS for targeted genes were crossed with virgin female flies of the selected driver line, unless otherwise noted.
For all behavioral experiments, non–balancer-bearing progeny from crosses of targeted protein RNAi male flies to Leo MB Gal80(ts) virgin female flies were used in order to drive temporal expression specifically during adulthood in the MBs.

***Drosophila* strains**

Wild type flies

- **W^{1118}**

Drivers

- **Leo MB Gal80(ts)**
  It drives expression predominantly in the MBs with a thermal shift from 18ºC to 30ºC.
- **Elav Gal4**
  An elav promoter predominantly regulates pan-neuronal expression of a GAL4 driver. It is used to drive pan-neuronal expression throughout development if flies are kept in 25ºC.
- **eElav $Gal80^{(ts)}$**
  An elav promoter predominantly regulates pan-neuronal expression of a GAL4 driver. It can drive pan-neuronal expression temporally with a thermal shift from 18ºC to 30ºC.

Other lines

- **UASZipper RNAi** (#37480 Bloomington Drosophila Stock Center)
  Expresses dsRNA for RNAi of zip under UAS control and segregates CyO balancer. (Map: Chr 2)
- **UASRyR RNAi** (#28919 Bloomington Drosophila Stock Center)
  Expresses dsRNA for RNAi of RyR under UAS control. (Map: Chr 3)
- **UASDlg1 RNAi** (#39035 Bloomington Drosophila Stock Center)
  Expresses dsRNA for RNAi of dlg1 under UAS control and segregates CyO balancer. (Map: Chr 2)
- **UASRhoGAP18B** (#6434 Bloomington Drosophila Stock Center)
  Expresses dsRNA for RNAi of RhoGAP18B under UAS control. (Map: Chr 2)
- **UASgprs RNAi** (#42630 Bloomington Drosophila Stock Center)
  Expresses dsRNA for RNAi of gprs under UAS control. (Map: Chr 2)
- **UASgprsRNAi/CyO;UAS-Flag-Drk (1-6M)**
  Overexpression of Flag-Drk in gprs mutant (#42630 Bloomington Drosophila Stock Center)

- **drk/CyO**
  Drk mutant
- **UAS_Flag-Drk (1-6M)**
- **UAS-Drk-Flag (2-7M)**
- **Control line for RNAi** (#36304 Bloomington Drosophila Stock Center)
  Control line for TRiP RNAi lines. (Map: Chr 2)
- **Control line for RNAi** (#36303 Bloomington Drosophila Stock Center)
  Control line for TRiP RNAi lines. (Map: Chr 3)

2. Behavioral assays

The non-balanced 2–4-day old flies (n=50) were used in all experiments and to maximally induce the transgenes, flies were moved from 18°C to 30°C for 3 days. Then, 1 h before the experiment flies are transferred into new fresh vials and into a dark box. All behavioral experiments were performed at 25ºC and 65–75% humidity under dim red light. Aversive olfactory conditioning utilized 90 Volt electric foot-shocks as unconditioned stimuli (US), paired with one of the aversive odorants 5% benzaldehyde (BNZ), or 50% octanol (OCT) diluted in isopropyl myristate as conditioned stimuli (CS). One training cycle consisted of 6 or 12 CS/US pairings of 1.25 s with a 4-s interstimulus interval, followed by 30 s of rest before presenting another odor in the absence of shock. Either odor was paired with shock, while the other served as control. For learning, flies were tested immediately after the training session. Massed conditioning (MC) involved five consecutive training cycles with 30 s between cycles and testing was performed 24h hours post-training. Flies remained in the dark until after the end of testing. Spaced training was identical to MC, except the interval between cycles was 15 min. In any case, memory testing involved simultaneous presentation of both odors for 90 s as described. [18]

Performance index in all graphs is calculated with the following way:

$$\textbf{Performance Index}: \quad \textbf{PI} = \frac{\left[\frac{BNZ}{2}\right]+\left[\frac{OCT}{2}\right]}{2} * 100$$

$\frac{BNZ}{2} = \frac{[OCT-BNZ^{**}]}{[OCT+BNZ^{**}]}$, $\frac{OCT}{2} = \frac{[BNZ-OCT^{**}]}{[BNZ+OCT^{**}]}$, BNZ : number of flies that chose benzaldehyde, OCT: number of flies that chose octanol.

To calculate Δ, the difference between uninduced and induced memories, the two groups of animals were simultaneously trained.

3. Western blotting

For detection of Drk in Western blots, total protein extract equivalent of two adult fly heads was loaded per lane of 10% acrylamide gels, transferred to PVDF membranes and probed with the primary rabbit a-Drk antibody [34] at 1:1000 and mouse anti-tubulin antibody (E7 anti-beta tubulin MIgG1, DSHB) at 1:5000. Secondary anti-mouse and anti-rabbit antibodies were used at 1:5000.

For detection of Zipper in Western blots a different protocol for larger proteins was used. Total protein extract equivalent of five adult fly heads was loaded per lane of 7,5% acrylamide gels, transferred to PVDF membranes and probed with the primary rabbit a-zipper antibody (kindly offered by Jeffrey H. Thomas, Ph.D, Associate Professor, School of Medicine Texas Tech University Health Sciences Center) at 1:20000 and mouse a-armadillo antibody at 1:250. Secondary anti-mouse and anti-rabbit antibodies were used at 1:5000. Running was performed at 100V, at 4ºC for 5h and transfer at same temperature O/N at 60V. Transfer Buffer was made with 10% concentration and 3,7 ml of SDS 10% was added per 1L of buffer.

4. Phalloidin Staining and Confocal Microscopy

Adult brains were dissected in cold PBS, fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.3% Triton X-100 in PBS. The brains were incubated with Rhodamine-Phalloidin (#R415; Invitrogen Molecular Probes) for 15 min at 25 °C to stain for F-actin. Confocal laser microscopy was performed using the Leica TCS SP8 system, and images at 40× magnification were obtained. Before acquisition, laser parameters were adjusted to obtain nonsaturating conditions, and samples were processed simultaneously using identical confocal acquisition parameters (laserpower, gain, and pinhole settings), as previously described. Quantification of fluorescent staining in the MB calyces was obtained from one z-stack of each calyx per brain, and each stack was taken approximately at the same depth. Image processing was performed with ImageJ software. In statistical analysis the ratio of mean fluorescence inside the region of MBs Calyces to mean fluorescence in a random brain area of controls is compared with the same ratio of subject brains.

5. Statistical analysis

Raw data analysis was performed with the JMP7 software (SAS Institute Inc., Cary, NC, USA). Statistical comparisons were performed as detailed in the Table 3 of appendix. Comparison between two groups was conducted with ANOVA or with non-parametric Wilcoxon T Test when variances of the measurements were unequal. Graphs were created with the GraphPad Prism 8.0.1 software and show means ± SEM.

F. RESULTS

A. Previous data from Skoulakis lab (unpublished data)

(People that conducted these experiments: Anna Bourouliti, Spiros Patelis, Katerina Papanikolopoulou)

Knowing the specific role of Drk in PSIM we wanted to investigate the proteins that may be involved in the PSIM formation pathway that Drk regulates. So, the initial purpose was to conduct a screening for genes interacting with *drk* that are specifically involved in PSIM. This project was more specifically placed within the mushroom bodies, since Drk was found to be selectively distributed there and they are also the center of olfactory learning and memory in *Drosophila*.

- **Uncovering protein interactors of Drk**

To uncover proteins that interact with Drk, the first step was the creation of transgenic flies. Through the construction of two flag-tagged Drk constructs, one tagged at the N terminus and the other in C-terminus, there was an overexpression of the Drk protein and specifically in the adult MBs with the help of a specific driver (Leo MB Gal80(^{ts})). Then, immunoprecipitation of Drk using an antibody against the flag and analysis with mass spectroscopy revealed proteins that may interact with Drk in adult MBs. (Fig. 14) Unsurprisingly, there are proteins that interact with only one of the two ends, such as SOS, which appeared in the C-Drk analysis.

