DEPARTMENT FOR INNOVATION IN BIOLOGICAL, FOOD AND FORESTRY SYSTEMS

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XXXV Cycle

"The eIF3d-RACK1 specialized protein translation in learning and memory formation in *Drosophila melanogaster*"

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Short Abstract

Canonical protein synthesis plays an important role in synaptic plasticity, learning, and memory formation.

The two main targets of the canonical initiation regulation are eIF4E, whose binding to the eIF4G protein is critical for initiation translation and controlled by many regulator proteins, and eIF2α, whose phosphorylation is crucial in amygdala-based memory.

Dysfunction of eIF4E or eIF2α leads to impairment in learning and learning-related synaptic plasticity.

Considering the existence of alternative protein translation mechanisms, the canonical protein synthesis might not be the only one involved in learning and memory processes.

Here, we show that the eIF3d-RACK1 specialized translation plays a fundamental role in *Drosophila melanogaster* Mushroom Bodies development and in learning and memory formation.

In fact, the downregulation of eIF3d and the overexpression of RACK1 cause defects in the MBs structure and, consequently, in learning abilities. This could be due to the increase in protein synthesis rate and in variations in Dlg1 expression. We have demonstrated also that double transgenic flies for eIF3d and RACK1 show a recovery in MBs structure, learning, and at the molecular level.

Thus, these results provide a new control mechanism in learning and memory formation, which could be pivotal in pathologies with impaired cognitive and mnemonic abilities.

Keywords: memory, learning, *Drosophila melanogaster*, Mushroom Bodies, protein translation, eIF3d, RACK1

Abstract

Canonical protein synthesis plays an important role in synaptic plasticity, learning, and memory formation.

The two main targets of the canonical initiation regulation are eIF4E, whose binding to the eIF4G protein is critical for initiation translation and controlled by many regulator proteins, and eIF2α, whose phosphorylation is crucial in amygdala-based memory.

Dysfunction of eIF4E or eIF2α leads to impairment in learning and learning-related synaptic plasticity.

Considering the existence of alternative protein translation mechanisms, canonical protein synthesis might not be the only one involved in learning and memory processes.

Therefore, the aim of this thesis work is to investigate the role of the eIF3d-RACK1 specialized translation in learning and memory formation. For this purpose, the Drosophila melanogaster Mushroom Bodies (MBs), which are the nervous structures deputed to olfactory reception, learning, and memory have been used. Precisely, because of the function they perform in memory, the Mushroom Bodies are comparable to the human hippocampus.

eIF3d is one of the factors involved in the eIF4F-independent translation. In fact, through its capability to bind the 5'UTR mRNA cap, it allows translation initiation without the eIF4F complex formation (Lee et al., 2016). Moreover, we have demonstrated that eIF3d interacts with RACK1, which plays a fundamental role in neuronal development, regulating axon growth and participating in the dendritic local translation of specific mRNAs to promote neuronal development. In addition, RACK1 recruits eIF3d on the ribosome, allowing the translation of specific mRNAs.

First, the expression of eIF3d and RACK1 was modulated in Mushroom Bodies. MBs consist mainly of about 2500 Kenyon cells (KCs), whose dendrites form the calyx structurein each hemisphere, while their axons constitute the peduncle and lobes. Lobes divide into vertical (α, α′) and horizontal (β, β, and γ) lobes, forming the typical lobular anatomical MBsstructure.

Data obtained revealed that the downregulation of eIF3d causes strong defects in the MBs structure. In fact, the Kenyon cells were reduced by about 50% and the α'/ α and the β'/ β lobes were thinner, more disorganized and elongated compared to those observed in control flies. These structural defects also cause impairments in learning abilities and behavior.

Flies overexpressing RACK1 presented the same impairments in the MBs structure and in behavior. Moreover, a larval developmental delay of about 24-48 hours has been observed both for larvae downregulating eIF3d and overexpressing RACK1.

The same phenotype with a larval developmental delay and strong defects in the MBs structure, that reflect impairments in learning abilities and behavior, have been observed also in flies downregulating eIF4E.

However, double transgenic flies for eIF3d and RACK1 showed a recovery in the MBs structure and consequently in learning and behavior. In addition, the larvae do not present a delay in their development and reached the pupal stage in 3-4 days.

To investigate the reasons behind this recovery, we have carried out some molecular investigations. First, protein synthesis was investigated, considering the role of eIF3d and RACK1 in eIF4F-independent translation.

Flies downregulating eIF3d and overexpressing RACK1 showed an increase in protein synthesis rate, which is recovered in the double transgenic flies for these proteins.

The expression levels of p-eIF2α, which is the other main target of translation control, were analyzed. Moreover, p-eIF2α plays a fundamental role in synaptic plasticity and memory formation.

Data obtained revealed that in single transgenic flies and in double transgenic flies for eIF3d and RACK1, the expression of p-eIF2α remains unchanged compared to the control flies.

Moreover, data acquired on single neurons showed that the downregulation of eIF3d and the overexpression of RACK1 causes smaller neurons and a fewer number of arborizations.

In addition, it is known that in neurons RACK1 interacts with the RNA binding protein Fragile X Mental Retardation protein (FMRP). The depletion of FMRP leads to dendritic andaxonal aberrant development, which are two main features of Fragile X syndrome patients. The binding of FMRP with RACK1 removes the translational repressive activity of FMRP and promotes the translation of the Post-synaptic Density (PSD-95) mRNA.

Considering these data, the expression of Dlg1, the PSD-95 ortholog, was investigated.

The results revealed that the overexpression of RACK1 caused an increase in the Dlg1 expression, while the downregulation of eIF3d determined a reduction in Dlg1 expression. Whereas Dlg1 plays an important role in synaptic plasticity, the variations in Dlg1 expression caused by RACK1 overexpression and eIF3d downregulation individually maybe another cause of the defects observed in MBs structure and consequently in learning and behavior. Indeed, in the double transgenic flies, in which the Dlg1 expression was restored to

wild-type levels, MBs impairments were recovered, and flies showed normal behavior and learning capabilities.

In conclusion, results obtained in this PhD work suggest that the eIF3d-RACK1 specialized translation could play a fundamental role in learning and behavior formation, such as demonstrated by the phenotypes obtained modulating the expression of these two proteins. From the data obtained, it appears that the eIF3d-RACK1 axis has a role in learning and memory formation comparable to that played by the eIF4E-dependent translation.

The eIF3d-RACK1 specialized translation acts on MBs development and consequently to learning and memory formation modulating protein synthesis rate and the expression of Dlg1.

Keywords: memory, learning, *Drosophila melanogaster*, Mushroom Bodies, protein translation, eIF3d, RACK1

Introduction

Protein synthesis in learning and memory formation

Canonical protein synthesis is fundamental for supporting memory-related synapseremodeling immediately after learning (Gindina et al., 2021). In the learning process, translation is finely regulated. In fact, learning requires not only new protein translation but also a fine balance between the mechanisms of translation regulation.

Different types of learning are associated with the strengthening (long-term potentiation) and weakening (long-term depression) of synaptic activity. New protein synthesis is required for both processes (Costa-Mattioli et al. 2009, Kandel 2001, Luscher & Huber 2010). Moreover, also the potentiation of synaptic activity can be divided into early long-term potentiation (E-LTP), which lasts about 2-3 hours, and does not need new protein synthesis, and more long-term potentiation (L-LTP), which requires new protein synthesis.

Also, memory requires synaptic activity and synaptic plasticity. It can be divided into short-term memory, which lasts only a few hours, and long-term memory, which lasts for much time (Davis and Squire, 1984; Steward and Schuman, 2001). Long-term memory requires modifications in gene expression, new protein synthesis, and new formation of synapses, unlike short-term memory. Indeed, the administration of protein synthesis inhibitors blocks long-term memory, but not short-term memory (Otani and Abraham, 1989; Stanton and Survey, 1984).

Time is necessary to stabilize and consolidate new memories before they become long-term memories. During this time, new information can be modified. The consolidation process requires time, and it passes through different stages of memory. Memory consolidation requires synaptic consolidation.

Translational control mechanisms in synaptic plasticity and memory

Protein translation plays a critical role in synapse and memory formation. Therefore, each step of the translation is finely regulated (Buffington et al., 2014).

Canonical protein synthesis

Protein synthesis occurs in four different steps: initiation, elongation, termination, and recycling (Figure 1).

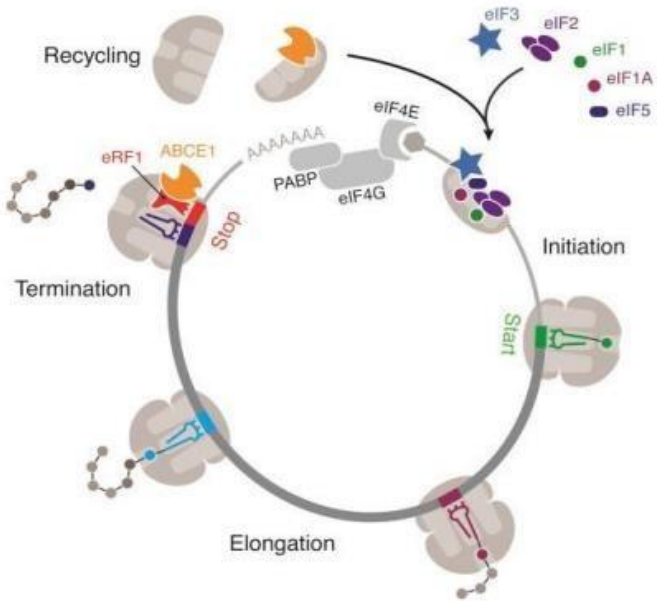


Figure 1. Protein translation is composed of 4 different steps: initiation, elongation, termination, and recycling. The translation initiation requires many eukaryotic initiation factors (eIFs) and represents the most rate-limiting step in protein translation (Schuller & Green, 2017).

The translation initiation is the most limiting-rate phase and thus the most regulated, even if the other translation phases also present different control mechanisms (Buffington et al., 2014). Canonical translation initiation requires the formation of the 43S pre-initiation complex, which is composed of the ternary complex, formed by Met-tRNA initiator bonded to the 40s ribosomal subunit and the eIF2-GTP, and many eukaryotic factors such as eIF1, eIF1A, eIF3, and eIF5. Then, with the binding of the 43S pre-initiation complex to the mRNA, through the formation of the eIF4F complex, the 48S complex is formed. The 48S pre- initiation complex scans the mRNAs until it reaches the AUG start codon and, at this point, after the release of some specific factors, the 60 ribosomal subunit can match with the 40S ribosomal subunit, forming the 80S. Then, the translation can start (Figure 2).

The eIF4F complex formation is the rate-limiting step for cap-dependent mRNA translation initiation. eIF4F complex contains eIF4E, which is a factor able to bind directly to the cap, eIF4G, which acts as a scaffold protein, and eIF4A, an ATP-dependent mRNA helicase (Gingras et al., 1999).

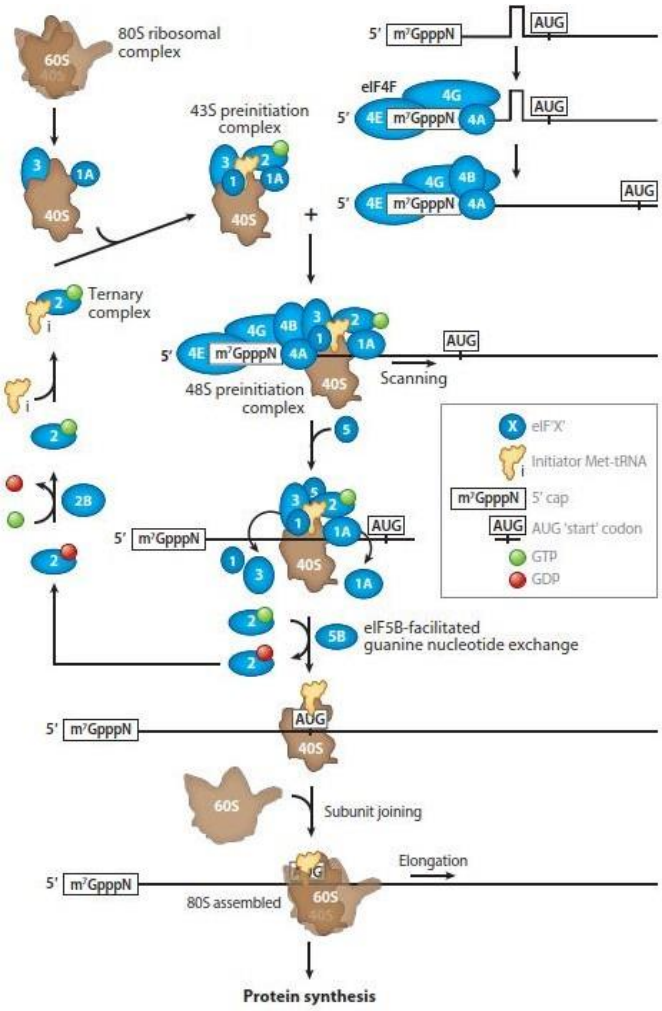


Figure 2. Schematic representation of the eIF4F-translation start mechanism dependent. This pathway is divided into 9 phases: - recycling of post-termination complexes, - formation of the ternary complex, - formation of the 43S complex, - activation of the mRNA made possible by the complex eIF4F, eIF4A, and eIF4B, - 43S complex attack to the mRNA, - scanning of the 5'-UTR region until the codon is reached, - recognition of AUG and subsequent formation of 48S and hydrolysis of GTP linked to eIF2, - release of a part of the starting and pairing factors with the subunit 60S, - hydrolysis of the GTP, linked to eIF5B and subsequent release of eIF5B and eIF1A, resulting in the formation of the 80S subunit (Buffington et al., 2014).

Translational control mediated by eIF4E

Factors involved in translation initiation control are the best characterized in synaptic plasticity and memory formation.

eIF4E is one of the main targets in translation initiation control. In addition to forming the eIF4F complex, eIF4E is also important for mRNA stability and for the mRNA correct cellular localization.

Previous studies have reported that during memory formation, eIF4E is more abundant that the other eIFs in dendritic spines. In fact, in animals trained using Pavlovian conditioning (also known as a threat or fear conditioning), which is an extensively studied behavioral paradigm that produces robust learning in a single training session (LeDoux, 2000; Maren, 2001), it is possible to observe a robust learning-induced upregulation of eIF4E in dendritic spines (Amorim et al., 2018). This is consistent with the importance of protein synthesis in learning and long-term memory.

However, an exaggerated protein translation can cause synaptic and behavioral aberrations. In fact, transgenic mice that overexpress eIF4E show an increased protein cap-dependent translation, which determines aberrant behaviors, such as those observed in autism spectrum disorders (ASD). eIF4E transgenic mice displayed repetitive and perseverative behaviors with cognitive inflexibility in simple behavioral tests (Hoeffer, 2008; Ehninger, 2008), together with an impairment in locating new positions. In addition, eIF4E transgenic mice exhibited reduced reciprocal interactions with a freely moving stranger mouse, which is further evidence of deficits in social behavior. Then, the eIF4E transgenic mice showed mild hyperactivity, but no impairments in motor coordination, motor learning, and sensorimotor gating (Santini et al., 2013).

Therefore, mice that overexpress eIF4E have deficits in synaptic plasticity and learning, as well as abnormal spine morphology like that seen in Fragile X and other intellectual disorders (Fiala et al., 2002; Grossman et al., 2006; De Rubeis et al., 2013; Santini et al., 2013).

In fact, eIF4E transgenic mice are a well-characterized model for ASD like The Fmr1 knockout (KO) mouse, which is a well-established mouse model of Fragile X Syndrome (FXS) (Santini et al., 2013).