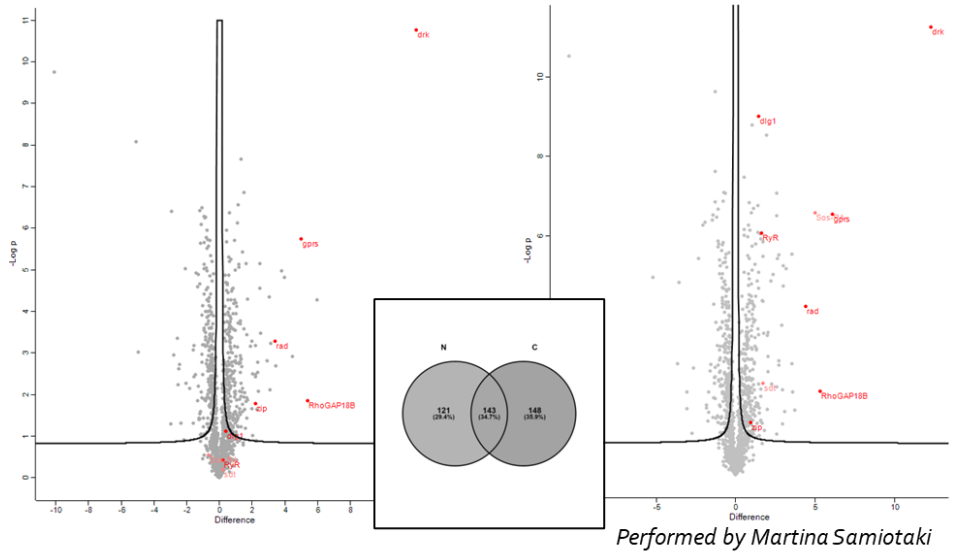


Fig. 14. Mass spectrometry analysis with antibody against flagged-Drk in both C- and N- terminals, revealing proteins that interact with Drk specifically within the MBs.

- **Selection of proteins as targets for further investigation**

From the list of all the proteins that were revealed via the mass spectrometry analysis, the most interesting candidates were selected, based on previously published knowledge on any of the following: expression pattern, engagement in cognitive functions, and involvement in processes linked to cytoskeletal organization. Actually, according to preliminary data, a few of them can now be placed in a hypothetical pathway (FIG. 15).

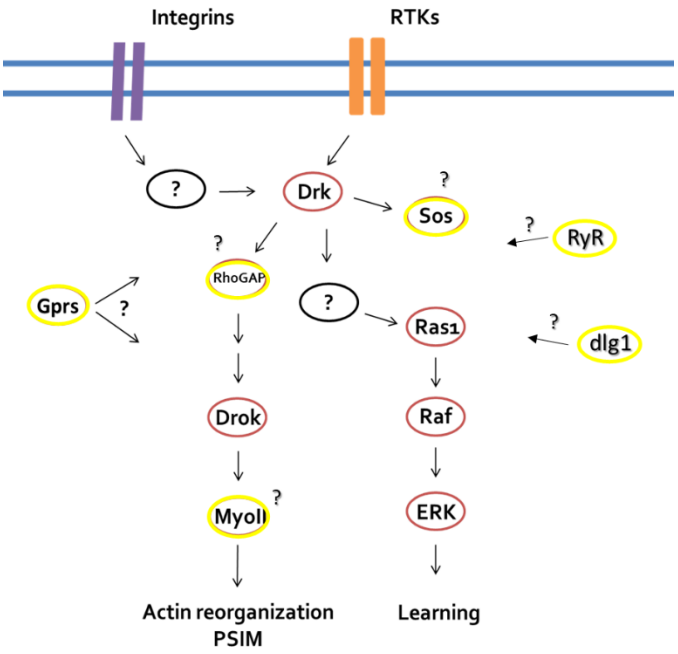


FIG.15 A proposed pathway introducing potential molecular components involved in Drk-mediated learning and PSIM formation.

➢ **Use of RNAis for specific reduction of protein levels**

A useful tool to examine if the candidate-proteins are involved in PSIM formation pathways is the RNAi. Especially, the interest has been focused on an adult-specific effect in the MBs, so the TARGET system was used to achieve a regional and temporal gene expression targeting. In the case of RNAis flies kept at 18ºC degrees act as control, since the Gal4 is under the control of the thermosensitive Gal80 molecule. On the other hand, upon a thermal shift at 30ºC, the RNAi is transcribed, and the protein levels are reduced. The effect of this genetic manipulation on the performance of flies in different behavioral assays is presented below.

1) Son of Sevenless (SOS)

One of the first proteins examined was the guanine exchange factor Son Of Sevenless, SOS, which was selected not only because it is appeared in the proteomic analysis but also because it is generally an element of the known Drk-mediated pathway. Drk protein binds to the guanine exchange factor SOS through its N-terminal SH3 domain and forms a complex essential for RAS pathway activation. [34] In addition, unpublished data show preferential localization of SOS in the MBs of adult flies.

Interestingly, expression of a SOS RNAi in the MBs of adult fly brains had no effect on either learning, or PSIM, and so it is concluded that SOS is not involved in PSIM pathways in adult fly brains.

2) Gprs

The *Gprs* gene codes a protein that little is known about. However, its protein product was found high in the proteomic analysis, a fact which made the need for its further examination prominent. Furthermore, preliminary data from the laboratory show that it

is expressed in MBs. In addition, staining of an endogenously GFP-expressing *gprs* mutant with an antibody to Drk revealed colocalization of the two proteins within the MBs and specifically in αβ neurons. So, the next question was whether it is involved in PSIM.

To assess the involvement of *gprs* in PSIM, two different gprs RNAis were used, which were individually expressed in adult fly MBs. The results showed that even though there are no deficits in learning, and LTM resulting from SC was not affected, PSIM after MC was significantly impaired. Therefore, *gprs* is a novel gene found to be specifically involved in PSIM.

3) RhoGAP18B

Knowing the role of Rho kinases in the PSIM pathways mediated by Drk [18], another protein found in proteomics that was examined is RhoGAP18B. Rho GTPase activating protein at 18B (RhoGAP18B) encodes a protein that can act as a GTPase activating protein for several Rho GTPase and is involved in the regulation of actin dynamics, affecting cell shape. It is also involved in regulating actin potentials by affecting cofilin activity and acting on Rac1 (and possibly Rho1) [39].

Data from the laboratory showed that decrease of RhoGAP18B protein levels led to PSIM deficits, without knowing yet the specificity of this effect on this memory alone.

4) Ryanodine receptor (RyR)

Ryanodine receptor (RyR) encodes an intracellular calcium channel localized in the sarcoplasmic reticulum of muscles and the endoplasmic reticulum of neurons and other cell types. It regulates the release of intracellular calcium stores and has a key role in muscle contraction [40]. Recent findings provide evidence supporting the involvement of RyR2 and RyR3 isoforms in the activation of postsynaptic pathways in hippocampal neurons, which play a crucial role in synaptic plasticity, learning, and memory. Calcium signals are known to trigger these pathways. Additionally, brain-derived neurotrophic factor (BDNF) is a critical signaling molecule involved in hippocampal synaptic plasticity and spatial memory. BDNF binds to specific receptors, initiating intricate signaling cascades that bring about modifications in synaptic structure and function. It has been discovered that the remodeling of dendritic spines in the hippocampus, induced by BDNF, requires functional RyR [41].

Reducing RyR levels in adult MBs by RNAi expression led to deficit in PSIM. However, specificity of this effect on PSIM remains to be investigated.

5) Discs large 1 (dlg1)

Discs large 1 (Dlg1) has guanylate kinase activity and is a key regulator of epithelial polarity, proliferation, assembly of junctions, and protein trafficking. Some of its isoforms are essential for proper neuronal differentiation and organization, while it is also involved in cellular adhesion as well as signal transduction to control cellular proliferation [42]. In addition, d*lg1* mutants display defects in short-term memory in the olfactory associative-learning paradigm [42], [43].

Reducing Dlg1 levels in the adult MBs did not affect learning with 6 pairings. However, the effect of reduced expression of dlg1 on PSIM remains to be investigated. It stands to reason, that since the learning performance of subject flies is similar to controls, testing performance at later time points will provide results specifically regarding memory.

6) Zipper

Zipper (zip) encodes a microtubule-binding protein involved in cytoskeleton-dependent intracellular transport. In mammals, three different genes encode the NMII heavy chain (NMHC II) proteins, while in *Drosophila*, only one gene encodes the *Drosophila* NMHC II protein called *zipper* (*zip*). The NMII molecule is composed of two identical heavy chains, along with a pair of essential light chains (ELC) and a pair of regulatory light chains (RLC).
According to the prevailing model for NMII recruitment, the assembly of new filaments takes place at the equatorial cortex through localized phosphorylation of RLC, which is stimulated by the Rho pathway. During late anaphase, the small GTPase RhoA (known as Rho1 in *Drosophila*) promotes the formation of F-actin filaments in the cortex. RhoA also controls the constriction of the contractile ring by activating Rho kinase, which in turn phosphorylates RLC [44]. Therefore, Zipper protein plays a significant role in the binding of filamentous actin and the remodeling of the cytoskeleton through the Rho kinase pathway and acts downwards of Rock which engages in the PSIM regulation pathway involving Drk [18]. The fact that Zipper was found in the proteomic analysis as a probable interactor of Drk, along with the previously published data regarding its function, render it an important candidate for the PSIM Drk-mediated pathway.

Interestingly, driving the RNAi expression for *zipper* in the MBs of adult fly brains led to significant deficits in PSIM whereas learning performance was normal. If SC-LTM remains unaffected as well, that would mean a specific involvement of *zipper* in the regulation of PSIM.

B. Data from experiments conducted for this thesis

Based on the previous findings and following the same experimental workflow, the purpose of this thesis was to further investigate the role of the selected proteins (RhoGAP18B, RyR, Dlg1, Zipper) that may interact with Drk within the MBs of adult flies in PSIM formation and to examine the specificity of their involvement in this pathway. Any undesired effects of the use of RNAis are eliminated with control experiments with two control lines for RNAi insertions while every transgene line (induced) is compared with a control one (uninduced) to ensure that RNAi insertion provokes no effect in performance of flies. In addition, some other control experiments were performed for the ability of flies to avoid both odors and electric shock when these are simultaneously presented versus air. The flies have again 90s to choose between the two options and a percentage of avoidance is calculated in each case. Possible avoidance deficits may influence our learning and memory assays, and so should be taken in account when interpreting behavioral data.
The results are presented below.

1. Behavioral part

i. Effect of reduced expression of RhoGAP18B in adult MBs in learning

Since previous data from the lab demonstrated a role of RhoGAP18B in PSIM, we next wanted to check how the reduction of this protein affects the learning process. While there was no significant difference between the two groups after a training consisted of 6 pairings of US/CS (Fig.16, A), we noticed that PIs were low for both the control and subject groups. For that reason, we also tried 12 US/CS pairings training with the same results (Fig.16, B), thus we moved on to an overtraining method of Massed Conditioning (MC-Learning), which consists of 5 consecutive cycles of 12 US/CS pairings, and tested flies immediately after training. In this case the performance of the RNAi expressing flies was significantly reduced compared to that of the controls (FIG.16, C). This suggests a role of RhoGAP18B in learning, which keeps us from further investigation of memory, since normal learning cannot be reached in the subject group after MC. However, the similar performance index observed in both groups after only one cycle, raises the question whether RhoGAP18B may be associated with memory acquisition that relies on processes which take place in the event of repetition of training.

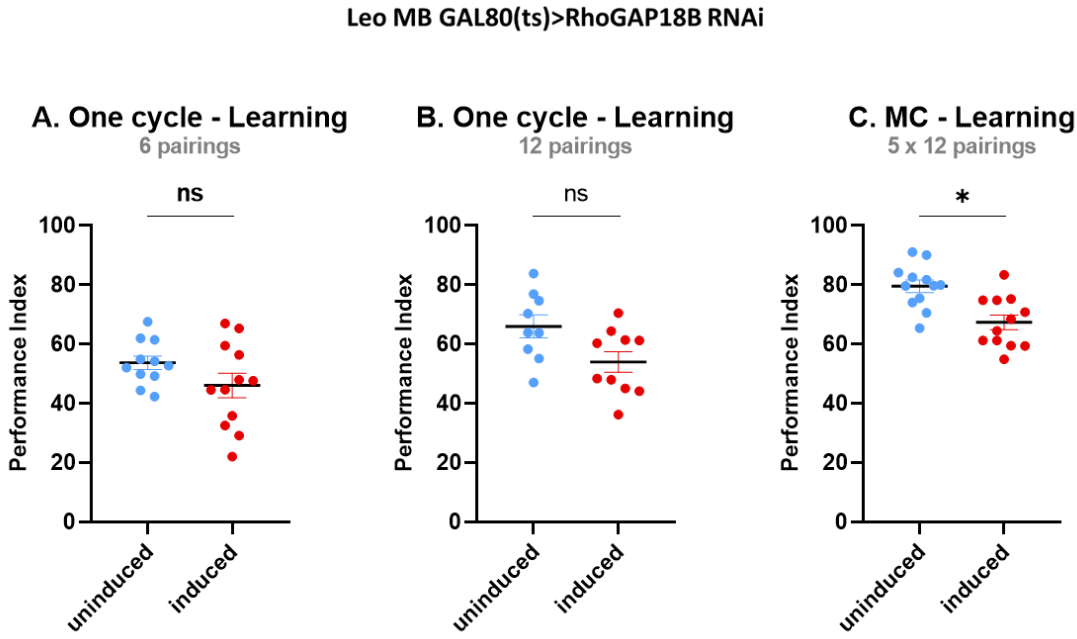


FIG.16 ***RhoGAP18B*** *is involved in learning during adulthood. The graphs show mean performance ± SEM. (A) Performance in learning was not significantly reduced neither after a 6 pairings training (ANOVA, p = 0.1280) nor (B) after a 12 pairings training (ANOVA, p = 0.0330). (C) Performance in learning was significantly reduced after MC-overtraining consisted of 5 cycles of 12 pairings (ANOVA, p = 0.0025).*

Avoidance experiments to electric shock and odors of subject groups are shown in Figure 17. Performance of RNAi expressing flies was not affected in any avoidance assay.

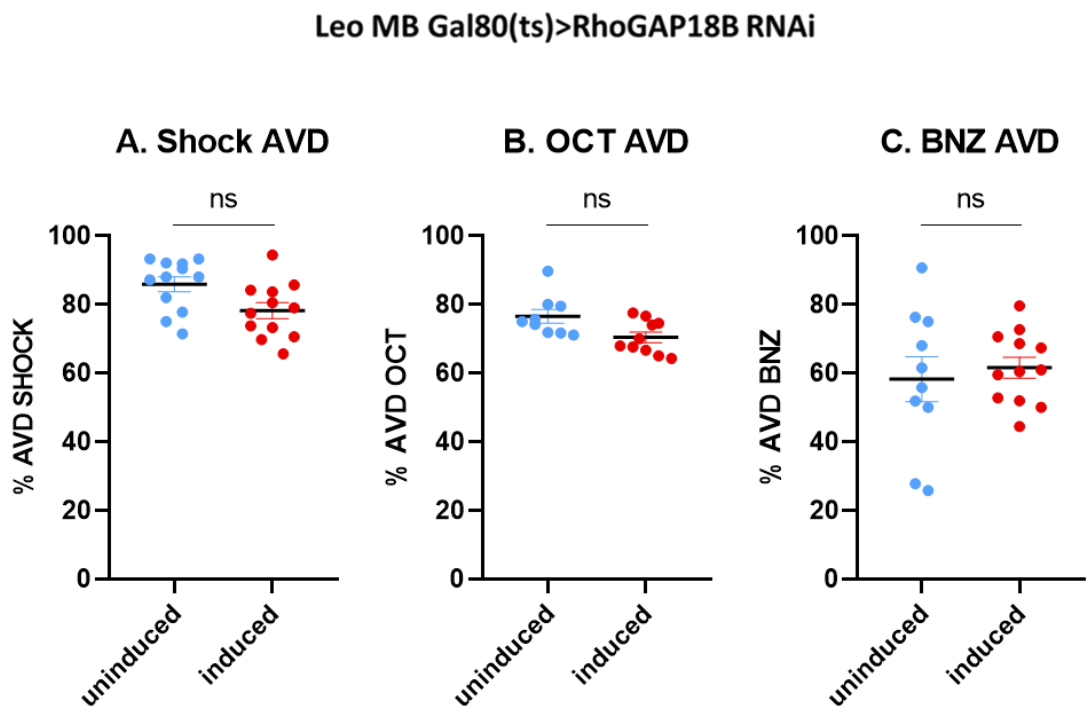


Fig.17 Odor and shock avoidance experiments of flies with reduced levels of RhoGAP18B. The graphs show mean performance ± SEM. There is not a statistically significant difference in any case of avoidance. (A) ANOVA, p=0.0250, (B) ANOVA, p=0.0241, (C) ANOVA, p=0.06320.

ii. Effect of reduced expression of RyR in adult MBs in learning

Knowing that reduced RyR levels led to deficit in PSIM formation, it was necessary to examine the effect of this reduction in learning as well. Both with one cycle learning and upon the MC-learning protocol, learning impairments were observed in RNAi expressing flies compared with the control uninduced ones (FIG.18).