In addition, it is reported from the literature that the blockage of the binding eIF4E/eIF4G in rat hippocampal slices affects the long-term potentiation (L-LTP), showing that the eIF4E activity is fundamental for the maintenance of protein synthesis-dependent L-LTP (Hoeffer

et al., 2013). Moreover, it has also shown that the interaction eIF4E/eIF4G is required for consolidation and not for reconsolidation of fear memory, suggesting a different requirement for cap-dependent translation in the consolidation and reconsolidation of fear memory (Hoeffer et al., 2011).

Therefore, dysfunction of eIF4E leads to impairment of various forms of learning and learning-related synaptic plasticity (Gkogkas et al., 2010; Buffington et al., 2014; Santini et al., 2014) as well as intellectual functions in humans (Kelleher and Bear, 2008; Darnell, 2011; Kapur et al., 2017).

Translational control mediated by the eIF4E activity regulators

Since the binding of eIF4E to the mRNA cap is crucial for translation initiation, its activity is finely regulated by a series of factors.

eIF4E activity is directly modulated in two different ways. One way is through the phosphorylation by the mitogen-activate protein kinase-interacting serine/threonine kinase 1 and 2 (Mnk1/2). Mnk1/2 is activated by the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways. The activation of this pathway allows the eIF4E phosphorylation, which reduces eIF4E affinity for the mRNA cap and allows the ribosome to scan for the AUG start codon (Sonenberg et al., 2009; Sonenberg, 2008; Marcotrigiano et al., 1999) (Figure 3).

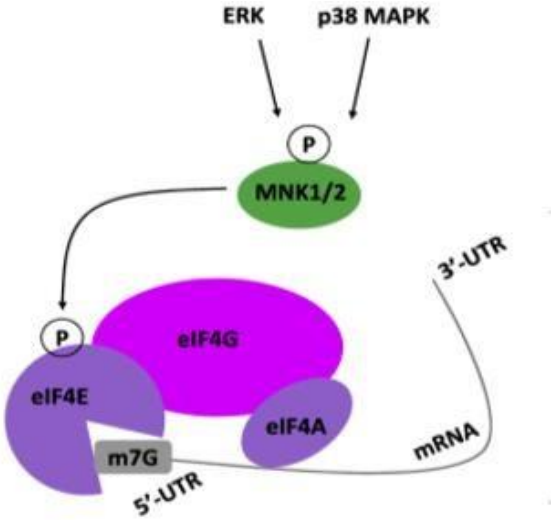


Figure 3. eIF4E represents one of the major targets in regulating translation initiation. eIF4E activity is controlled by a series of kinases, such as mTOR and Mnk1/2 (Kosciuczuk et al., 2017).

The other way is through the mammalian target of rapamycin (mTOR), which can regulate indirectly the eIF4E activity. In fact, mTOR can phosphorylate the eIF4E binding protein 1 (4EBP1) which in its phosphorylated state is not able to bind the eIF4E, allowing the eIF4F complex formation (Figure 4).

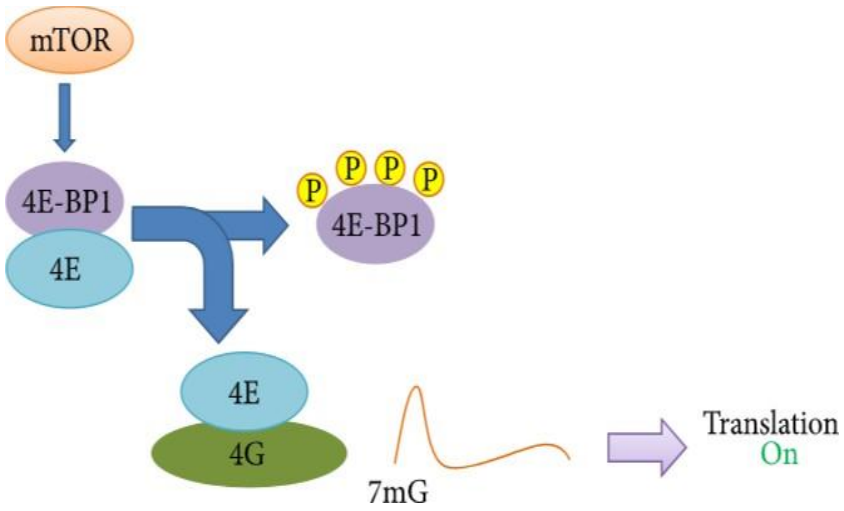


Figure 4. mTOR promotes the translation initiation, phosphorylating 4E-BP1. Once phosphorylated, 4E-BP1 cannot bind the eIF4E, allowing the eIF4F-complex formation (Showkat and Beigh, 2014).

Also, mTOR is involved in synaptic plasticity and memory. In this context, its function is the regulation of protein synthesis, acting on the two targets S6K1 and 4EBP1, which are involved in the initiation and elongation phases of protein translation. Indeed, S6K1 phosphorylates eIF4B, which stimulates the activity of helicase eIF4A (Roesler, 2017).
mTOR is activated by a series of neurotransmitters and neurotrophic factors such as N-methyl-D-aspartate receptors (NMDA-R), dopamine receptors (DRs), metabotropic glutamate receptors (mGluRs), and BDNF receptors (i.e. TrkB-R), but also the extracellular signal-regulated kinase (ERK) activates mTOR (Ma et al., 2005; Roux et al., 2004). In addition, mTOR is activated by amino acids and energy availability. All these signals are fundamental for the control of cap-dependent protein synthesis.
From previous experiments conducted on rodent hippocampal slices, it emerged that the administration of Rapamycin, an inhibitor of mTOR, abolished the late protein synthesis-dependent phase of long-term potentiation (L-LTP), demonstrating that the mTOR activity is fundamental for the maintenance of L-LTP. However, the early LTP was not affected by the administration of rapamycin (Tang et al., 2022)
Moreover, mTOR blockage damages long-term memory (LTM) formation (Dash et al., 2006), confirming that mTORC is fundamental for LTM formation in mammals.

Specifically, long-term memory, but not short-term memory (STM), is blocked by rapamycin treatment (Bekinschtein et al. 2007; Blundell et al. 2008).

mTOR also promotes protein synthesis activating the phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K/Akt) and the extracellular-regulated kinase (ERK) pathways in LTP and memory (Horwood et al., 2006; Giovannini et al., 2015).

Also, the 4E-BP family is important for synapse formation, plasticity, and memory formation. Indeed, mice knockout for 4EBP2, which is the isoform more expressed in the brain, displayed an increased interaction between eIF4G and eIF4E, which determined an enhanced cap-dependent protein translation rate and increased synaptic plasticity. However, behavioral assays conducted on these KO mice revealed some defects.

These results suggest that there is an optimal level of protein synthesis fundamental for synaptic plasticity and memory formation, and an exaggeration in protein synthesis activity is harmful to cognitive function (Santini et al., 2014).

Translational control mediated by p-eIF2α

In addition to eIF4E the second major target of translational control mechanisms is eIF2α, whose regulation has been implicated in amygdala-based memory (Trinh and Klann, 2013; Jian et al., 2014).

eIF2α is required for the exchange of GDP-bound eIF2 with active GTP-bound eIF2 at the recycling phase. P-eIF2α can block the recycling of ribosomes, inhibiting eIF4B, which is the guanine nucleotide exchange factor (GEF) that catalyzes the conversion of eIF2-GDP in the active eIF2-GTP. The phosphorylation of eIF2α is tightly regulated. Kinases responsible for this phosphorylation, such as kinase-RNA regulated (PKR), heme-regulated initiation factor 2α kinase (HRI), eIF2α kinase 3 (PERK), and general control non-derepressible 2 (GCN2), are all present in the brain. They are activated by different cellular stress: double- stranded RNA activates PKR, low heme levels HRI, endoplasmic reticulum stress, and unfolded proteins PERK and amino acid starvation activate GCN2 (Klann and Dever, 2004) (Figure 5).

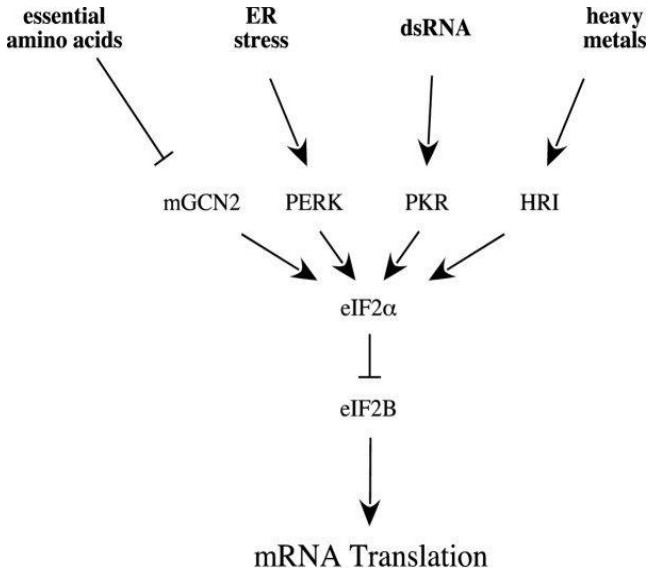


Figure 5. Regulation of eIF2α phosphorylation. Phosphorylation of eIF2α is mediated by four known protein kinases that are regulated by diverse cellular stresses. Phosphorylation of eIF2α inhibits eIF2, blocking the mRNA translation (Kimball and Jefferson, 2004).

Several studies have demonstrated the importance of the phosphorylation of eIF2α in memory formation. In these studies, the use of KO mice of GCN2 KO mice (Costa-Mattioli et al., 2007), PKR KO mice (Trinh et al., 2012), PERK KO mice (Dever et al., 1998), and eIF2α s51 mutant mice have demonstrated that the regulation of phosphorylation of eIF2α is important for synaptic plasticity and for long-term memory.

In fact, stimuli are known to induce a long-lasting change in synaptic strength, such as BDNF pathway activation, repeated synaptic stimulation, or even behavioral training, reduceeIF2α phosphorylation (Costa-Mattioli et al. 2009, Takei et al. 2001).

Moreover, in eIF2α knock-in heterozygous mice, in which eIF2α cannot be phosphorylated because the Ser51 residue is replaced by alanine, L-LTP, and spatial and gustatory LMT are favored. The same situation has been observed also for GCN2 or PKR KO mice, in which the eIF2α phosphorylation is reduced in the hippocampus (Costa-Mattioli et al. 2005, 2007; Stern et al. 2013; Zhu et al. 2011). On the contrary, increasing the p-eIF2α expression, for example through the inhibition of eIF2α phosphatases, L-LTP and LTM are damaged (Costa-Mattioli et al. 2007).

Therefore, these data demonstrate that eIF2α dephosphorylation is necessary for L-LTP and LTM storage.

Treatments with eIF2α phosphorylation inhibitors, such as the integrated stress response inhibitor B (ISRIB), which blocks the translational p-eIF2α effects, or PKRi, a selective PKR inhibitor, increase the spatial and fear-associated LTM (Sidrauski et al. 2013, Zhu et al. 2011). Among the translation effects caused by the eIF2α phosphorylation, there is the activation of two specific mRNAs translation, that encode for BACE1 and ATF4 (De Pietri et al., 2004; Vattem and Wek, 2004). BACE1 is an aspartic-acid protease whose mRNA is present in dendritic spines of hippocampal pyramidal neurons (Smalheiser et al., 2008). Its expression is fundamental for normal memory formation (Laird et al., 2005; Ma et al., 2007). In fact, it is reported that BACE1 expression is aberrantly upregulated in sporadic AD (Holsinger et al., 2002).

Then, ATF4 is locally translated into dendrites and is a repressor of CREB-mediated transcription, which is fundamental for memory formation (Bartsch et al., 1995).

Lastly, the eIF2α phosphorylation determines impairment in memory formation. However, whether the eIF2α phosphorylation decrease occurs by promoting the eIF2α phosphatase activity or by blocking eIF2α kinases remains to be still clarified. As it still remains to be clarified if eIF2α phosphorylation regulates other forms of synaptic plasticity (Buffington et al., 2014).

eIF4F-independent translation

eIF4F-independent translation mechanisms mean all those translation mechanisms that do not involve the eIF4F complex formation but adopt alternative strategies for 43S attack on the mRNA.

Among these mechanisms, there are the internal entry sites for ribosomes, the so-called IRES. These elements are mainly localized at the 5'-UTR on RNA and often adopt complex secondary structures, which serve as an anchor site for the ribosome.

Many viruses depend on IRES sites for viral protein synthesis. These sites, in fact, allow to proceed with the translation by recruiting and manipulating the ribosome using a set of eIFs and mRNA binding proteins (RBPs) without the binding to the cap and without the formation of the eIF4F complex.

IRES initiation translation is also present in cancer cells, particularly in conditions in which cap-dependent translation is inhibited, such as in case of DNA damage (Qin et al., 2004).

Also, the upstream open reading frames (uORF) are an example of alternative protein translation.

Many cancerous cells, but also not, exploit the translation of specific transcripts in an eIF4F-independent manner using the uORF sites (upstream open reading frames), which differ from the classic ORF sites (open reading frames).

Numerous genes coding for growth factors and cellular oncogenes present various uORF sites, which suggests that this is an important pathway in tumorigenesis (Ye et al., 2015).

Specific stresses activate specific pathways that can cause changes in mRNAs. Among them, adenosine methylation in position 6 (m6A) along the 5'-UTR is one of the best known. This is a reversible modification in the adenosine base that under normal conditions can be observed mainly at 3'-UTR and in the coding sequence and only in small numbers to 5'-UTR.However, under certain stress conditions, the number of m6A sites at 5'-UTR is significantly increased compared to a control situation (Domissini et al., 2012; Meyer et al., 2012).

Recently it has been shown that the m6A at 5'-UTR works as an alternative to the 5' cap in stimulating the translation of mRNAs, which presents this modification, promoting a cap-independent translation mechanism (Figure 6).

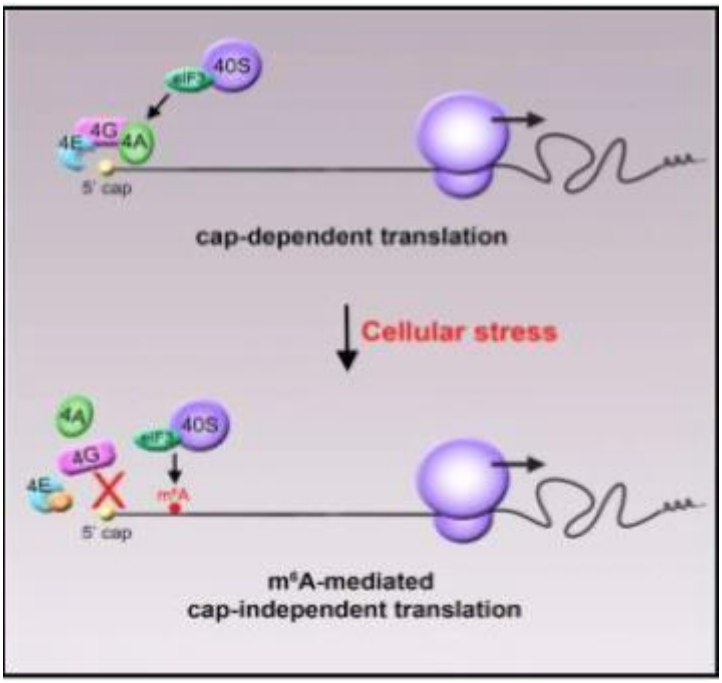


Figure 6. The m6A at 5'-UTR works as an alternative to the 5' cap in stimulating the translation of the specific mRNA. Methylation of adenosine 6 to 5'-UTR can promote an eIF4F-independent translation initiation mechanism, in which the presence exclusively of the factors eIF1, eIF1A, ternary complex, subunit 40S, and eIF3 multiprotein complex, which makes direct contact with the methylated site (Meyer et al., 2015).

Therefore, the initiation translation in the mRNAs with m6A does not require the formation of the complex eIF4F, but only the presence of eIF1, eIF1A, of the ternary complex, eIF3, and the 40S subunit.