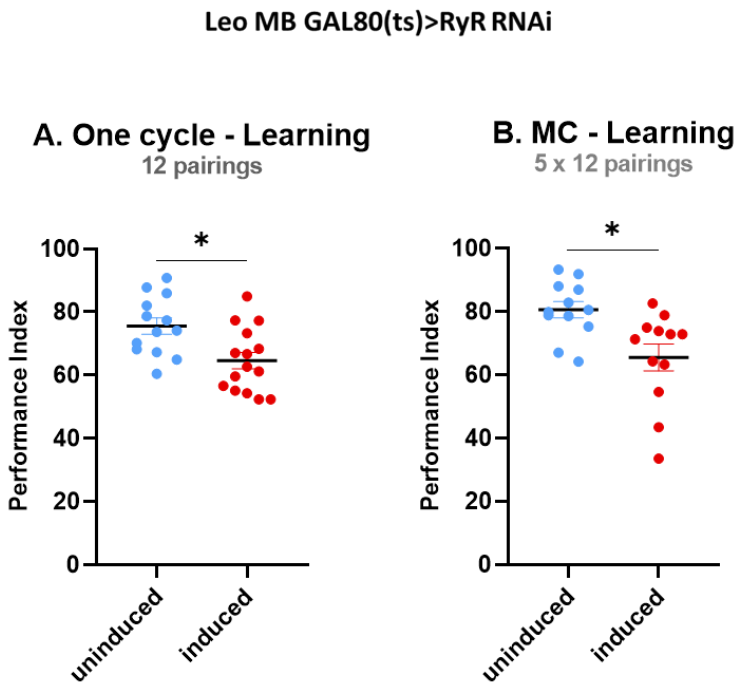


FIG.18 ***RyR*** *is involved in learning during adulthood. The graphs show mean performance ± SEM. Performance in learning was significantly reduced both after a 12 pairings training (A) (ANOVA, p = 0.0068) and upon overtraining consisted of 5 cycles of 12 pairings (B) (ANOVA, p = 0.0062).*

Avoidance experiments to electric shock and odors for each genotype are shown in Figure 19. Performance of RNAi expressing flies was affected only in octanol avoidance.

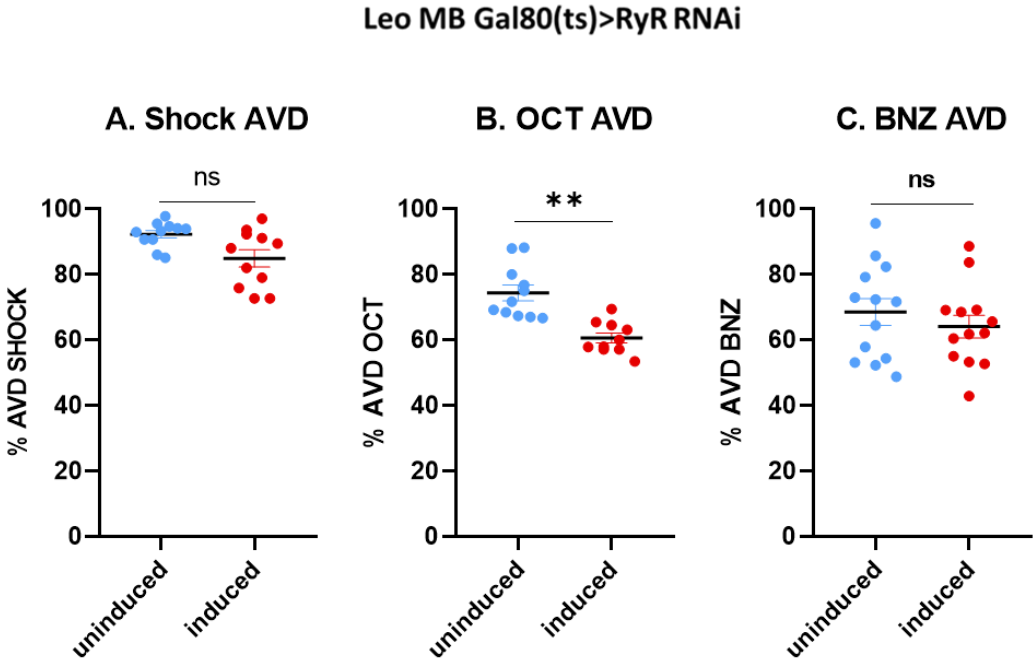


Fig.19 Odor and shock avoidance experiments of flies with reduced levels of RyR. The graphs show mean performance ± SEM. (A) Experimental flies avoid shock as the controls (ANOVA, p=0.0192), (B) Avoidance of octanol is significantly different between control and experimental flies (ANOVA, p=0.0002), (C) Experimental flies avoid benzaldehyde as the controls (ANOVA, p=0.4150).

iii. Effect of reduced expression of *dlg1* in adult MBs in learning upon overtraining and MC-PSIM

Dlg1 was selected as a promising protein for having a role in PSIM pathway. However, reduced expression of *dlg1* had no effect on performance of flies in the PSIM assay (FIG.20, B). On the other hand, learning upon MC-learning was significantly impaired in adult flies with reduced levels of dlg1 in adult MBs (FIG.20, A).

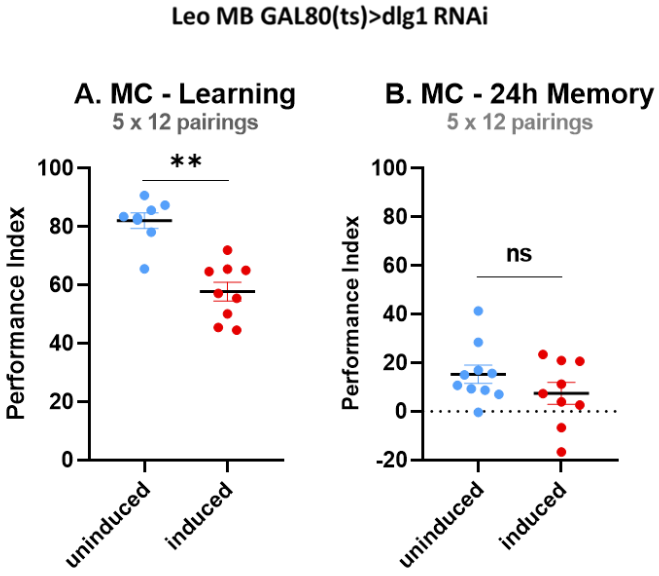


*FIG.20 Reduction of **dlg1** levels during adulthood disrupts learning upon MC-overtraining while leaving MC-PSIM unaffected. The graphs show mean performance ± SEM. (A) Performance in learning is significantly reduced after overtraining consisted of 5 cycles of 12 pairings (ANOVA, p<0.0001). (B) Performance in MC – 24h Memory is not significantly different between subject flies and controls (ANOVA, p = 0.2350).*

Avoidance experiments to electric shock and odors for each genotype are shown in Figure 21. Performance of RNAi expressing flies was affected only in octanol avoidance.

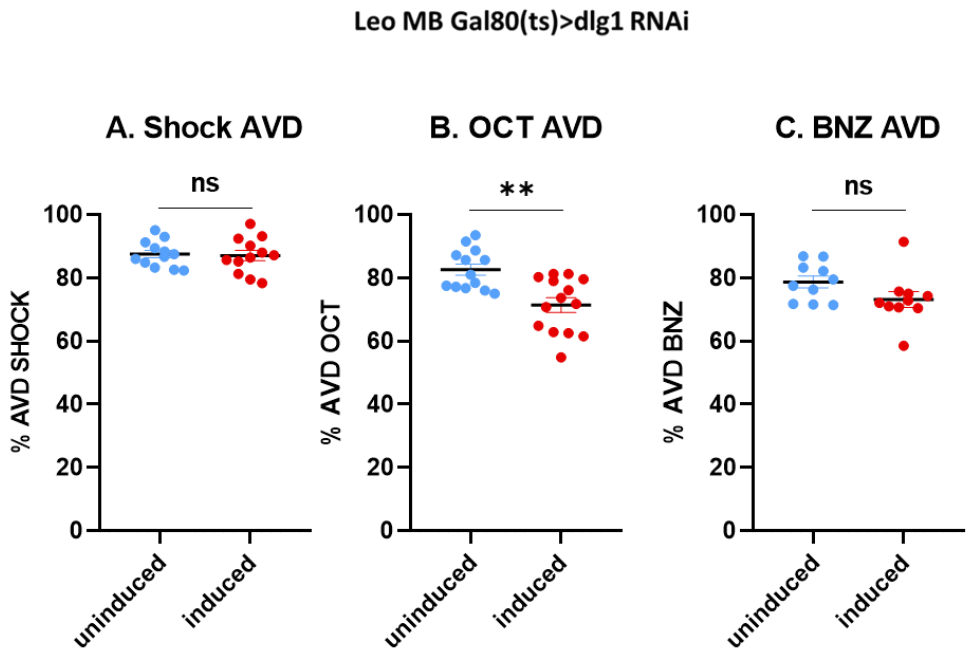


Fig.21 Odor and shock avoidance experiments of flies with reduced levels of dlg1. The graphs show mean performance ± SEM. . (A) Experimental flies avoid shock as the controls (ANOVA, p=0.8082), (B) Avoidance of octanol is significantly different between control and experimental flies (ANOVA, p=0.0009), (C) Experimental flies avoid benzaldehyde as the controls (ANOVA, p=0.0976).

iv. Effect of reduced expression of Zipper in adult MBs in SC-LTM

Since previous data from the lab demonstrated a role of Zipper in PSIM (FI.22, B), but not learning, spaced conditioning-elicited memory (SC-LTM) was still to be examined. Reduced levels of *zipper* had no effect on SC-LTM formation (FIG.22, A), indicating a specific role of *zipper* in PSIM pathways.