Moreover, in this type of translation, the presence of other binding proteins is not required to the ribosome, in stark contrast to what happens with m6A residues in the stop codon or to 3'-UTR (Meyer et al., 2015).

eIF3d specialized translation

Recent studies suggest that the eIF3 starting factor, in addition to its general role of scaffold protein for beginning complex formation can perform other functions in eukaryotic translation.

Among the 13 subunits forming the eIF3 complex only eIF3a, eIF3b, eIF3d, and eIF3g can interact directly with RNA. The eIF3d subunit represents, among these four, the subunit of greatest interest in the field of protein translation of specific mRNAs.

EIF3d is one of the 5 subunits (along with eIF3b, eIF3g, eIF3i, and eIF3j) of the eIF3 complex which is not part of the octameric core but occupies a more peripheral position.

It can be defined as an mRNA cap-binding protein due to its ability to bind directly to the cap (Lee et al., 2016).

From the crystallographic structure, it emerged that the binding site for the cap is in the C-terminal domain of eIF3d. Here it presents, in fact, a central binding tunnel for RNA whose closing or opening depends on an insertion of about 15 aa, evolutionarily preserved, commonly known as the "RNA gate" (Figure 7).

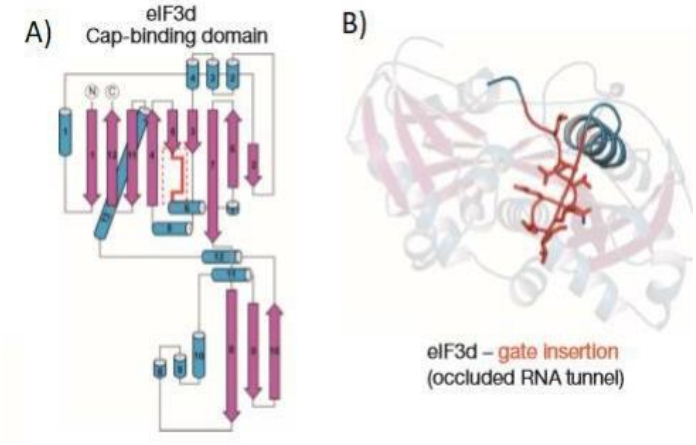


Figure 7. The structure of eIF3d reveals a binding domain to the conserved cap. A) Topological map of the cap-binding domain of eIF3d. B) Structure of the binding domain to the cap together with the RNA gate, which is the binding channel for the mRNAs (Lee et al., 2015).

Only after eIF3 has contacted the mRNA, eIF3d can interact with the cap. It is known that mRNAs with a stem-loop to the 5'UTR require this type of translation, in which eIF3d is necessary for the binding to the cap (Sonenberg et al., 1979; Lindqvist et al., 2008).

EIF3 can act in this case both as an activator and as a translation inhibitor based on the bond type that is formed. During translation activation, eIF3 recognizes the mRNA by binding directly to the stem-loop, while during the inhibition of translation, this binding of eIF3 requires the presence of additional factors or modifications.

In this way, eIF3d guides a cap-dependent translation, which is completely independent of the eIF4F complex formation (Figure 8).

The eIF3d-dependent translation mainly occurs for the translation of cell proliferation mRNAs (Lee et al., 2016).

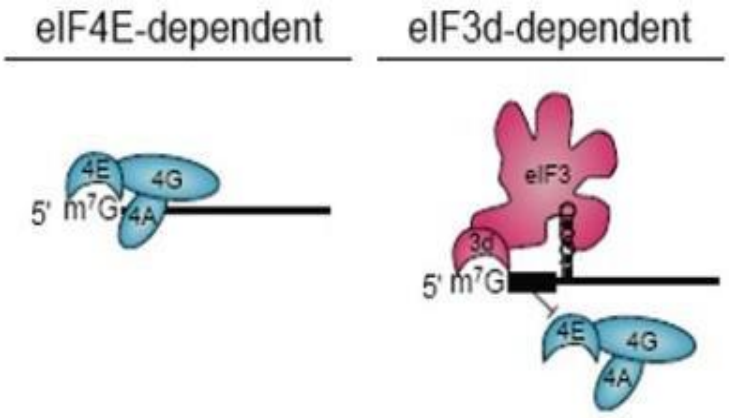


Figure 8. eIF3d promotes an eIF4F-independent translation. eIF4F-dependent translation initiation compared to eIF3d-dependent translation initiation. The presence of the stem-loop ensures that translation takes place via the specialized pathway of eIF3d (Lee et al., 2016).

As mentioned before, the mRNAs that require this type of translation are mainly involved in cell proliferation, but it is also known that the eIF3 complex, probably in the eIF3d subunit, can modulate the translation of specific mRNAs under stress conditions. It is reported that the translation of specific mRNAs with residues m6A to 5'-UTR could be modulated by eIF3d.

In this case, the direct interaction of eIF3d with 5'-UTR m6A residues would stimulate the translation initiation recruiting directly the pre-initiation complex 43S in the 5'-UTR of the mRNA, independently of the eIF4F complex.

To translate mRNAs, it is necessary that eIF3d be recruited on the ribosome (Meyer et al., 2015).

Receptor for Activated C Kinase 1 (RACK1)

Another eIF4F-independent translation mechanism is mediated by the Receptor for Activated C-Kinase (RACK1).

RACK1 is the leading member of the family of intracellular binding receptors for PKCβII (Activated Protein Kinase C). In fact, it has been isolated for the first time as the scaffold protein for PKCβII, whose active conformation is stabilized by RACK1, such as its transport at certain substrates (Ron et al., 1994).

RACK1 is encoded by the GNB2LI gene, which is highly conserved and expressed in all tissues, particularly in nerve tissue (Chou et al., 1999). It has 7 WD40 domains, which are sequences of about 40 aa ending mostly with the dipeptide Trp-Asp [W-D], which allows interaction simultaneously with different molecules, integrating the signaling of numerous pathways (Adam et al., 2011) (Figure 9).

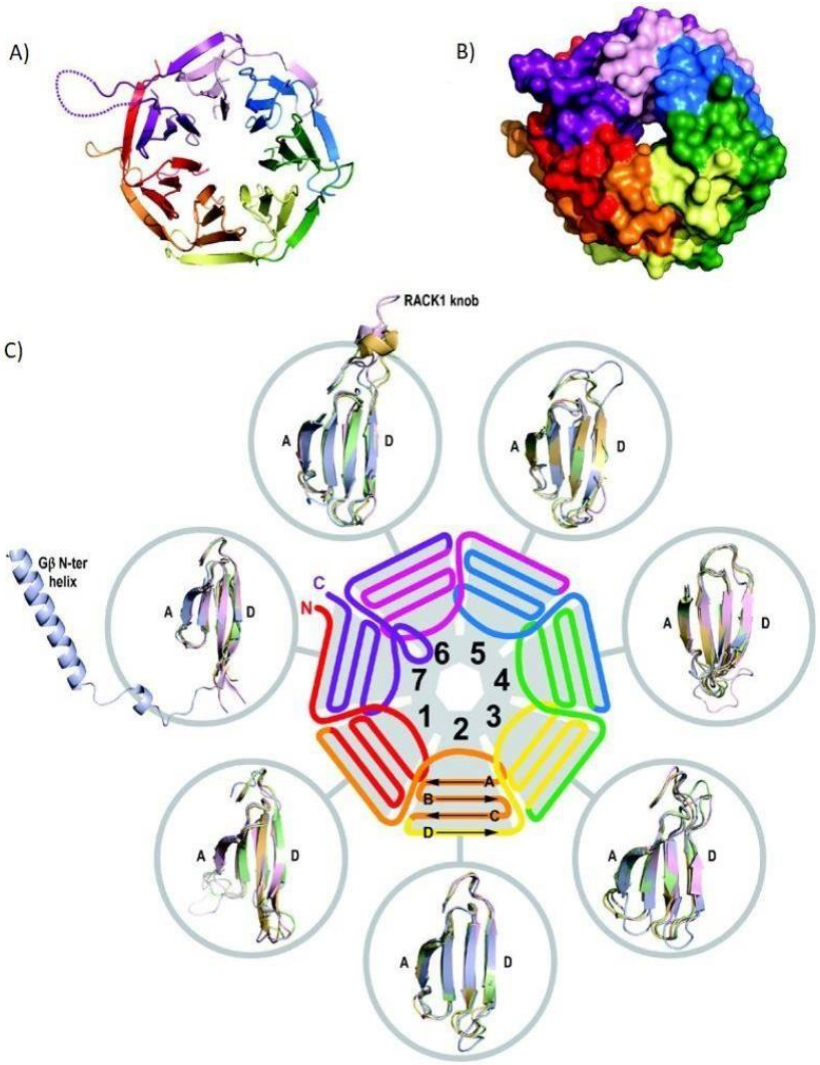


Figure 9. RACK1 structure. Both in figure A) and in figure B) the RACK1 crystallographic structure is reported, allowing to see the 7 WD40 domains. C) Schematic representation of RACK1 structure and organization of WD40 domains (Adam et al., 2011).

There are numerous cellular functions performed by RACK1. Cell growth and tumorigenesis, but also apoptosis, and cell migration are some of the processes regulated by RACK1, as shown in detail in Figure 10.

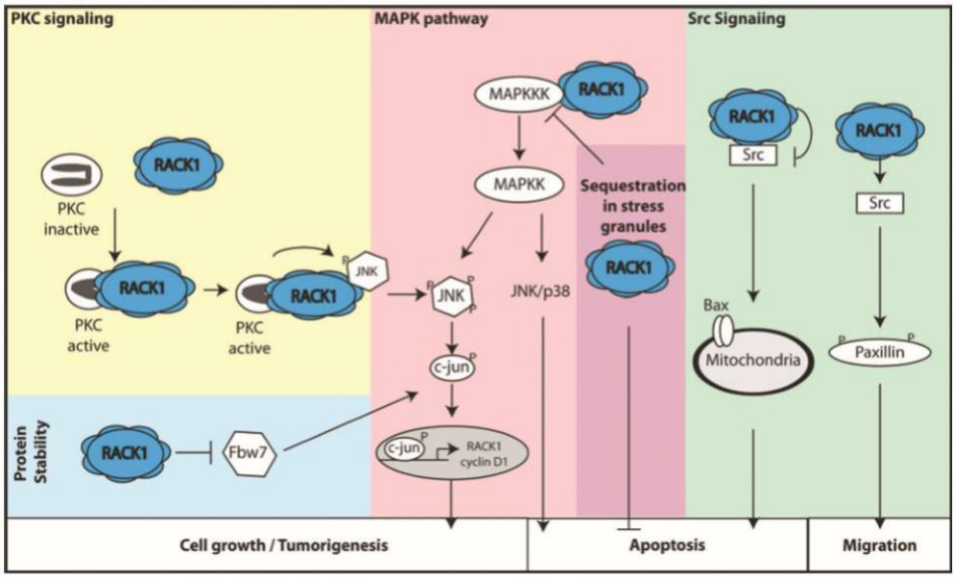


Figure 10. RACK1 modulates a series of cellular processes. RACK1 is involved in a series of cellular processes including cell growth and tumorigenesis, apoptosis, and cell migration.
Phosphorylation of JNK by PKCβII leads to partial activation of kinase, which then determines the expression of cyclin D1 by activation of c-Jun. RACK1 also stabilizes c-Jun, inhibiting proteasome degradation. Apoptosis is promoted by RACK1 by activation of P38 and JNK following binding with MTK1 kinase. However, the recruitment of RACK1 on stress granules, reduces its binding capacity with MTK1and this prevents apoptotic response. Bax oligomerization, moreover, induced by the inactivation of Src by RACK1, activates cell death signals. Finally, RACK1 modulates most of the characteristic phases of the cell migration process also through the regulation of the phosphorylation of paxillin (Gandin et al., 2013).

Ribosomal RACK1

RACK1 was also identified as a component of the smaller subunit of ribosomes, present in a 1:1 ratio with other ribosomal proteins (Ceci et al., 2003; Sengupta et al., 2004).

As seen before for eIF3d, also RACK1 is located on the 40S ribosomal subunit. It is located behind the head region of the 40S subunit, near the mRNA exit site and the mRNA entry site.

The mRNA entry site is surrounded by uS3, uS4 e eS30. It is known that the RACK1 interacts with the uS3 protein (Nielsen et al., 2017) (Figure 11).

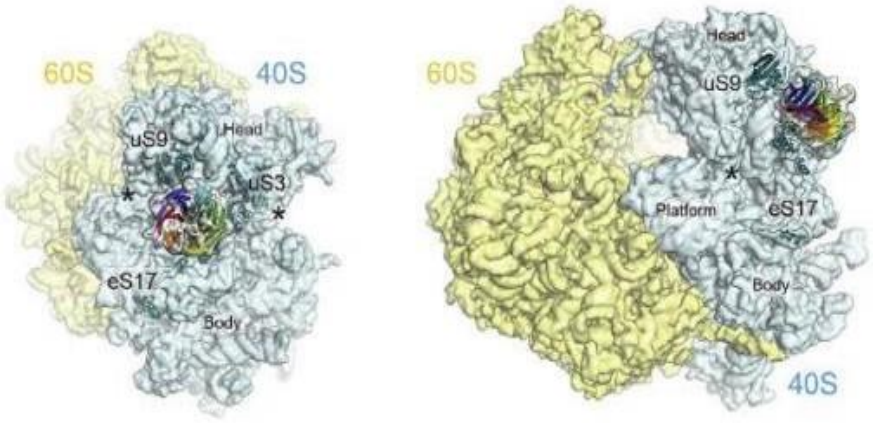


Figure 11. RACK1 is located on the small ribosomal subunit near the back of the head. The asterisk marks the mRNA entry and exit tunnel on the ribosome. Both of these points are close to RACK1, and this could then allow the ligands of this protein to regulate mRNA passage through the ribosome.

In ribosomes, RACK1 promotes the 80S formation and modulates the subsequent stages of translation. In fact, RACK1 recruits the kinase PKCβII on the ribosome, which phosphorylates eukaryotic starting factor 6 (eIF6) on serine 235 (Sharma et al., 2013). eIF6 binds the 60S subunit, preventing its association with the 40S subunit. Only after its phosphorylation, eIF6 is released from the 60S and the 80S ribosome can be assembled onthe mRNA. RACK1 carries the PKCβII on the ribosome and then, PKCβII can phosphorylate eIF6 (Ceci et al., 2003).

On ribosomes, RACK1 interacts also with eIF3 and probably through this interaction, can anchor the ternary complex to the subunit 40S. It is known, in fact, that the loss of RACK1 prevents the recruitment of the ternary complex, suggesting an involvement in the translation initiation (Kouba et al., 2012).

In addition, while eIF3 is released to allow assembly of the 80S, RACK1 remains associated with polyribosomes.

Among the various cellular processes in which it is involved, RACK1 modulates also global protein synthesis. In fact, it is known that in neuroblastoma cells the upregulation in the expression of RACK1 determines an increase in protein translation through the activation of the kinase PKCβII, independently of the Akt/mTOR pathway.

In fact, both the upregulation and downregulation of RACK1 do not cause changes in the Akt and mTOR phosphorylation. This type of regulation occurs independently from the eIF4E phosphorylation (Romano et al., 2019).

RACK1 in neuronal development

RACK1 is fundamental for neuronal development since mice lacking RACK1 are embryonic lethal. In addition, RACK1 interacts with PTK7 to regulate neural tube closure (Wehner et al., 2011). Moreover, it is known that in primary neuronal cultures, RACK1 regulates axon growth, modulating the formation of neuronal-specific adhesions, which are point contacts (Kershner and Welshhans, 2017). RACK1 recruits at the ribonucleoprotein particles (RNPs) also numerous RNA-binding proteins and a series of kinases, including PKCβII (Ceci et al., 2003), Src (Ceci et al., 2012), and JNK (Gandin et al., 2013).