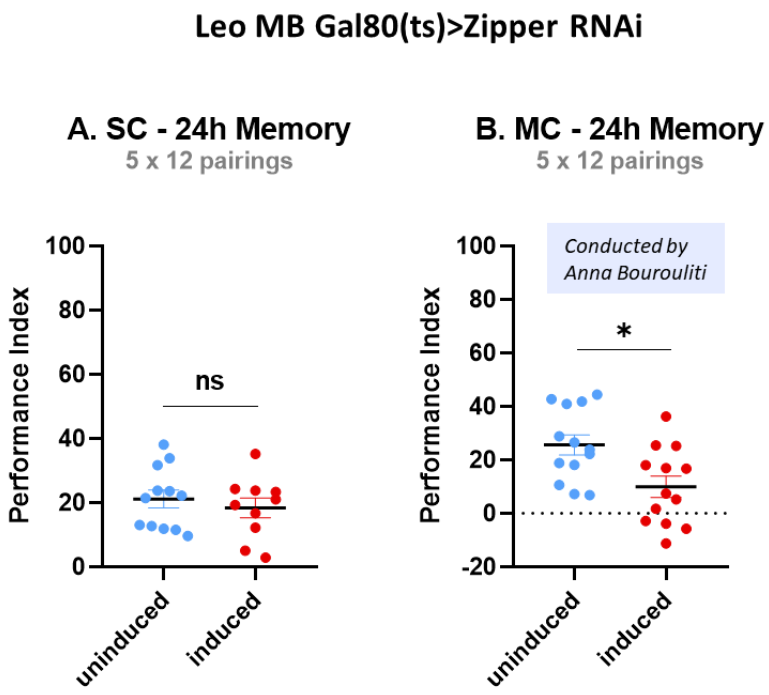


FIG.22 Reduction of Zipper levels during adulthood specifically affects MC-24 memory while leaving SC-24h memory unaffected. The graphs show mean performance ± SEM. (A) Performance in SC – 24h Memory is not significantly different between RNAi-expressing and controls (ANOVA, p = 0.5131). (B) Performance in MC – 24h Memory is significantly reduced in RNAI-expressing flies (ANOVA, p=0.008425). This experiment was conducted by Anna Bourouliti.

Avoidance experiments to electric shock and odors of subject groups are shown in Figure 23. Performance of RNAi expressing flies was not affected in any avoidance assay.

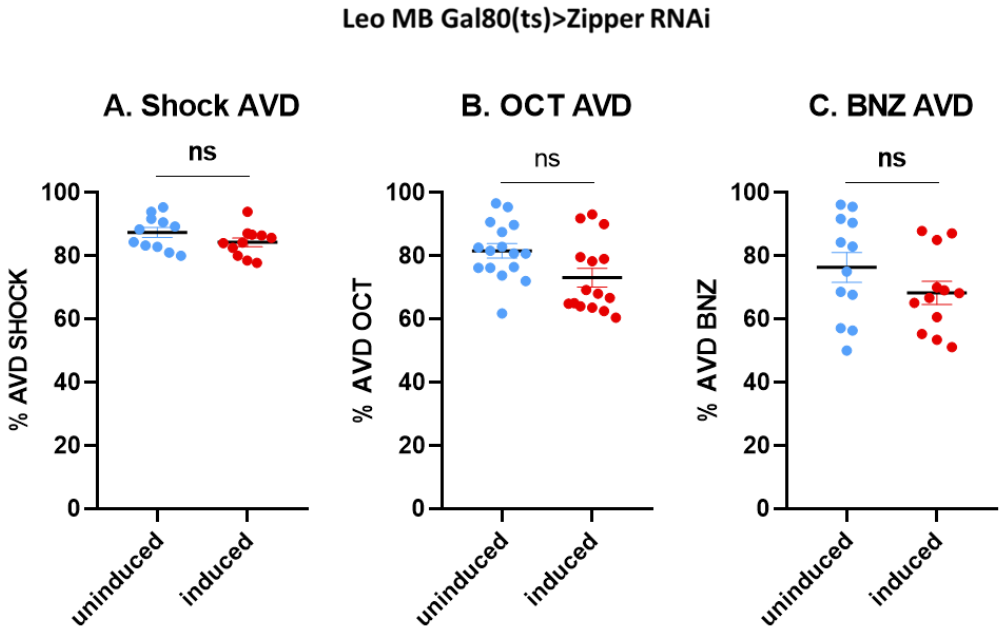


Fig.23 Odor and shock avoidance experiments of flies with reduced levels of zipper. The graphs show mean performance ± SEM. There is not a statistically significant difference in any case of avoidance. (A) ANOVA, p=0.1597, (B) ANOVA, p=0.0285, (C) ANOVA, p=0.1907.

v. Control experiments to investigate undesired effect of RNAi insertion in subject lines.

All of the RNAi-expressing lines have the insertion in the same locus, thus to ensure that the insertion of RNAi does not itself affect in any way the performance of flies, we performed control experiments using two different control lines for TRiP RNAi lines in chromosomes II and III crossed in the same way with Leo MB Gal80(ts) driver.

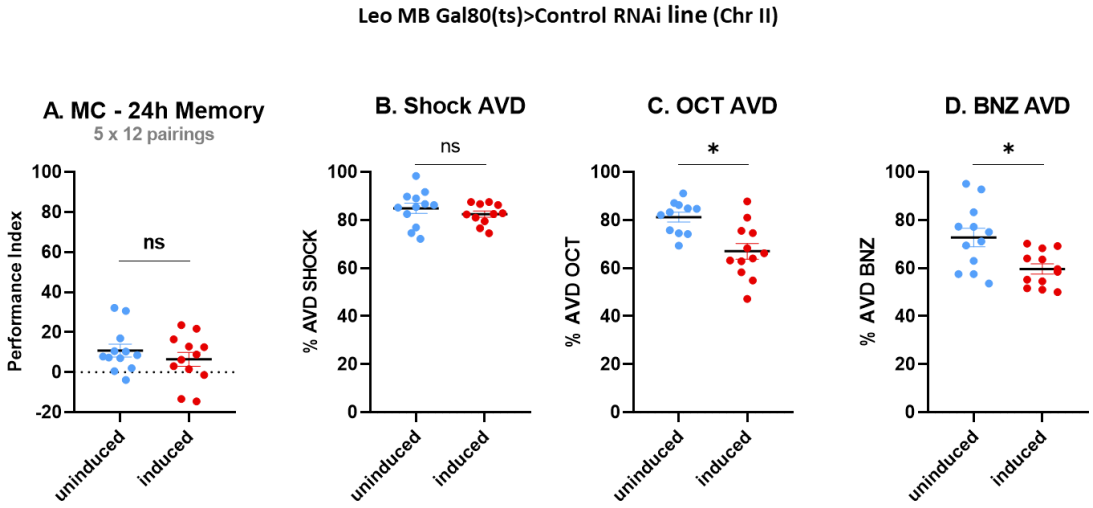


Fig.24 Control experiments for RNAi insertion in Chr II. The graphs show mean performance ± SEM. (A) Performance of RNAi-expressing flies in MC-PSIM (ANOVA, p=0.3649) and (B) shock avoidance remain unaffected (ANOVA, p=0.3605), while in (C) octanol avoidance (ANOVA, p=0.0017) and (D) benzaldehyde avoidance (ANOVA, p=0.0072) was affected.