The interaction of RACK1 with ZBP1 leads to the translation of β-actin mRNA, which is essential for neurite outgrowth (Krichevsky and Kosik, 2001). During the formation of the growth cone in neurons, ZBP1 binds the β-actin mRNA, repressing it translationally and transporting it near the growth cone (Zhang et al., 2001; Huttelmaier et al., 2005; Sasaki et al., 2010).

RACK1 anchors the complex formed by the β-actin mRNA and ZBP1 on the ribosome and binds it at the RACK1 site Y246F. The binding of RACK1 with this complex is crucial for the β-actin mRNA release and translation. In fact, RACK1 and ZBP1 repress the mRNA translation on the RNPs during the transport to specific sites. Once reached the target sites, activated Src phosphorylates ZBP1, which releases the β-actin encoding mRNA, allowing the translation in loco (Sasaki et al., 2010).

Therefore, RACK1 may participate in the local translation of specific mRNAs through the binding to other RBPs to promote neuronal development. Another RBP involved in dendritic and axonal development is the Fragile X mental retardation protein (FMRP). FMRP modulates the mRNA transport and local protein synthesis of specific mRNAs, which are crucial for neuronal development and synaptic plasticity in dendrites (Romano et al., 2022). Among these, PSD-95 is important in neuronal development. It is known that the FMRP loss in neurons causes first the formation of immature dendritic spines and then an up-regulation of the PSD-95 mRNA translation (Muddashetty et al., 2014). High expression of PSD-95

and an increase in the number of dendritic spines are reported to be features of neurodevelopment disorders such as autism and Fragile X syndrome (Romano et al., 2022). As already known, the FMRP phosphorylation represses the translation of PSD-95 mRNA. The binding of FMRP to RACK1 removes the translational repressive activity of FMRP and promotes the translation of PSD-95 mRNA. In fact, RACK1 favors the FMRP dephosphorylation and, therefore, the PSD-95 translation is increased, determining impairments in axons and dendrites.

Moreover, when RACK1 mutant with a low binding affinity for the ribosome is expressed in neurons, a decrease in dendritic arborization and an increase in spine density are observed. This suggests that ribosomal RACK1 is involved in neuronal differentiation (Coyle et al., 2009). The expression of this RACK1 mutant recovers the impairments in the dendritic spine and neurite determined by the loss of FMRP. Therefore, RACK1 may recruit FMRP on ribosomes, promoting PSD-95 mRNA translation.

Furthermore, RACK1 mislocalization has been observed in Amyotrophic Lateral Sclerosis (ALS) patients (Russo et al., 2017). RACK1 recruits the TDP-43 protein on the translational machinery. TDP-43 is an RNA-binding protein involved in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTLD) pathogenesis. It is also known that RACK1 recruits TDP-43 in stress granules, promoting the specific mRNA translation under stress conditions.

In addition, increased interaction between huntingtin protein and RACK1 is reported in a mouse model of Huntington's disease (Culver et al., 2012).

Drosophila melanogaster

Recently, *Drosophila melanogaster* is emerging as a good model for studying synaptic plasticity, memory, and neurodegenerative diseases, but also for the study of cancer progression (Mundorf & Uhlirova, 2016).

Drosophila melanogaster is a simple, rapid, and less expensive system. in fact, the reproductive life cycle of *Drosophila* is about 10–12 days at 25 °C. A laid egg takes approximately 24 hours to hatch as a first instar larva. In about 24 more hours, the larva reaches the second larval stage. After another day, the larva reaches the third larval stage. After about 2 days at this stage, the larva comes out of the food substrate and develops intoan immobile pupa. During the next 3–4 days, the pupa undergoes a dramatic physical transformation into the adult form when it emerges from the pupal case (eclosion). During this period, many adult structures develop from 19 imaginal discs present at the larval stage. Imaginal discs are a set of tissue-specific progenitor cells that develop during the embryonic and larval stages and subsequently, give rise to most adult structures (such as eyes, legs, and wings) during the pupa phase (Hales et al., 2015) (Figure 12).

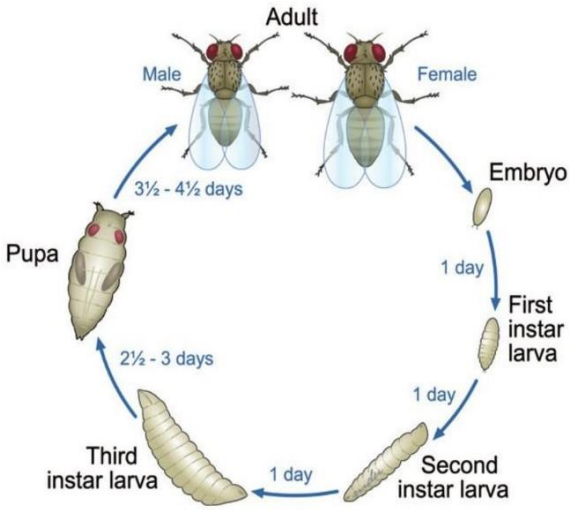


Figure 12. *Drosophila melanogaster* life cycle includes different stages: embryo, first instar larva, second instar larva, third instar larva, pupal stage, and then, after the eclosion, the adult flies (Ong et al., 2014).

The adult female is mature to mate about 12 hours after eclosion. Importantly, a single female fly can lay hundreds of eggs within a few days (Nichols et al., 2006). The short generation time and the large number of progenies are just some of the advantages which *Drosophila melanogaster* offers (Nichols et al., 2006).

Drosophila melanogaster has four pairs of chromosomes, and the introduction of balancing chromosomes, which prevent gene recombination, allows the maintenance of stocks for a long time without a recurring selection (Deng & Lambris, 2019).

Despite the different sizes of their genomes, over 60% of human genes have orthologs in *Drosophila melanogaster* (Bernards & Hariharan, 2001). Although the overall nucleotide identity of orthologs is around 40%, and similarities are between 40% and 50% for orthologous proteins, specific regions that include catalytic domains and other features can exhibit greater than 80% similarities. Therefore, molecular pathways which involve these orthologs are greatly conserved (Nichols, 2006).

To study neurodegenerative diseases, and those connected to impairment in learning and memory, the *Drosophila melanogaster* Mushroom Bodies have been used as a model.

The Mushroom Bodies (MBs)

The Mushroom Bodies (MBs) are the nervous structure deputed to olfactory reception, learning, and memory in *Drosophila melanogaster* (Ito et al., 1998). They are in the central nervous system lobes.

MB is generated by four equipotent Mushroom Bodies neuroblasts (MBNBs), which are not quiescent during the first and second waves of neurogenesis. Therefore, they directly generate the final structures of the Mushroom Bodies (Yasugi & Nishimura, 2016).

MBs consist mainly of about 2500 Kenyon cells (KCs), whose dendrites form the calyx structure in each hemisphere while their long and thin axons fasciculate together and project to the anterior brain face (Guven-Ozkan and Davis, 2014). KCs axons form the peduncle and lobes.

Once reached the anterior face, they divide into vertical (α, α′) and horizontal (β, β, and γ) lobes, forming the typical lobular anatomical MBs structure (Guven-Ozkan and Davis, 2014) (Figure 13).

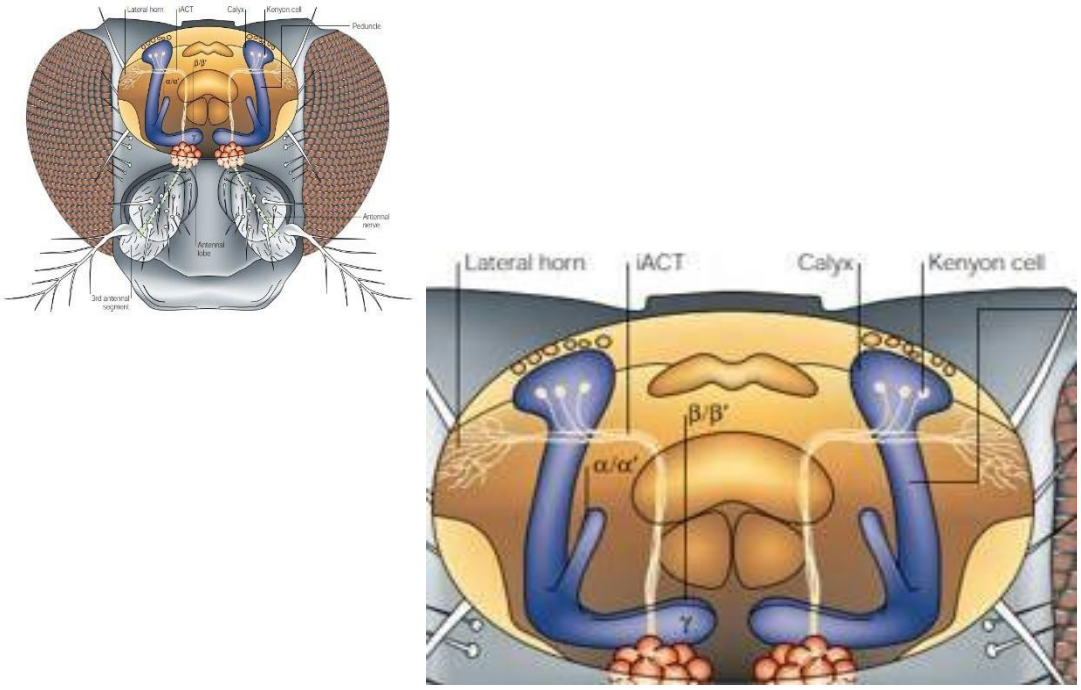


Figure 13. Schematic representation of the MBs position inside the Central Brain (CB) in the two CNS lobes. In B the calix, which is composed of the Kenyon cells dendrites, and the peduncle, which is formed by the Kenyon cells axons, can be seen (Mochizuki et al., 2011).

The axons of the α'/β' and α/β MBs neurons divide into two separate branches, while the γ axons remain unbranched (Lee et al., 1999). These three different types of neurons form at different development stages; in fact, from the embryo stage to the middle of the third larval stage, MBNBs generate γ neurons, between the third larval stage and the pupa formation generate the α'/β' and after pupal stage, generate the α/β neurons (Yasugi & Nishimura, 2016). To regulate these transitions of MBs neurons are both extrinsic and intrinsic cellular factors. Chinmo, i.g., is a zinc-finger protein highly expressed in MB neurons during the early stages and its expression gradually decreases during development (Kuo et al., 2006). Still, the zinc-finger protein Abrupt (Ab) is highly expressed during all the larval stages (Kucherenko et al., 2012).

Also, the steroid hormone ecdysone acts as an extrinsic factor for the transition from α'/β' neurons to neurons α/β. Ecdysone induces the expression of microRNA let-7, necessary for this final transition, which acts through negative regulation of the expression of Chinmo and Ab (Yasugi & Nishimura, 2016).

The peculiarity of MB is that γ neurons undergo a pruning axonal and dendritic process like that observed for sensory neurons C4da. In fact, about 18 hours after the puparium formation (APF), neurons complete dendritic and axonal pruning (Yu & Schuldiner, 2014).

Mushroom Bodies in the olfactory associative learning and memory circuits

As mentioned before, the Mushroom Bodies (MB) play a critical role in olfactory memory formation, such as in some types of visual and courtship memory (McBride et al., 1999; Vogt et al., 2014).

The MBs are part of the olfactory pathway, as the tertiary structure, like the mammalian amygdala or piriform cortex (Su et al., 2009). To confirm this, most of the proteins required for olfactory learning and memory are mainly expressed in the MBs (e.g., Dnc, Rut, DC0) (Skoulakis et al., 1993).

Olfactory receptor neurons (ORNs) receive first the olfactory stimuli. Then, they transmit the information to projection neurons (PNs), and, finally, to the MBs and the lateral horn (Davis, 2005; Fiala, 2007). The KCs, which represent the intrinsic MBs neurons, transmit the information to the body output neurons (MBONs) (Aso et al., 2014a; Tanaka et al., 2008) through cholinergic synapses (Barnstedt et al., 2016). The MBs are innervated by modulatory neurons, such as the dopaminergic neurons (DANs), which play a critical role in learning and memory (Tanaka et al., 2008). Moreover, the MBs lobes are divided into zones and each zone receives dopaminergic stimuli from specific dopaminergic neurons (Aso et al.,2014a; Aso et al., 2014b; Tanaka et al., 2008) (Figure 14).

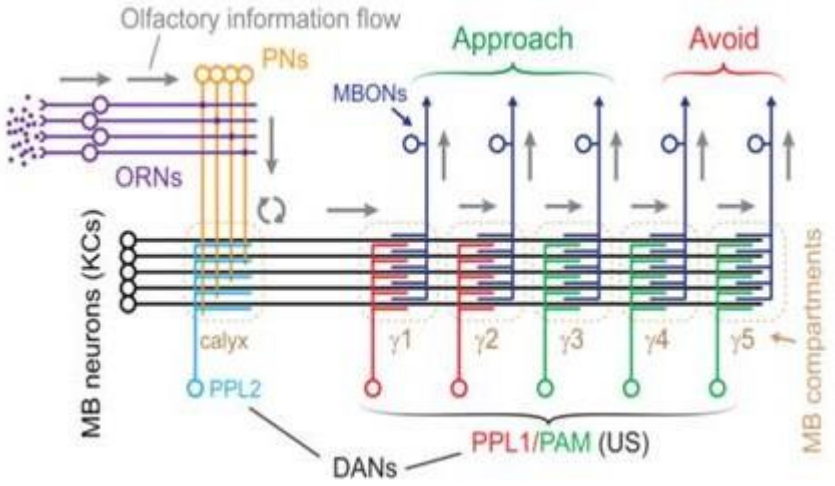


Figure 14. Schematic representation of olfactory pathway through the mushroom body (MB) and mushroom body output neurons (MBONs). Gray arrows indicate the inward information flow from peripheral olfactory sensory receptors. ORNs are the olfactory receptor neurons, PNs are the projection neurons, KCs are the Kenyon Cells, MBON are the MBs output neuron and DAN are the dopaminergic neuron(s) divided into PPL1/2, which are the paired posterior lateral (dopaminergic) neurons, and PAM, which are the protocerebral anterior medial (dopaminergic) neurons.

It is important to note that the information flow is not only unidirectional but there is also a bidirectional communication, conferring complexity to the behavioral pathway (Christiansen et al., 2011; Pauls et al., 2010; Rolls et al., 2007).

The MBs integrate information about olfactory stimuli, which is transmitted by modulatory neurons (Burke et al., 2012; Claridge-Chang et al., 2009; Gervasi et al., 2010; Schroll et al., 2006; Tomchik and Davis, 2009; Ueno et al., 2017).

As confirmation of this, flies with abnormal MBs morphology show memory defects, such as flies in which the MBs are chemically ablated (De Belle and Heisenberg, 1994).

During conditioning, a specific subset of KCs responds to odors with depolarization, which determines an increase in the cAMP and PKA expression in KCs (Gervasi et al., 2010; Tomchik and Davis, 2009). Downstream effectors generate plasticity through local excitability, compartmentalized signaling, and modulation of synaptic vesicle and release, such as synapsin protein.

Therefore, MBs are critical for olfactory-induced learning. About synaptic plasticity, also antennal lobe local neurons (Scheunemann et al., 2012), KCs (Louis et al., 2018; Zhang and Roman, 2013), dorsal paired medial (DPM) neurons (Cervantes-Sandoval and Davis, 2012; Yu et al., 2005), GABAergic anterior paired lateral (APL) neurons (Liu and Davis, 2009), dorsal anterior lateral (DAL) neurons (Chen et al., 2012), and MB output neurons (MBONs) play an important role in this context, showing changes following conditioning.

At the circuit level, DANs neurons can be divided, based on their role in memory formation, into proto cerebral posterior lateral1 (PPL1) neurons and proto cerebral anterior medial (PAM) DANs neurons. PPL1 neurons, which innervate the MB vertical lobes, are deeply involved in aversive olfactory learning (Galili et al., 2014). PAM DANs, instead, innervate the MB horizontal lobes and are important for US processing during appetitive olfactory classical conditioning.

PPL1 and PAM neurons can be further distinguished into different classes, based on their function. The γ1-pedc neurons belong to the PPL1 neurons are necessary for aversivelearning in olfactory classical conditioning (Aso et al., 2012). Other PPL1 neurons innervate the MB vertical dorsal lobes region, which is particularly important for taste learning (Maseket al., 2015).

Again, the PAM-β2β′2a neurons modulate aversive memory (Aso et al., 2012) and PAM DANs neurons innervate the γ3 compartment of the MB function, activating aversive memory and block driving appetitive memory (Yamagata et al., 2016).

In addition, PPL2ab DANs innervate the dendritic MB region in the calyx, playing a fundamental role in memory and plasticity (Kuo et al., 2015; Mao and Davis, 2009). Large responses are observed in the γ2/γ3 (proximal) compartments than in the γ4/γ5 (distal) compartments (Figure 15).

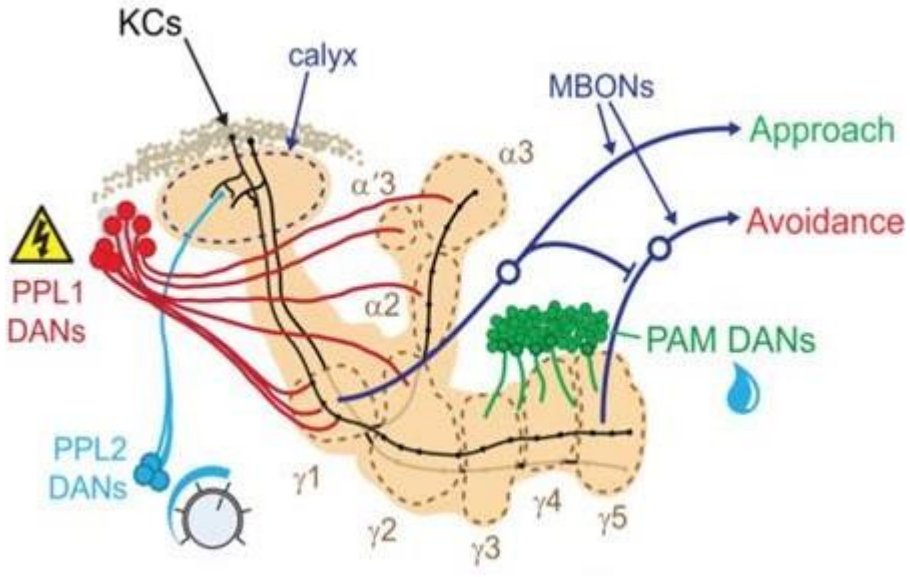


Figure 15. Image of Mushroom Bodies, in which the major anatomical subdivisions and classes of neurons relevant to learning are shown. Anatomical compartments γ1-γ5, α2, α3, α′3 are outlined with dashed lines (Boto et al., 2020).

Therefore, the olfactory circuit predicts that antennal lobe PNs form bidirectional connections with the KC dendrites in the calyx. Then, DANs neurons activated to release dopamine, which acts on MBs and enhances the cAMP concentration (Boto et al., 2014). Through the release of dopamine, DANs regulate learning and memory.

To olfactory learning, memory formation follows. In *Drosophila melanogaster*, 4 different phases in memory formation can be observed. Short-term memory (STM), which decays in less than an hour, middle-term memory (MTM), from one to three hours, and two different forms of long-term memory. These are distinguished based on different training procedures. One is independent of protein synthesis, and it is called the anesthesia-resistant memory (ARM) and the long-term memory (LTM), observed after training that has been repeatedly interrupted by rest periods (Tully et al., 1994).

Also, in this case, each neuron plays a different role. Experiments conducted to investigate the long-term water-reward memory in thirsty flies, for example, revealed that the output from α'/β' neurons is only required for memory consolidation, while the output from γ and α/β neurons is only required for memory recovery. This suggests that long-lasting water memory is first elaborated in α'/β' neurons and eventually formed in α/β and γ neurons

through the stabilization of α'/β' neurons activity during memory consolidation (Shyu et al., 2016).

Mushroom Bodies in locomotor behavior control

Among the functions in which MBs are involved, there is also the control of locomotor behavior and reactivity. In this system, locomotor activity and locomotor reactivity are regulated by different mechanisms (Sun et al., 2018).

A sudden external stimulus, known as a startle, usually determines the inhibition or the arrest of spontaneous locomotion together with an appropriate response.

Startle-induced reactivity has long been investigated using various behavioral assays, such as phototaxis (Benzer, 1967) and negative geotaxis (Miquel et al., 1972). In this context, the innate reflex called startle-induced negative geotaxis (SING) is generally investigated. It is based on the fast climbing of flies entrapped in vials induced by a mechanical shock. SING performance progressively declines with age (White et al., 2010; Jones and Grotewiel, 2011; Vaccaro et al., 2017), unlike spontaneous locomotion, which is not influenced by the age of the flies (White et al., 2010). Alterations in SING reflex are present also under neuropathological conditions, such as in Parkinson's disease *Drosophila* model (Feany and Bender, 2000; Coulom and Birman, 2004; Chaudhuri et al., 2007; Riemensperger et al., 2013; Bou Dib et al., 2014).

It has been well-established that the MBs inhibit locomotion, suppressing locomotor behavior. In this context, the dopaminergic neurons (DANs) prominently control the locomotor activity in *Drosophila*. In fact, it has been reported that DANs degeneration causes a rapid decline in the SING performances in aging flies (Riemensperger et al., 2013; Vaccaro et al., 2017).

It is also known that PPL1 DANs in the PPL1 act as inhibitory neurons in the SING-modulating circuits, while the PAM DANs play both an inhibitory and excitatory role.

Moreover, experiments conducted using GAL4 driver strains specific for certain MBs regions have revealed that the SING reflex is controlled mainly by the activity of the α'β' KCs (Riemensperger et al., 2013), but also γ KCs are implicated in SING control. In particular, the SING modulation performed by the α'β' and γ neurons is hampered by the αβ KCs co-activation.

Lastly, also the MBONs M4/M6 and V2 are part of this circuit. In fact, the activation of cholinergic MBON-V2 and glutamatergic MBON-M4/M6 neurons determines a decrease in the SING performance of the flies (Aso et al., 2014b).

eIF3d in *Drosophila melanogaster*

In *Drosophila melanogaster*, little is known about the functions performed by eIF3d. It seems that eIF3d plays an important role in embryogenesis. In fact, eIF3d modulates the translation of *Nanos*, an mRNA that encodes for a fundamental posterior determinant required for abdominal segmentation and development of the germline. In particular, the Aubergine protein, which is one of the PIWI proteins, which are proteins essential in germ cells to repress transposons and regulate mRNAs, physically interacts with eIF3d. This interaction allows the *Nanos* translation (Ramat et al., 2020).

Moreover, eIF3d is also involved in *Drosophila melanogaster* dosage compensation, which is the fundamental mechanism that allows equalizing the expression of X-linked genes between males (XY) and females (XX), which depends on translation regulation (Graindorge et al., 2011).

In this context, eIF3d modulates the translation of *msl-2* mRNA, which is retained in the nucleus and translational inhibited by the female-specific RNA binding protein Sex-lethal (SXL), which inhibits the MSL2 expression. EIF3d binds to *msl-2 5′* UTR, is required for *msl-2* translation, and is necessary for SXL-mediated translational repression. EIF3d depletion, indeed, determines a consistent *msl-2* de-repression in female flies (Szostak et al., 2018).

Lastly, the eIF3 complex, especially in the figure of eIF3d, together with the eIF4A factor, modulates the translation of mRNAs in dendritic pruning, which occurs in the metamorphosis from larvae to the pupal stage. Dendritic pruning is the controlled degeneration of synapses and neurites without the loss of the parent neuron. It is a fundamental process in the correct neuronal development. During the dendritic pruning, canonical eIF4F-dependent translation is blocked, since the 4EBP1 binds to eIF4E, preventing the formation of the eIF4F complex. Considering its cap-binding ability, eIF3d binds to the mRNA and allows the translation of specific mRNAs fundamental in pruning (Rode et al., 2018) (Figure 16).

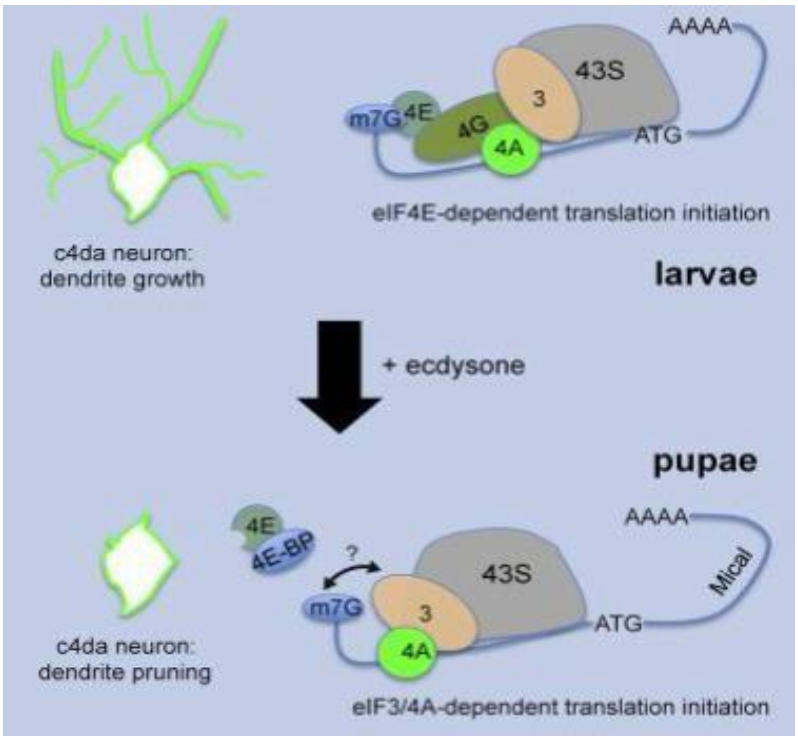


Figure 16. C4da neurons perform the dendritic pruning during the metamorphosis between the third larval stage to the pupal stage. In this context, the translation of mRNAs necessary for dendritic pruning, such as Mical, required an eIF4F-independent translation, which is mediated by eIF3 complex and eIF4A (Rode et al., 2018).

RACK1 in *Drosophila melanogaster*

The role of RACK1 has been mainly studied in cell cultures, but in model organisms, its many activities have not yet been fully described.

Both the human and *Drosophila melanogaster* genomes encode for a single RACK1 gene. The alignment of the RACK1 protein reveals high conservation between humans and *Drosophila*. Therefore, RACK1 shows in both cases 7 WD domains with 77% of identity and 87% of similarity.

In *Drosophila melanogaster*, RACK1 plays an important role in embryogenesis. In fact, RACK1 is expressed in both the germ tissue and somatic tissue of each egg chamber, which it has a cytoplasmic localization. Then, RACK1 transheterozigous mutants show

larval lethality in 75% of cases, and, when larvae reach the pupariation stage, about 85% die before adult eclosion. Moreover, *Drosophila* lacking RACK1 are sterile, suggesting an important role of this protein in the production of gametes.

From previous studies, it seems that RACK1 in *Drosophila* performs most of the functions described in mammalian cell cultures, such as apoptosis, cell survival, cell migration, and protein synthesis (Kadrmas et al., 2007a).

RACK1 was also identified as an interacting partner of many proteins, including kinases, phosphatases, and adhesion molecules. This suggests that RACK1 acts as a scaffold protein also in *Drosophila melanogaster* (Gibson, 2012; Long et al., 2014; Li and Xie, 2015).

RACK1 plays also a role in muscle homeostasis. In fact, RACK1 has been identified as a novel substrate of p38 MAPK in aging muscle. Moreover, the genetic interaction between RACK1 and p38 has been demonstrated to control muscle aggregate formation, locomotor function, and longevity. Studies conducted on aging and stressed muscles revealed that in these situations, p38 MAPK causes a redistribution of RACK1 between a ribosome-bound pool and a putative translational repressor complex.

In addition, ribosomal RACK1, as a component of the 40S ribosomal subunit, is also required for the IRES translation, which are elements present in the genome of *Drosophila* viruses, but also of humans (viruses of hepatitis C) (Kuhn et al., 2017). In addition, RACK1 is required for non-canonical protein synthesis (Majzoub et al., 2014).

Outline of the thesis

Canonical protein synthesis is fundamental for supporting memory-related synapse remodeling immediately after learning (Gindina et al., 2021). In fact, both the learning process and memory require not only new protein translation but also a fine balance between the mechanisms of translation regulation.

Factors involved in translation initiation control are the best characterized in synaptic plasticity and memory formation. Among these, it is known from the literature that the eIF4E factor and p-eIF2α play a fundamental role in modulating protein synthesis in memory formation. Also, mTOR and 4EBP1, which are the eIF4E activity main regulators, are involved in learning and memory formation, and therefore, impairments in the expressions of all these factors cause defects (Buffington et al., 2014).

While the role of canonical protein translation is well known, little is known about the role of eIF4F-independent translation in synaptic plasticity and memory formation.

In recent years, the cap-dependent eIF3d-specialized translation is emerging in the context of eIF4F-independent translation.

eIF3d is located on the 40S ribosomal subunit near to RACK1, as shown in the figure 17.

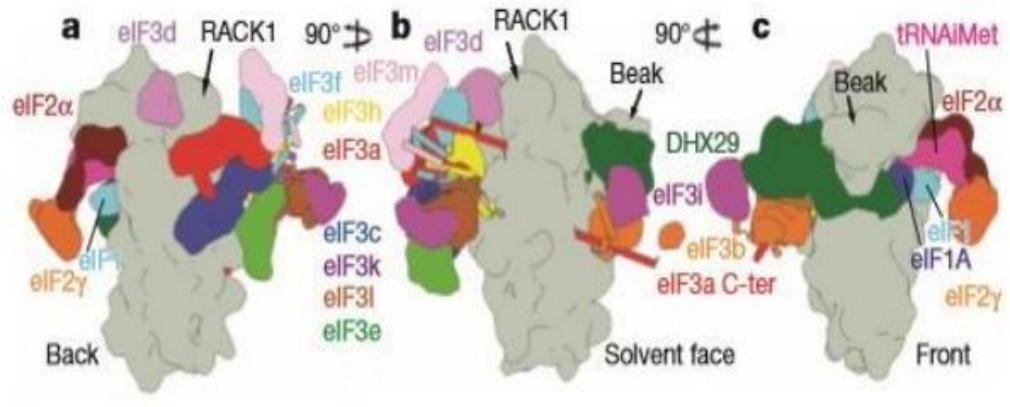


Figure 17. RACK1 and eIF3d are located near to each other on the 40S ribosomal subunit. The image shows 43S pre-start complex with the required start factors for 43S formation seen from behind (a), from the side exposed to solvent (b) and from the front (c). As is evident RACK1, ingrey, encounters some eIF3 subunits, including eIF3a, in red, eIF3c, in blue, eIF3e, in green, and eIF3d, in lilac (Des Georges et al., 2015).

Moreover, it is also known from the literature that both RACK1 and eIF3d are involved in the eIF4F-independent cell proliferation mRNAs translation.

Considering all these data, it has been hypothesized that there might be an interaction between RACK1 and eIF3d.

Therefore, the aim of this PhD thesis was to investigate the role of the eIF3d-RACK1 specialized translation in synaptic plasticity and memory formation.

For this purpose, *Drosophila melanogaster* has been used as a model system, due to the ease with which genetic manipulation can be carried out. Experiments were conducted mainly on the Mushroom Bodies, which are the *Drosophila nervous* structures deputed to learning and memory, comparable for their function to the human hippocampus.