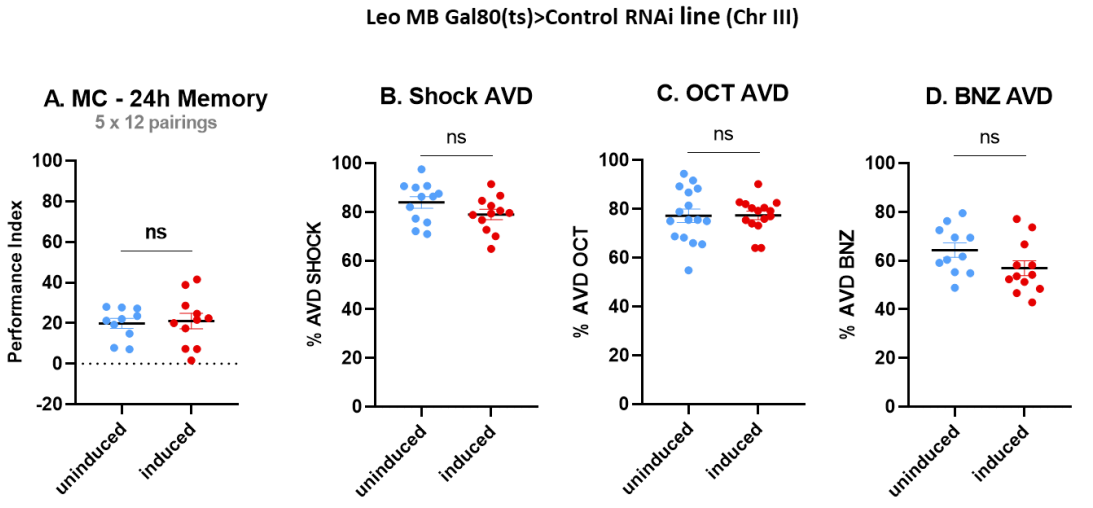


Fig.25 Control experiments for RNAi insertion in Chr III.. The graphs show mean performance ± SEM. Performance of RNAi-expressing flies was unaffected in MC-PSIM and every avoidance assay. (A) ANOVA, p=0.8047, (B) ANOVA, p=0.1367, (C) ANOVA, p=0.9601, (D) ANOVA, p=0.0974.

2. Molecular part

The behavioral screen described in the first part of the results, revealed two novel proteins that have a role in PSIM. Previous data from Anna Bourouliti indicate that *gprs* specifically regulates PSIM processes and with the contribution of this thesis one more was found; *zipper*, which is also specifically involved in PSIM. Therefore, we wanted to check the relation between the levels of these two proteins with Drk attempting to draw a possible pathway involved in PSIM.

So, at first we performed a Western blotting analysis for Zipper levels in flies with reduced levels of Drk (Drk/CyO). (Fig. 26). The results presented significantly reduced levels of Zipper in flies with lower levels of Drk compared to control W1118 flies.

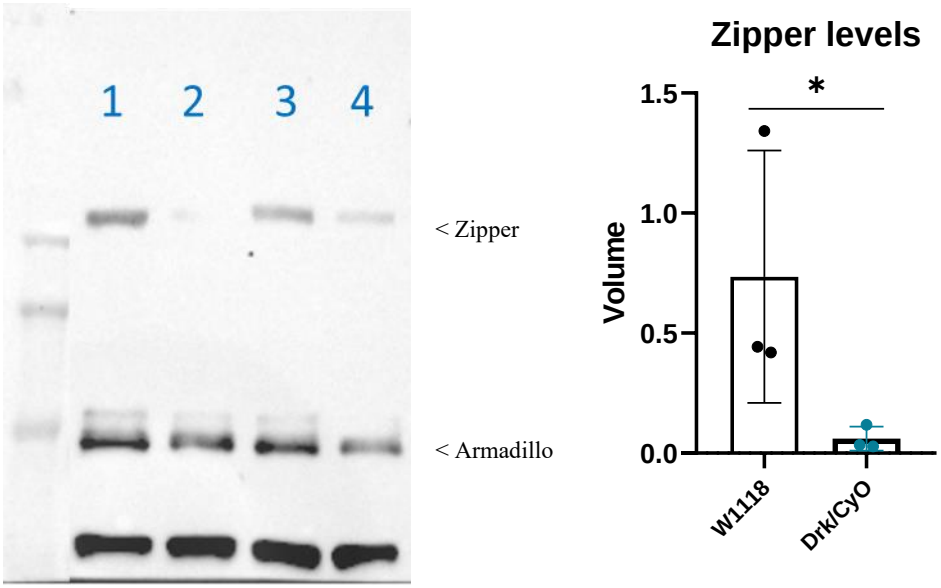


Fig. 26 Western blot analysis for Zipper levels.
(1) W1118, (2) Drk/CyO, (3) W1118, (4) Drk/CyO.
Zipper levels are significantly reduced in Drk mutant flies comparing with the control ones. (Wilcoxon T Test, x^2=3.8571, p<0.0495)

Given the specific role of Zipper in PSIM, found out from our experiments and the relation of Drk and Zipper levels we wanted to investigate the effect of Zipper in filamentous actin, since actin cytoskeleton dynamics is a hallmark of PSIM formation and Zipper protein plays a significant role in the binding of filamentous actin as well. Therefore, we performed a confocal microscopy analysis after rhodamine-conjugated phalloidin staining to identify F-actin levels in whole-mount brains of Zipper-RNAi expressing flies, induced for 3 days in 30^{O}C compared with the control uninduced ones (Fig. 27). The results presented significant reduced levels of F-actin in Zipper RNAi-expressing flies compared with the control ones.

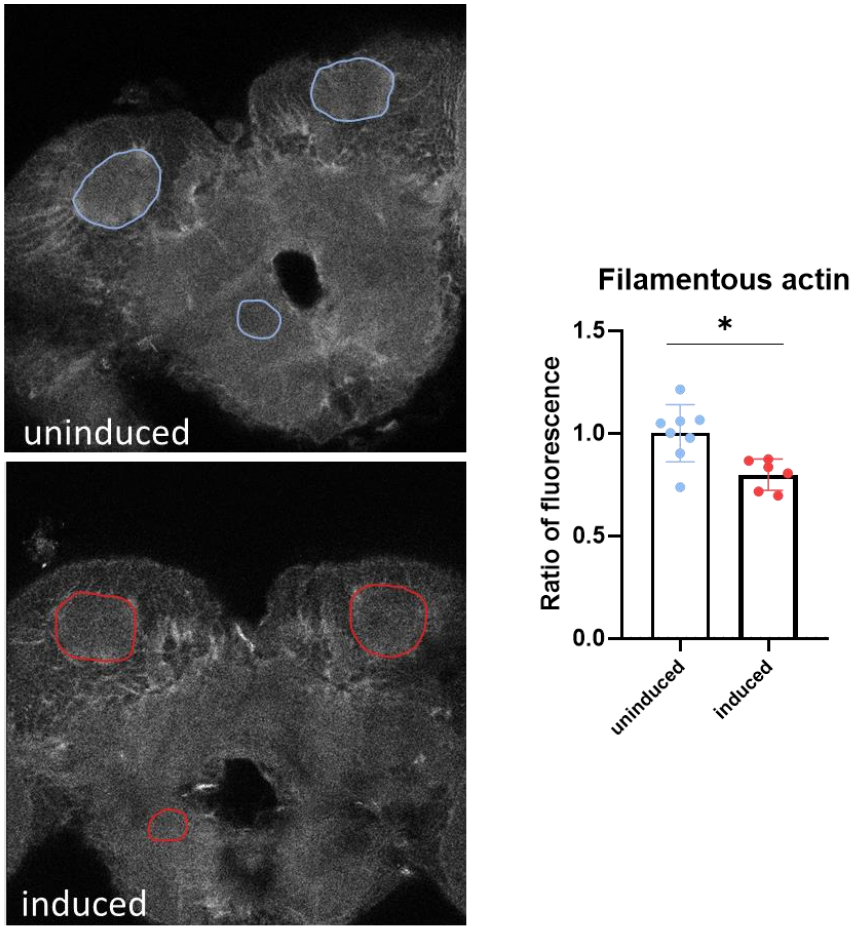
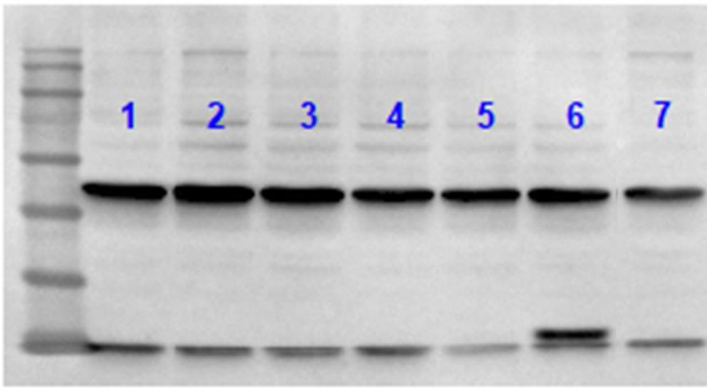


Fig. 27 Decreased filamentous actin in the MBs of Zipper-RNAi expressing flies. Representative confocal images of whole-mount brains at the level of the calyces used to quantify the ratio of fluorescence from the marked regions of interest (ROI) inside the calyces to a random area of the same brain after rhodamine-conjugated phalloidin staining. Quantification (Right) of multiple experiments revealed significant differences in fluorescence in the calyces of control and transgenic animals (Wilcoxon T test, ,x^2=6.6667, $p < 0.0098$).