Materials and methods

D. Melanogaster husbandry and lines

Flies were raised at low density, at 25°C, on a standard food medium containing 9 g/l agar (ZN5 B&V, Italy), 75 g/l corn flour, 60 g/l white sugar, 30 g/l brewer yeast (Acros Organic), 50 g/l fresh yeast, and 50 ml/l molasses (Biosigma), along with nipagin (Sigma- Aldrich).
These ingredients were mixed and dissolved by adding hot water in a final volume of 1.5l, the hydration source of *Drosophila.*
The mix was autoclaved and cooled down slowly. When the temperature reached about 50°C, Nipagina, a broad-spectrum antifungal (3g dissolved in 16 ml of absolute ethanol) was added, and finally, the gruel was separated into various plastic containers.
Stocks used were partly obtained from Dr. Paola Bellosta of the University of Trento and partly purchased by the Bloomington Drosophila Stock Center (BDSC, Indiana University Bloomington, IN, USA).
The driver stocks are: Elav-Gal4/Fm7, UAS-GFP/Cyo, Ok107-Gal4, UAS-GFP/cyo;mkrs/tm6b and Uas-5'Utr Mical::GFP/CyO weeP; ppk-Gal4, Uas-Td::tomato/TM6b. The UAS stocks are UAS-RACK1-HA, UAS-eIF3d RNAi, UAS-eIF3d WT-HA, and UAS-eIF4E RNAi. The W1118 strain has been used as a control.

UAS-GAL4 system

One of the most widely used genetic tools for gene expression manipulation is the UAS-GAL4 system. It is a bipartite system that uses the yeast transcriptional factor GAL4, known to regulate the galactose metabolism in yeast, and the upstream activation sequence UAS. In fact, a transgenic driver line expressing GAL4 upstream of a specific-tissue promoter is crossed with a second transgenic line carrying an UAS-dependent transgene (Figure 18).

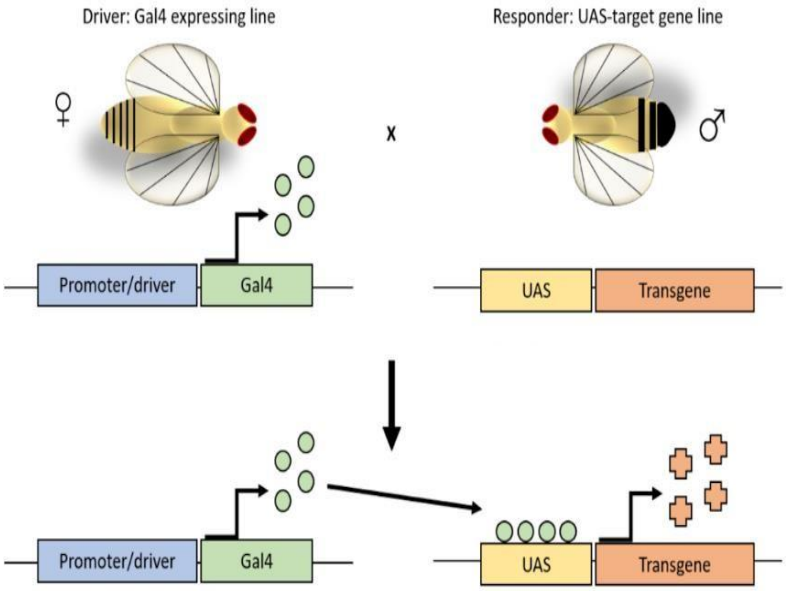


Figure 18. The UAS-GAL4 system. GAL4 driver and UAS-target gene fly lines are generated and maintained as separate stocks. In the absence of GAL4, there is no expression of the target gene. The cross with these two transgenic lines results in a progeny which expresses the gene of interest in a specific tissue (Caygill and Brand., 2016).

Therefore, once the Gal4 has been expressed, this binds the UAS sequence (Upstream Activating Sequences) that allows the expression of a transgenic element downstream.

The Gal4/UAS system allows the expression of a transgene of interest, in a specific region of the animal and with the same pattern of expression throughout the tissue, if the Gal4 transcription factor is downstream of a specific tissue promoter (Brand and Perrimon, 1993).

Learning test

Flies were placed inside tubes containing agarized gruel + 30% molasses, which was the positive stimulus. They were subjected three times to training tests. 37°C thermic shock represented the negative stimulus.

In this assay, the ability of flies to associate a positive stimulus, which is represented by the molasses-containing gruel (C+), with a negative stimulus, represented by 37°C heat shock (U-), has been investigated. They were subjected to three training cycles with the positive stimulus, each for two minutes. Then, a single training cycle consists of 30 seconds of thermic shock at 37°C (negative stimulus) followed by 20 seconds of resting. All these steps were repeated three times.

Finally, the flies' associative capability was tested by placing flies in a box containing molasses-containing gruel at 30%.

For each sample, the performance index (P.I) has been defined. P.I. is calculated using the following formula: P.I. = (CS+) – (US-)/ tot. of flies, in which the number of animals responding negatively to the stimulus is subtracted from the number of animals responding positively to the stimulus, divided by the total number of animals.

Climbing assay

Geotaxis was assessed using a climbing assay (negative geotaxis reflex in opposition to the Earth's gravity). *D. melanogaster* was separated into cohorts (empty vials) consisting of 10–30 flies. A horizontal line was drawn 5 cm above the bottom of the vial. After a 10 min rest period, the flies were tapped to the bottom of the vials: all flies were forced to start climbing (vertical walking). The number of flies that climbed up to the 5 cm mark after 10 s was recorded as the percentage success rate. A camera was recording fly movement during the experiment. Each trial was performed thrice, at 1 min intervals, and the results were averaged.

Immunofluorescences

D. melanogaster larvae

Third instar larvae were selected and dissected in cold PB 0.1M (Na2HPO4 8 mm, KHPO4 1.4 mm). Subsequently, the larval samples, put in stirring, were set for 20 minutes at RT in PFA 4% (16% EM grade Thermofisher diluted in 1:4 PBS). After fixing, the PFA 4% was removed, and the samples were washed twice with cold PBTween (0.04%) 0.1M. Having used Drosophila strains with GFP, after fixation, the samples were stained directly with DAPI and diluted in PB 0.1M for 10 minutes at RT stirring. Finally, the samples were washed 3 times with PBTween (0.04%) 0.1M and mounted with the mounting solution. Images were acquired with a Zeiss confocal microscope and processed using the Fiji software.

D. melanogaster adult flies

The adults were selected, beheaded, and fixed in PFA 4% (16% EM grade Thermofisher diluted in 1:4 PBS, then dissected in PB 0.1M cold (Na2HPO4 8 mm, KHPO4 1.4 mm).
The samples were then stirred and set again for 20 minutes at RT in PFA 4% + PBTriton 0,5% (Triton X diluted 1:20 in PB). After fixing the PFA 4% and the PBTX 0.5% were removed, and the samples were washed twice with cold PBTween (0.04%) 0.1M.
The samples were then stained with DAPI and diluted in PB 0.1M for 10 minutes at RT stirring. Finally, the samples were washed 3 times with PBTween (0.04%) 0.1M and mounted with the mounting solution. Images were acquired with a Zeiss confocal microscope and processed using the Fiji software.

Co-immunoprecipitation assay

Adult *Drosophila melanogaster* heads were lysed in buffer lysis at a low salt concentration (Tris Hcl pH 7.4 10 mm, MgCl2 10mm, NaCl 100 mm, Triton 0.1%). The lysate was recovered and then centrifuged at 14000 rpm for 5 minutes at 4° C; pellets were discarded.30 µl of supernatant was used as input while the remaining was incubated for 6h in ice with

an anti-HA antibody. After incubation with the antibody, the proteins of interest were incubated with Protein A Sepharose (Biorad), diluted in ethanol, for 1:30h. After centrifugation at 14000 rpm at 4° C for 15 minutes, the supernatant was eliminated and the precipitated proteins were eluted from Protein A Sepharose using Laemmli Sample Buffer 1X (Tris-HCl pH 6.8 0.125 M, Glycerol 20%, SDS 10%, DTT 1 M, Bromophenol blue 0.004%), and loaded on an acrylamide gel to 10% (Stacking gel: Bis-acrylamide 5%, tris- HCl pH 6.8 0.3 M, SDS 5%, Ammonium Persulphate 10%, TEMED 0.2%; Running gel: Bis-acrylamide 10%, Tris-HCl pH 8.8 0.3 M, SDS 5%, ammonium persulfate 10%, TEMED 0.2%), together with the input diluted in Laemmli Sample Buffer 1X. We then proceeded with Western Blotting analysis as described in Protein extraction, SDS-PAGE and Western Blotting section.

Cell cultures

Neuroblastoma SHSY5Y cells were used for some experiments. They were purchased by ATCC (ATCC % CRL-2266^{T}M) and used to produce stable lines. All cell lines were grown in DMEM-F12 culture medium Glutamax (1:1 v/v) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin (100U/l), and Streptomycin (100 U/l). The cells were kept in an incubator at 37°C and 5% carbon dioxide (CO2) up to about 90% confluence.

Once the established confluence is reached, the culture medium was discarded, and two washings with salt phosphate buffer (PBS 1X; Na2HPO4 8 mm, KHPO4 1,4 mm, NaCl 140 mm, Kcl 1.7 mm) without calcium and magnesium ions were done to eliminate any residual trace of the medium. To detach the cells from the plates, in which they are placed in culture, Trypsin-EDTA 1X (trypsin 0.05% and EDTA 0.02%) was used. After about 3-4 minutes the enzyme was inactivated with the addition of 10% serum. The detached cells were collected and centrifuged at 1000 rpm for 5 minutes. Then, the supernatant was discarded, and the cells were sown at a concentration of $2x10^5$ cells per cm ^{2}. All reagents for cell cultures were acquired by Corning cellgro.

Production of the MRWT and MRDE cell clones

Two stable lines have been produced through the introduction of plasmids containing RACK1 protein constructs. To produce these two lines, two cDNAs carrying one the Myc- RACK1WT construct, which contains the RACK1 Wild Type gene, while the other the Myc-RACK1DE construct (R36D and K38E).

The myc-RACK1WT construct was used to obtain the overexpression of RACK1 while the myc-RACK1DE construct was used to express the RACK1 protein incapable of binding the ribosome.

All the transfections have been done following the datasheet provided by SIGMA. The cells were seeded to obtain a confluence of $1x10^6$ cells in each well of a 6-well. In an eppendorf, 14 µg of DNA were diluted in 150 µl of medium without serum and without antibiotics while, in another eppendorf, 28 µl of Lipofectamine were diluted in 150 µl of medium without serum and without antibiotics, in a Lipofectamine: DNA ratio of 2:1.

Five minutes after the introduction of Lipofectamine, the two eppendorf contents were united and incubated for 20 minutes to allow the formation of micelles, which contain the plasmid. After 20 minutes, the mix was added to 1,7 ml of medium without serum and antibiotics previously placed in the plate containing the cells. After 24h from the addition of the mixture in the plate, the medium was discarded and 450 µg/ml of G418 was added to select plasmid-positive cells.

Histidine pull-down

After removing the medium and 2 washes in PBS 1x (Na2HPO4 8 mm, KHPO4 1,4 mm, NaCl 140 mm, Kcl 1,7 mm), the cells were lysed in buffer lysis at a low salt concentration (Tris Hcl pH 7.4 10 mm, MgCl2 10mm, NaCl 100 mm, Triton 0.1%). The lysate was recovered and then centrifuged at 14000 rpm for 5 minutes at 4°C; pellets were discarded. 30µl of supernatant were used as input while the remaining was incubated for 1:30h in ice with an affinity resin containing nickel (Biorad) ions, previously pre-equilibrated with the lysis buffer already mentioned above. After incubation, the mixture was transferred to suitable columns for purification (Biorad); here were carried out before 3 washes of 500 µl with the buffer of lysis and then the proteins of interest were eluted with imidazole 0.3 M dissolved in

lysis buffer. The eluted proteins were precipitated with 10% TCA and left in incubation for 30 minutes on ice. After incubation, the proteins were centrifuged at 14000 rpm for 15 minutes at 4 ºC; the supernatant was discarded while the pellet was resuspended in 30 µl of Tris Base 1M. Both the precipitated eluate and the input were then diluted in Laemmli Sample Buffer 1X (Tris-HCl pH 6.8 0.125 M, Glycerol 20%, SDS 10%, DTT 1 M, Bromophenol blue 0.004%) and loaded on an acrylamide gel to 10% (Stacking gel:
Bis-acrylamide 5%, tris-HCl pH 6.8 0.3 M, SDS 5%, Ammonium Persulphate 10%, TEMED 0.2%; Running gel: Bis-acrylamide 10%, Tris-HCl pH 8.8 0.3 M, SDS 5%, ammonium persulfate 10%, TEMED 0.2%). We then proceeded with Western Blotting analysis as described in the Protein extraction, SDS-PAGE and Western Blotting section.

Ribosomal profiling

SHSY5Y cells both transfected with the HA-eIF3d construct and were lysed using a polysomal buffer (10mm Tris-HCl, 50mm kcl, 10mm MgCl2, and 0.5% NP-40). The lysate was then centrifuged at 14000 rpm for 5 minutes at 4 ºC; the pellet was discarded while the supernatant was loaded on a continuous gradient of sucrose 50% in 10 mm Tris-HCl, 50 mm KCl, 10 mm MgCl2. After centrifugation in an ultracentrifuge (Beckman) at 27000 rpm for 4h to 4° C, the gradient was collected in fractions and the profile was obtained by analysis of total RNA at 254 nm using Bio-rad Biologic LP. The fractions were then precipitated with TCA (trichloroacetic acid) at 10% and incubated in ice for 30 minutes; then we proceeded with centrifugation at 14000 rpm for 15 minutes at 4° C. The pellet precipitated was resuspended in Tris-Base 1M and used for the immunoblotting analysis described in Protein extraction, SDS-PAGE and West,ern Blotting section.

Protein extraction, SDS-PAGE, and Western Blotting

Protein extraction was performed from 10 *Drosophila* heads and placed in ice to inhibit the enzymatic activity of protease and phosphatase. The low-concentration lysis buffered salt (Tris Hcl pH 7.4 10 mM, MgCl2 10 mM, NaCl 100 mM, Triton 0.1%). The tissues were thenlysed using pestles and the lysate recovered was centrifuged at 14000 rpm for 5 minutes at

4°C; the pellet is discarded while the supernatant, which contains proteins, is used for SDS-PAGE analysis. The protein extracts were diluted in Laemmli Sample Buffer 1X (Tris-HCl pH 6.8 0.125 M, Glycerol 20%, SDS 10%, DTT 1 M, Bromophenol blue 0.004%) and loaded on acrylamide gel 10% (Stacking gel: Bis-acrylamide 5%, tris-HCl pH 6.8 0.3 M,SDS 5%, Ammonium Persulfate 10%, TEMED 0.2%; Running gel: Bis-acrylamide 10%,
Tris-HCl pH 8.8 0.3 M, SDS 5%, ammonium persulfate 10%, TEMED 0.2%). The run was carried out in Running buffer 1X (Tris Base 3.03 g, Glycine 14.4g, SDS 1g in 1000 ml of H2O) at 180 v constant for 1h. The proteins were then transferred to the PVDF membrane with a Bio-Rad Wet/Tank Blotting System in Transfer Buffer containing 20% methanol at 100V constant per 1h. Once the transfer was complete, the non-specific sites of the membrane were blocked in 5% milk in TBST1X (Tris-HCl 0.1 M pH 7.4, NaCl 0.8 M) at 37 Incubation is then carried out with primary antibody diluted half in TBST 1X (TBS 1X and 0.1% Tween) and half milk at 5% in TBST 1X overnight at 4 months. After 3 washes of 5 minutes each with TBST, the membrane is incubated with the secondary antibody diluted half in TBST 1X and 5% milk in TBST 1X conjugated to HPR (horseradish peroxidase) for 1h. After having done the 3 washes again, we proceed with the incubation with ECL (Enhanced Chemioluminescence) and the chemoluminescence produced can be detected through a specific apparatus, the Chemidoc. The quantification was done with the help of theImageJ program.

Primary antibodies:

- Anti-HA; Rabbit (1:1000)
- Anti-RACK1; Rabbit (Cell signaling, diluizione 1:1000)
- Anti-RACK1; Rabbit ibridoma (prodotto dal CNR, diluizione 1:1000)
- Anti-Puromicina; Mouse monoclonale (DSHB, diluizione 1:10000)
- Anti-β actina; Rabbit monoclonale IgG (Cell signaling; diluizione 1:1000)
- Anti-Dlg; Mouse monoclonale IgG (Cell signaling; diluizione 1:1000)
- Anti-p-eIF2α; Rabbit monoclonale IgG (Cell signaling, diluizione 1:1000)
- Anti-eIF3d; Rabbit monoclonale IgG (Cell signaling, diluizione 1:1000)

Secondary antibodies:

- Goat anti-Mouse, policlonale IgG coniugato a perossidasi di rafano HRP (BD, diluizione 1:5000)
- Goat anti-Rabbit, policlonale IgG coniugato a perossidasi di rafano HRP (BD, diluizione 1:5000).

Drosophila Lobes and Kenyon Cells size analysis

IF images were visualized using a Nikon Ti-2E microscope at a magnification of 20X (0.45 N.A.). Images were captured with a Hamamatsu sCMOS camera (ORCA Flash4.0 V3), using NIS Elements software. Lobes analysis was carried out by measuring the vertical (α, α′) and horizontal (β, β, and γ) lobes thickness and lenght while the Kenyon cells area analysis was carried out measuring the area occupied by Kenyon cells cellular bodies usingin both of cases Fiji software.

SUnSET assay

The SUnSET (Surface Sensing of Translation) assay was done to examine the proteinsynthesis rate. The SUnSET technique specifically uses an anti-puromycin antibody for the detection of puromycin-labeled peptides. Indeed, puromycin, which is an aminonucleoside antibiotic, is a structural analog aminoacyl-transfer RNA and can be incorporated into elongated peptide chains through the formation of a peptide bond (Goodman C. and Hornberger T., 2013). About 15 adult heads were administrated 20 µg/mL of puromycin for 40 minutes in stirring at room temperature. Then, adult heads were lysate and used for Western Blotting analysis.
The same concentration of puromycin and the same time of administration was used for the SUnSET assay on the larval nervous system.

Statistical analysis

The results are presented as the mean ±standard error of the mean (SEM) or mean ±deviation standard (SD). IBM SPSS Statistics for Windows, version 23.0 (IBM Corp.), and GraphPad Prism software (5.0 version; GraphPad Software, Inc.) were used for the statistical analyses. One-way analysis of variance (ANOVA) with Tukey's post hoc test were used for comparisons between multiple groups, respectively. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Error bars represent the standard error of the mean from a minimum of three independent experiments.

Results

Canonical protein synthesis plays an important role in synaptic plasticity and in the correct neuronal development. Also, learning and memory are particularly susceptible to the manipulation of translational control mechanisms.
One of the major targets of the initiation regulation is eIF4E, whose binding to the eIF4G is critical for protein translation initiation (Gindina et al., 2021).
Considering the existence of alternative protein translation mechanisms, canonical protein synthesis might not be the only one involved in learning and memory processes.
Among the alternative initiation translation mechanisms, the eIF3d specialized translation has emerged in recent years.
The translation of eIF3d is not affected by the eIF4E inactivation (Lee et al., 2016).

The downregulation of eIF4E and eIF3d respectively in the MBs determines a delay in larval development

First, to understand if the eIF3d specialized translation could have a role like that of eIF4E-dependent translation in learning and memory processes, the expression of eIF4E and eIF3d has been modulated in the Mushroom Bodies (MBs). For this purpose, the OK107-gal4 driver strain, which carries the Mushroom Bodies specific promoter OK107, was used and crossed with the UAS-eIF4E RNAi strain and the UAS-eIF3d RNAi strain, respectively. In the first case, the expression of eIF4E has been downregulated in the MBs, while in the second case, the expression of eIF3d has been reduced in the MBs. The W1118 strain crossed with the OK107-gal4 has been used as a control.
A larval developmental delay has been first observed for both crosses. Indeed, larvae downregulating eIF4E reached the third larval stage with a delay of about 72 hours, while larvae downregulating eIF3d showed a developmental delay of about 48 hours compared to the control larvae, which, instead, reached the third larval stage in 3-4 days (Figure 21).

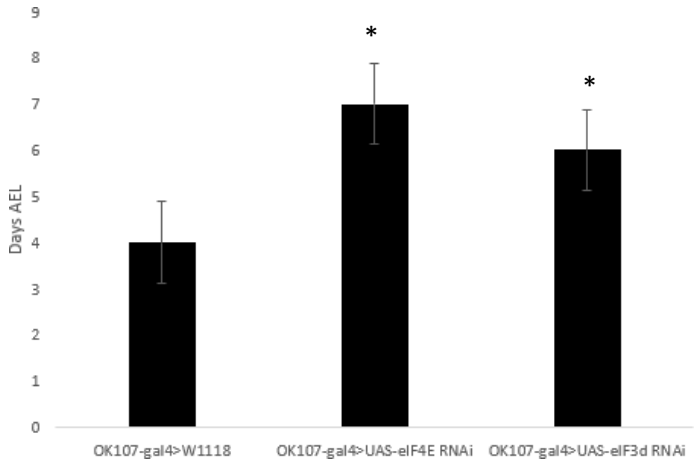


Figure 21. Larvae downregulating eIF4E and eIF3d respectively showed a developmental delay. For eIF4E, the developmental delay is about 72 hours while for eIF3d is 48 hours. The larvae control takes 3-4 days to reach the third larval stage after the eggs laying (AEL). Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118.

The downregulation of eIF4E and eIF3d determines impairments in learning and locomotion behavior

Next, flies were subjected to a simple learning test to investigate learning abilities. In the assay, the ability of flies to associate a positive stimulus, which is represented by the molasses-containing gruel (C+), with a negative stimulus, represented by 37°C heat shock (U-), has been investigated.

For each sample, the performance index (P.I) has been defined. P.I. is calculated using the following formula: P.I. = (CS+) – (US-)/ tot. of flies, in which the number of flies responding negatively to the stimulus is subtracted from the number of flies responding positively to the stimulus, divided by the total number of flies.

The control flies, obtained from the crossing of the W1118 strain with the OK107-gal4 driver strain, respond positively to the test, associating molasses-containing gruel with 37°C heat shock and not moving from normal gruel. Flies downregulating eIF4E, instead, move

quickly toward the molasses-containing gruel, probably failing to associate molasses with 37°C heat shock and only a small part of the flies remains on the control gruel. In fact, the performance index (P.I) is greatly reduced for flies downregulating eIF4E compared to the control flies, which respond positively to the assay and have a positive P.I.

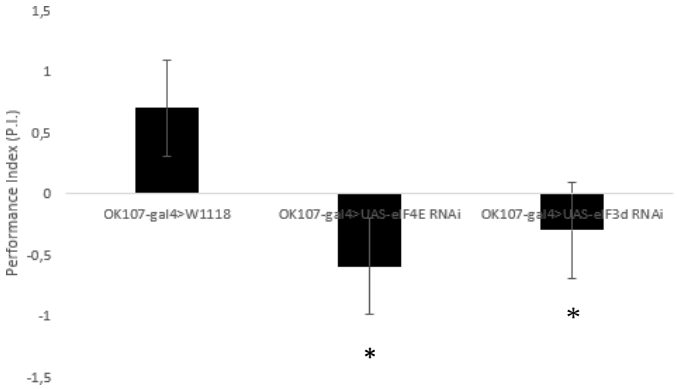
Flies downregulating eIF3d showed the same behavior observed for eIF4E downregulation. Indeed, flies failed in associating molasses with 37°C heat shock and they move quickly toward the molasses-containing gruel. In fact, the P.I. is a negative value (Figure 22 A).

Moreover, it is reported in the literature that among the functions in which they are involved, MBs control locomotor behavior and reactivity (Sun et al., 2018).

To investigate this aspect, the climbing test has been performed. In this case, this assay allows not to examine the locomotion abilities of flies, but especially their behavior in locomotion. Data obtained from the climbing test revealed that flies downregulating eIF4E had severe problems in locomotion, remaining on the bottom of the tube. Moreover, they seemed to slow in movements. This is an anomalous behavior, since flies spontaneously move upwards. The control flies, instead, reached quickly the top of the tube, following a linear direction and with coordinated movements.

In contrast to that observed for flies in which eIF4E expression is reduced, flies downregulating eIF3d quickly reached the top of the tube with disorganized movements and jumping from one side to the other. Once they reached the top of the tube, they are unable to maintain their position, but continue to move with uncoordinated movements (Figure 22 B).

A



B

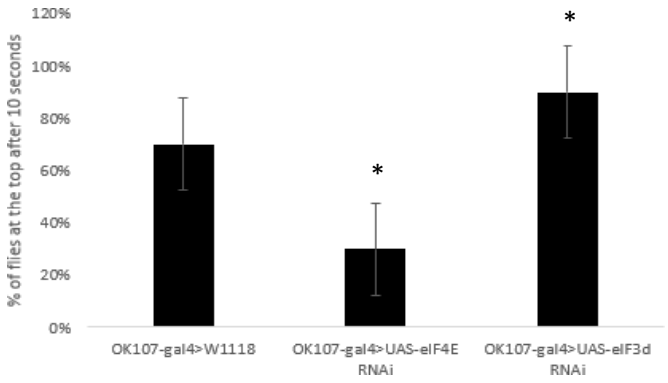


Figure 22 A Learning test revealed impairments in associating the positive stimulus with the negative stimulus for flies downregulating eIF4E and eIF3d, showing a P.I. of about -0,6 and - 0,4 respectively. The control flies, instead, had a P.I. of about 0,7. **B** In the climbing test flies reducing the expression of eIF4E remain on the bottom of the tube, while flies downregulating eIF3d move quickly toward the top of the tube, with disorganized movements and jumping from one side to the other. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118.

The downregulation of eIF4E and eIF3d causes defects in the MBs structure

Considering these impairments in learning and locomotion behavior, the structure of the Mushroom Bodies (MBs) has been investigated. For this purpose, the strain UAS-GFP/cyo; mkrs/tm6b, which carries the GFP, has been used and crossed with the OK107-gal4 driver strain before the crossing with the UAS- eIF4E RNAi and the UAS-eIF3d RNAi strains.

In adult flies, the Mushroom Bodies are completely formed, and they include the Kenyon cells (KCs), the γ lobes, the α'/α lobes, and the β'/β lobes. The Kenyon cells, the γ lobes, andthe α'/ β' lobes are already formed at the third larval stage. Instead, the α/ β lobes are formed during the metamorphosis from larva to pupa (Yasugi & Nishimura, 2016).

Both the downregulation of eIF4E and eIF3d respectively determines a strong reduction in Kenyon cells area, which is more than 50% compared to the control for eIF4E and eIF3d (Figure 23), and consequently, the α'/β' lobes and the α/β lobes structures are altered, since the α'/ α lobes are thinner and more elongated, while the β'/ β lobes are more disorganized compared to the control (Figure 24).

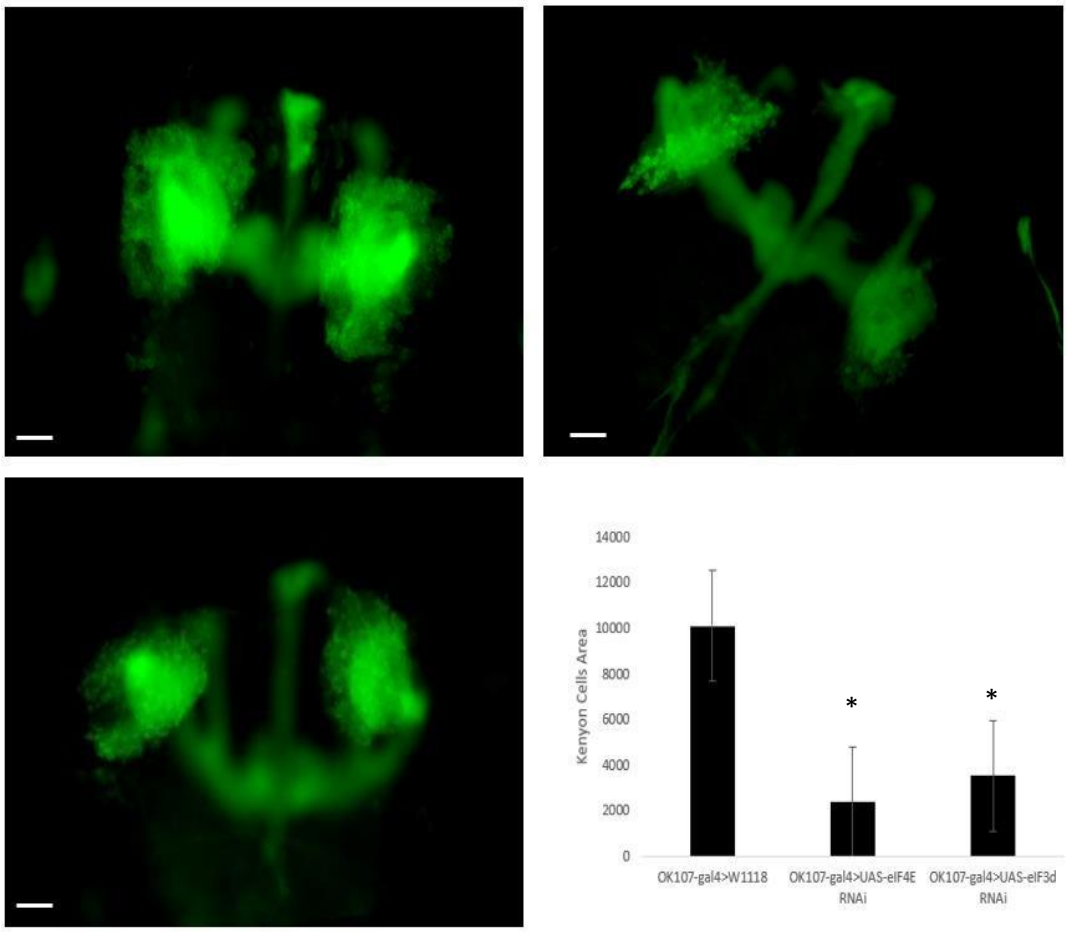


Figure 23. OK107-gal4>W1118 (top left)**,** OK107-gal4>UAS-eIF4E RNAi (top right)**,** OK107-gal4>UAS-eIF3d RNAi (bottom left). Both the downregulation of eIF4E and eIF3d respectively determines a strong reduction in Kenyon cells area, which is more than 50% compared to the control for eIF4E and eIF3d as it is reported in the graph**.** Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118.