Then, we performed a Western blotting analysis for Drk levels in flies with reduced levels of Gprs and upon overexpression of Drk in the same flies (Fig.28). The results presented below indicate no differences in Drk levels between different subjects.



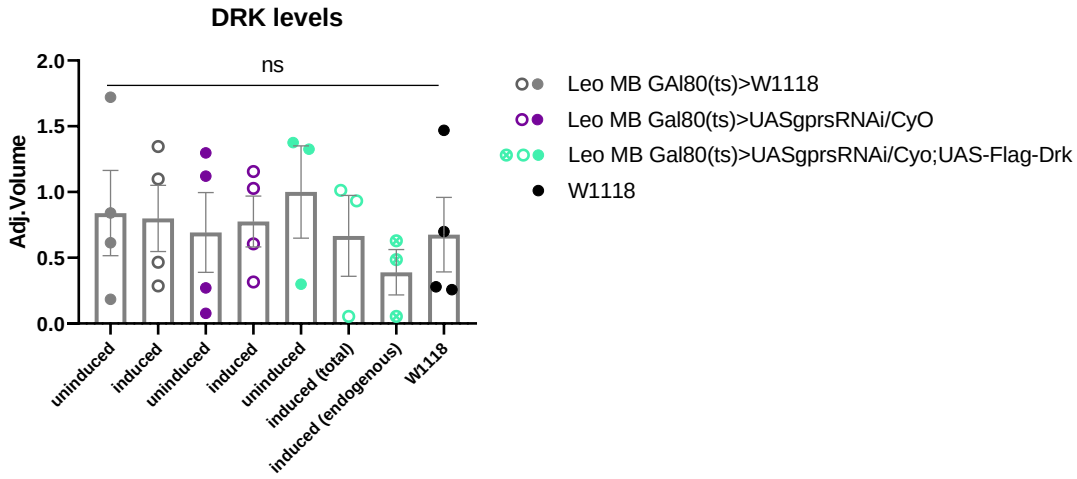


Fig. 28 Western blot analysis for Drk levels.
(1) Leo MB Gal80(ts)>W1118 (uninduced), (2) Leo MB Gal80(ts) (induced), (3) Leo MB Gal80(ts)>UASgprsRNAi/Cyo (uninduced), (4) Leo MB Gal80(ts)>UASgprsRNAi/Cyo (induced), (5) Leo MB Gal80(ts)>UASgprsRNAi/Cyo;UAS-Flag-Drk (uninduced), (6) Leo MB Gal80(ts)>UASgprsRNAi/Cyo;UAS-Flag-Drk (induced), (8) W1118.
No statistically significant differences were detected comparing the levels of Drk.

G. DISCUSSION

Protein Synthesis Independent Memory (PSIM) is an interesting type of memory described in *Drosophila* that can be elicited via Massed Conditioning in the classical negatively reinforced olfactory conditioning task. The molecular mechanisms that govern this type of memory as well as any other function towards PSIM regulation remain mainly unknown. Previous data from the lab reveal Drk, a protein necessary in the Mushroom Bodies (MBs), the center of learning and memory in *Drosophila*, for normal PSIM. As such, later studies focused on finding probable protein interactions of Drk within the adult MBs, in order to uncover the molecular pathway of PSIM formation. Bourouliti and Skoulakis presented evidence that indicates specific involvement of a novel protein, namely gprs, in the mechanisms that regulate PSIM. Also, they demonstrated that three other proteins, namely RyR, RhoGAP18B, and Zipper, may have a role in PSIM regulation. The present thesis aims to define if these

proteins are involved specifically in processes of PSIM formation, investigate the role of a new protein, Dlg1, in PSIM, and to find out whether any of these proteins are involved in a common signaling pathway along with Drk.

1. Behavioral part

First, we performed avoidance experiments to shock and odors to ensure that subjective flies are able to sense the stimuli presented during conditioning (Table 1). The data show that insertion of RNAi at a certain genetic locus of chromosome II may provoke a reduced performance in octanol avoidance. This fact could explain any tendency of the experimental lines for lower performance in octanol avoidance, suggesting that this effect is not genotype-specific. On the other hand, the reduced performance of RyR RNAi-expressing line in octanol avoidance cannot be obviously explained in the same manner, thus it could be an effect produced by this specific insertion itself. In any case, avoidance experiments consist of simultaneous presentation of shock and odors with air, so we cannot say they represent the exact conditions of the behavioral experiments for learning and memory, but we ought to take them into consideration.

Table.1 Summary of results from avoidance experiments on adult MBs RNAi-expressing lines for each gene of interest.

Genotype	Shock AVD	OCT AVD	BNZ AVD
RhoGAP18B RNAi (II)	-	-	-
Dlg1 RNAi (II)	-	Affected	-
Zipper RNAi (II)	-	-	-
RNAi Control line (II)	-	Affected	Affected
RyR RNAi (III)	-	Affected	-
RNAi Control line (III)	-	-	-

Based on previous findings that implicate a possible participation of specific proteins (RhoGAP18B, RyR, Dlg1, Zipper) in PSIM processes, the main purpose of this thesis was to further investigate the role of the selected proteins that may interact with Drk within the MBs of adult flies in PSIM formation and to examine the specificity of their involvement in this pathway. These results are presented below and are summarized in Table 2.

1) Reduction in levels of **RhoGap18B** had no significant difference between the two groups after a training consisted of 6 or 12 pairings of US/CS (Fig.16, A,B). However, we noticed that PIs were low for both the control and subject groups. For that reason, we also tried MC-Learning. In this case the performance of the RNAi expressing flies was significantly reduced compared to that of the controls (FIG.16, C). This suggests a role of RhoGAP18B in learning, which keeps us from further investigation of memory, since normal learning cannot be reached in the subject group after MC. However, the similar performance index observed in both groups after only one cycle, raises the question whether RhoGAP18B may be associated with memory acquisition that relies on processes which take place in the event of repetition of training. This could be further validated by training subject flies with more training cycles than the controls.

2) Knowing that reduced **RyR** levels led to deficit in PSIM formation, it was necessary to examine the effect of this reduction in learning as well. Both with one cycle learning and upon the MC-learning protocol, learning impairments were observed in RNAi expressing flies (FIG.18). This suggests that the role of RyR is not specific in PSIM, but the deficit in this type of memory may be a result of inefficient learning ability.

3) On the other hand, even though **Dlg1** was revealed in the proteomic analysis as a probable interactor of Drk within the adult MBs, it seems that it does not affect PSIM. However, it engages in learning since reduction in its levels led to deficit in learning performance after MC-learning (FIG.20). This indicates that Dlg1 and Drk may synergize in a common pathway governing learning in the adult MBs instead of in one that regulates PSIM.

4) Finally, it was investigated if **Zipper**, which has been found to be implicated in PSIM (FIG.22, B) but not learning, affects the SC-elicited LTM. It is clear that reduction in zipper levels left LTM intact (FIG.22, A), revealing a specific role of zipper in the regulation of PSIM.

Table.2 Summary of results from behavioral part analysis on adult MBs RNAi-expressing lines for each gene of interest. (Data in parentheses are from previous laboratory study)

Genotype	Learning 6 pairings	Learning 12 pairings	MC-learning 5 x 12 pairings	MC-24h memory	SC-24h memory
RhoGAP18B RNAi (II)	No effect	No effect	Deficit	(Deficit)	-
RyR RNAi (III)	-	Deficit	Deficit	(Deficit)	-
Dlg1 RNAi (II)			Deficit	No effect	-
Zipper RNAi (II)	(No effect)	-	-	(Deficit)	No effect
RNAi Control line (II)	-	-	-	No effect	-
RNAi Control line (III)	-	-	-	No effect	-

As a conclusion from the behavioral part, we could say that RhoGAP18B may be implicated in the step of acquisition, but this needs further validation with training consisted of different number of cycles for control and subject flies. When it comes to RyR, it could be proposed as a learning mutant with the reduced performance of subject flies in PSIM being caused by the learning deficits, but we have to take also into consideration the reduced performance in octanol avoidance which could be a possible cause of behavioral deficits as well. On the other hand, Dlg1 is suggested to participate along with Drk in a pathway regulating learning instead of another one that governs PSIM. Finally, Zipper has a specific role in the regulation of PSIM. Therefore, experiments with overexpression of the protein either in Zipper RNAi-expressing flies or Drk mutant background to examine a possible rescue of the phenotype in PSIM could be proved important, validating the present results.

When aggregating the data from both the control experiments and the various behavioral experiments, it becomes evident that in certain instances, any impairment in odor avoidance is not transferred to the performance in MC-24h memory or in SC-24h memory respectively. Take, for instance, the case of *dlg1*, where a deficiency in octanol avoidance is observed, yet the performance of RNAi-expressing flies in MC-24h memory remains unaffected. It is worth noting, however, that the extensive repetition which takes place in both of memory assays could be the reason of this deficit's overcome. Furthermore, we note a deficit in MC-learning in this case, which interestingly does not translate into an impairment in MC-24h memory. A possible explanation could be the timing of the testing, since in the first assay the flies are tested right after the training whereas in the other there is a resting period of 24 hours. Consequently, it is conceivable that fatigue may play a role in this deficit, implicating the subject gene in sensory fatigue.
In any case, we always have to take into consideration that behavioral experiments may suggest possible roles of genes and proteins in specific behaviors such as learning and memory but given the complexity of these processes and their dependence on various parameters it is better if these results get further validated upon other techniques, including molecular assays.

2. Molecular part

The next step regarding the specific role of zipper and gprs in PSIM, was to examine whether they act in the same pathway along with Drk.
So, at first, we investigated if reduction in gprs levels inside the MBs of adult flies has an effect on Drk levels, and if overexpression of Drk could reverse any possible effect. Wester Blotting analysis confirmed that there was no significant difference in Drk levels among gprs RNAi expressing lines and control flies or with overexpression of Drk (Fig. 26).

On the other hand, Western Blotting analysis for Zipper levels revealed that reduction in Drk levels led to reduced Zipper levels (Fig.27). This indicates a possibility that Zipper act downstream of Drk in A PSIM-regulating pathway. In any case, such a claim needs to be further validated maybe with histochemical analysis inside the MBs, investigating the effect of reduction or overexpression of one of these proteins to the levels of the other and *vice a versa.* Also, it would be interesting to evaluate the effect of overexpression of Zipper in Drk mutant flies in behavioral assays to see if this overexpression could rescue the deficits caused by the reduction of Drk, especially in PSIM performance.

Finally, since Zipper has a specific role in PSIM, it may act downstream of Drk and it regulates the actin cytoskeletal dynamics, a hallmark in PSIM formation we investigated its effect in F-actin. Thus, confocal microscopy revealed decreased levels of F-actin inside the MBs of adult flies with reduced Zipper levels. These results are consistent with previous data from the lab showing that reduction in Drk levels leads to reduced F-actin in MBs calyces of adult brains [45]. Therefore, we could propose that these two proteins act in the same PSIM formation pathway by regulating somehow the F-actin organization.

Overall, the findings of the present study offer informative evidence regarding the understanding of the mechanisms that govern PSIM in *Drosophila*. Further investigation of molecular pathways and genes that are involved in this unique type of memory will be continued to reveal new proteins and their role along with known ones like Drk in the regulation of PSIM not only in *Drosophila*, but also in mammals.

H. APPENDIX

Table 3. Statistical comparisons.
*For behavioral experiments: *:p value<0.01, **:p value<0.001*
*For Western Blotting and Confocal Microscopy: *:p value<0.05*

Group	Mean ± SEM	p Value
Figure 16A	**ANOVA**	
Uninduced	53.70 ± 3.50	
Induced	46.02 ± 3.35	0.1280
Figure 16B	**ANOVA**	
Uninduced	65.97 ± 3.76	
Induced	53.94 ± 3.57	0.0330
Figure 16C	**ANOVA**	
Uninduced	79.51 ± 2.22	
Induced	68.47 ± 2.32	0.0025*
Figure 17A	**ANOVA**	
Uninduced	75.19 ± 2.26	
Induced	85.89 ± 2.26	0.0250
Figure 17B	**ANOVA**	
Uninduced	76.55 ± 1.79	
Induced	70.42 ± 1.70	0.0241
Figure17C	ANOVA	
Uninduced	58.28 ± 5.04	
Induced	61.59 ± 4.60	0.6320
Figure 18A	ANOVA	
Uninduced	75.51 ± 2.71	
Induced	64.63 ± 2.52	0.0068*
Figure 18B	ANOVA	
Uninduced	80.62 ± 3.53	
Induced	65.55 ± 3.53	0.0062*
Figure 19A	ANOVA	
Uninduced	92.27 ± 2.04	
Induced	84.90 ± 2.04	0.0192
Figure 19B	ANOVA	
Uninduced	74.40 ± 2.03	
Induced	60.62 ± 2.12	0.0002**
Figure 19C	ANOVA	
Uninduced	68.53 ± 3.78	
Induced	64.09 ± 3.78	0.4150
Figure 20A	ANOVA	

Uninduced	82.03 ± 3.10	
Induced	57.77 ± 2.93	<0.0001**
Figure 20B	**ANOVA**	
Uninduced	14.08 ± 3.38	
Induced	8.10 ± 3.53	0.2350
Figure 21A	**ANOVA**	
Uninduced	87.55 ± 1.42	
Induced	87.06 ± 1.42	0.8082
Figure 21B	**ANOVA**	
Uninduced	82.65 ± 2.13	
Induced	71.46 ± 2.06	0.0009**
Figure 21C	**ANOVA**	
Uninduced	78.74 ± 2.24	
Induced	73.21 ± 2.24	0.0976
Figure 22	**ANOVA**	
Uninduced	21.13 ± 2.79	
Induced	18.39 ± 3.05	0.5131
Figure 23A	**ANOVA**	
Uninduced	87.35 ± 1.49	
Induced	84.27 ± 1.49	0.1597
Figure 23B	**ANOVA**	
Uninduced	81.57 ± 2.56	
Induced	73.09 ± 2.64	0.0285
Figure 23C	**ANOVA**	
Uninduced	76.34 ± 4.21	
Induced	68.30 ± 4.21	0.1907
Figure 24A	**ANOVA**	
Uninduced	10.84 ± 3.35	
Induced	6.46 ± 3.35	0.3649
Figure 24B	**ANOVA**	
Uninduced	84.90 ± 1.78	
Induced	82.49 ± 1.86	0.3605
Figure 24C	**ANOVA**	
Uninduced	81.22 ± 2.85	
Induced	67.00 ± 2.72	0.0017*
Figure 24D	**ANOVA**	
Uninduced	72.76 ± 3.13	
Induced	59.66 ± 3.13	0.0072*
Figure 25A	**ANOVA**	
Uninduced	19.90 ± 3.33	
Induced	21.05 ± 3.18	0.8047
Figure 25B	**ANOVA**	
Uninduced	83.92 ± 2.26	
Induced	78.98 ± 2.26	0.1367
Figure 25C	**ANOVA**	
Uninduced	77.21 ± 2.31	
Induced	77.38 ± 2.39	0.9601
Figure 25D	**ANOVA**	

Uninduced	64.33 ± 3.08	
Induced	56.94 ± 2.95	0.0974
Figure 26	**WILCOXON T TEST**	
W1118	1.00000 ± 0.29356	
Drk/CyO	0.08232 ± 0.29356	0.0495* (x^2=3.8571)
Figure 27	**WILCOXON T TEST**	
Uninduced	1.00000 ± 0.04131	
Induced	0.079807 ± 0.04770	0.0098* (x^2=6.6667)
Figure 28	**WILCOXON T TEST**	
1st bar	0.83974 ± 0.25465	
2nd bar	0.79895 ± 0.25465	
3rd bar	0.69177 ± 0.25465	
4th bar	0.77611 ± 0.25465	
5th bar	1.0000 ± 0.29404	
6th bar	0.83203 ± 0.29404	
7th bar	0.38975 ± 0.29404	
8th bar	0.67607 ± 0.25465	0.8878 (x^2=3.6426)

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