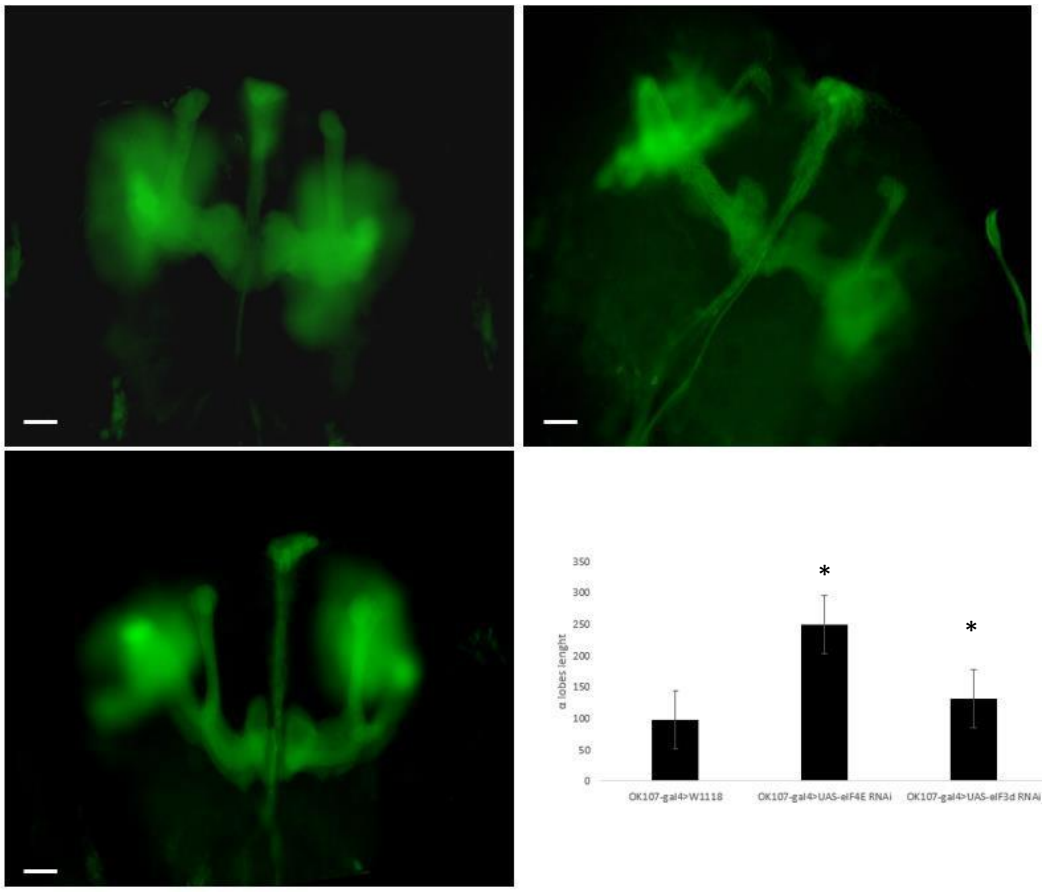


Figure 24. OK107-gal4>W1118 (top left), OK107-gal4> UAS eIF4E RNAi (top right), OK107-gal4> UAS eIF3d RNAi (bottom left). The downregulation of eIF4E and eIF3d respectively causes impairments in MBs structure. The graph shows that α' and α lobes are thinner and more elongated compared to the control. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118.

Therefore, the reduction in the expression of eIF4E and eIF3d causes important defects in the Mushroom Bodies structure, altering learning abilities and behavior locomotion. These aspects suggest that also the eIF3d specialized translation could play an important role in learning and memory formation, such as the eIF4E-dependent translation.

eIF3d interacts with ribosomal protein RACK1

To modulate the translation of specific mRNAs, which could be critical for learning and memory formation, eIF3d must be recruited on the 40S ribosomal subunit. As seen before (figure 17), analyzing the crystallographic analysis of the 40S ribosomal subunit, it was observed that on ribosomes eIF3d is located near RACK1, which is a well-known scaffold protein. Moreover, both eIF3d and RACK1 modulate the translation of specific cell proliferation mRNAs.

These data led to think that there could be an interaction between eIF3d and RACK1.

To investigate this aspect, co-immunoprecipitation assays have been done. For this purpose, transgenic fly strains expressing exogenous eIF3d and RACK1 conjugated to the hemagglutinin tag (HA) in the Mushroom Bodies have been used.

Co-immunoprecipitation experiments have been done using the antibody anti-HA, which is able to recognize the tag conjugated to exogenous eIF3d and RACK1. Therefore, eIF3d-HA has been immunoprecipitated and the interaction with endogenous RACK1 has been investigated. From data obtained from Western Blotting analysis, it emerged that the expression of exogenous eIF3d was enriched in the immunoprecipitated sample. Moreover, immunoprecipitating eIF3d, endogenous RACK1 is co-immunoprecipitated too (Figure 25 A).

Then, also the exogenous RACK1-HA has been immunoprecipitated and the interaction with endogenous eIF3d has been investigated. Western Blotting analysis revealed that, in this case, immunoprecipitating RACK1-HA, eIF3d is co-immunoprecipitated (Figure 25 B).

These data revealed that there is an interaction between eIF3d and RACK1 in *Drosophila* MBs.

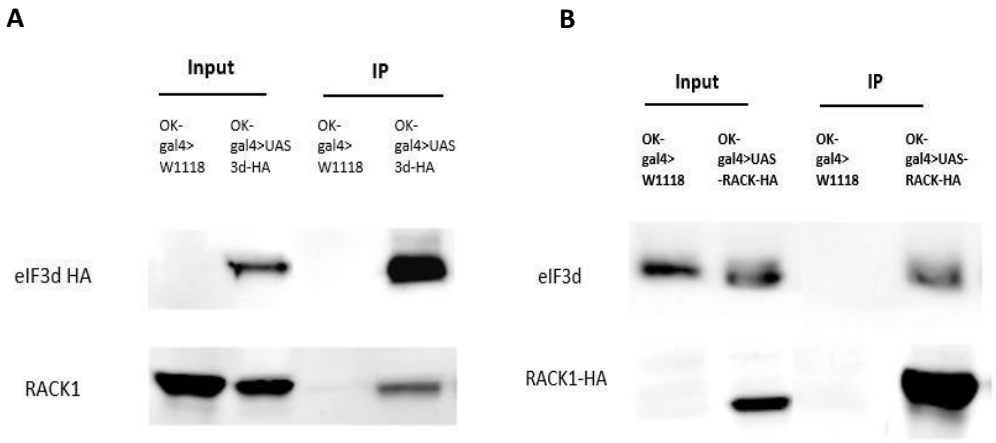


Figure 25. eIF3d and RACK1 interact on 40S ribosomal subunit in Drosophila melanogaster. To investigate this interaction, a co-immunoprecipitation assay has been done. **A** The Western Blotting analysis revealed that immunoprecipitating for eIF3d-HA, endogenous RACK1 is co-immunoprecipitated. **B** Immunoprecipitating for RACK1-HA, also endogenous eIF3d is co-immunoprecipitated.

eIF3d and RACK1 interact also in SHSY5Y neuroblastoma cells

From the data obtained it emerged that eIF3d and RACK1 interact in *Drosophila melanogaster*.

Moreover, being RACK1 a scaffold protein, it could be RACK1 to recruit eIF3d on the ribosome. To investigate this hypothesis, given the difficulty of obtaining ribosomal transgenic lines for RACK1 in *Drosophila*, human neuroblastoma cells SHSY5Y have been used. Neuroblastoma SHSY5Y cells have been transfected with a specific plasmid, in which the cDNA used presents the construct Myc-RACK1DE (MRDE), which contains the gene codifying for RACK1 mutated at two different sites (R36D and K38E), conjugated to the Myc tag. These mutations reduce the binding affinity for the ribosome. As a control, a cDNA presenting the construct Myc-RACK1WT (MRWT) has been used.

First, the interaction between eIF3d and RACK1 has been confirmed also in neuroblastoma cells. For this purpose, the co-purification of RACK1-eIF3d has been performed using a histidine affinity resin that can retain the myc tag associated with the exogenous protein RACK1. Western Blotting analysis using an antibody anti-myc showed enrichment in the

expression of RACK1 both for SHSY5Y cells transfected with RACK1-WT and RACK1- DE. Next, the use of a specific antibody for eIF3d on the same proteins eluted following purification, showed that in MRWT and MRDE there is a band specific for eIF3d, not found in control SHSY5Y. Therefore, eIF3d co-purifies with RACK1 (Figure 26).

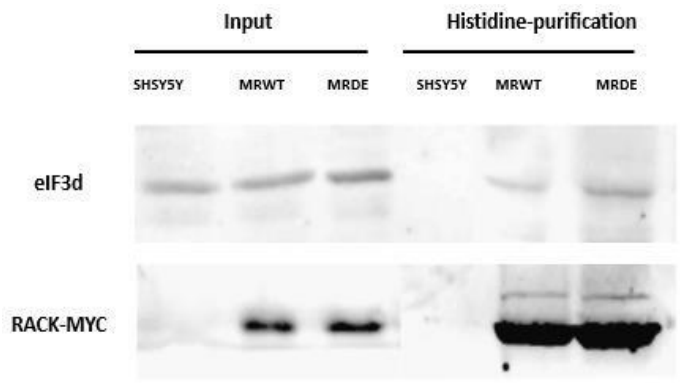


Figure 26. eIF3d and RACK1 interact also in neuroblastoma cells. Lysates from neuroblastoma SHSY5Y cells transfected stably with the constructs Myc-RACK1 WT and Myc-RACK1DE respectively have been purified using a histidine affinity resin, that can retain the myc tag associated with the exogenous protein RACK1. Western Blotting analysis using an antibody anti-myc showed enrichment in the expression of exogenous RACK1 both for SHSY5Y cells transfected with RACK1-WT and RACK1-DE. eIF3d is present also in MRWT and MRDE purified samples, but not in control SHSY5Y.

eIF3d is recruited on the ribosome by RACK1

Once it was established that eIF3d and RACK1 interact with each other, it was verified whether it was RACK1 to recruit eIF3d on the ribosome. For this purpose, a ribosomal profiling analysis has been performed on SHSY5Y transfected both with MRWT and MRDE.

As already known from the literature, RACK1WT correctly localizes on ribosomal fractions, while for RACK1DE there is a strong reduction in the amount of protein present in free ribosomes and polyribosomes (Romano et al., 2019).

The Western Blotting analysis conducted on ribosomal fractions showed that, in the MRWT line, eIF3d is present both on free ribosomes and polyribosomes.

In contrast, eIF3d is present only in the first ribosomal fractions in SHSY5Y transfected with RACK1DE (Figure 27). Therefore, it is RACK1 to recruit eIF3d on the ribosome.

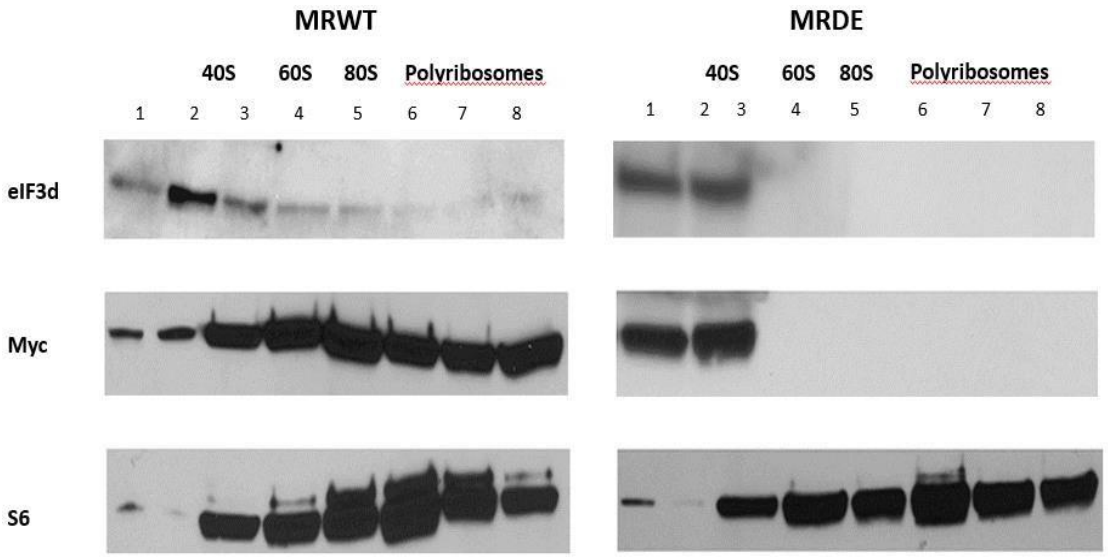


Figure 27. RACK1 recruits eIF3d on the ribosome. The Western Blotting analysis conducted on ribosomal fractions showed that, in the MRWT line (on the left side), eIF3d is present both on free ribosomes and polyribosomes. In contrast, eIF3d is present only in the first ribosomal fractions in SHSY5Y transfected with RACK1DE (on the right side).

Double transgenic flies for eIF3d and RACK1 show a normal time of development

After having confirmed the interaction between eIF3d and RACK1 and having demonstrated that RACK1 recruits eIF3d on ribosomes, it was asked how the eIF3d-RACK1 complex can act in the Mushroom Bodies development.

Since the downregulation of RACK1 in MBs is lethal in the earliest stages of development, RACK1 has been overexpressed. For this purpose, the UAS-RACK1-HA strain, in which the gene codifying for *rack1* is fused to the hemaglutinin tag (HA), has been crossed with the OK107-gal4 driver strain. For the double-crossing, instead, the UAS-RACK1-HA flies were first crossed with the OK107-gal4 driver strain flies, and the offspring obtained was then crossed with flies of the strain UAS-eIF3d RNAi.

First, a larval developmental delay was observed. In fact, larvae overexpressing RACK1 reached the third larval stage with a delay of about 24 hours compared to the control. The downregulation of eIF3d, as seen before, caused a developmental delay of 48 hours.

Larvae coming from the double-crossing showed, instead, a normal timing of larval development with a slight delay of about 8 hours, very similar to that observed for the control (Figure 28).

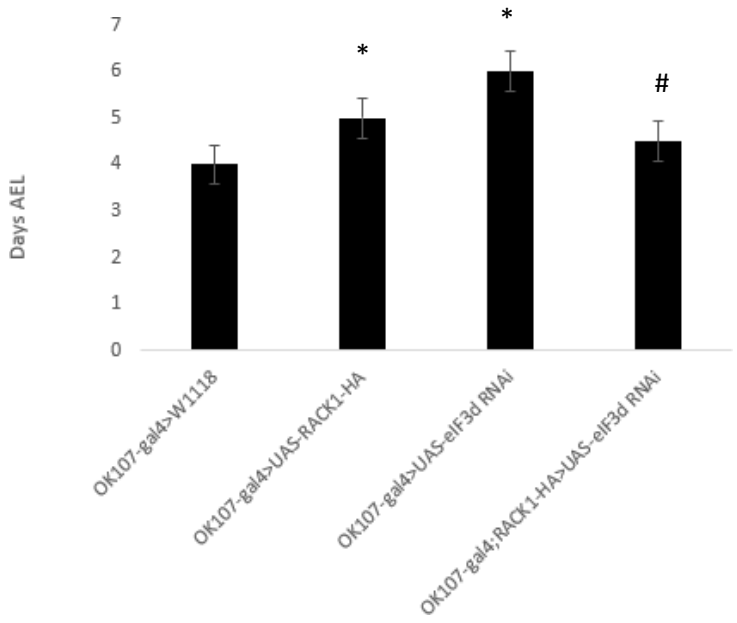


Figure 28. The larval developmental delay caused by the expression of RACK1 and eIF3d in the MBs, respectively, is restored in double transgenic flies for these two proteins. OK107-gal4>W1118 reach the third larval stage in 3-4 days, larvae from OK107-gal4>UAS-RACK1-HA crossing show a delay of about 24 hours, while larvae obtained from OK107-gal4>UAS eIF3d RNAi crossing have a delay of about 48 hours. The double transgenic flies, instead, show a slight delay of about 8 hours compared to the control. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi.

Double transgenic flies for eIF3d and RACK1 showed a recovery in learning and locomotion behavior

Later, flies were subjected to the learning test previously seen. Adult flies overexpressing RACK1-HA were not able to associate the molasses with the 37°C heat shock, as previously

observed for the downregulation of eIF3d. But, compared to eIF3d, the performance index (P.I) is higher, suggesting that fewer flies responded negatively to the learning test.

Also, most of the flies downregulating eIF3d and overexpressing RACK1 at the same time were able in associating the positive stimulus with the negative one, remaining on the control gruel. Only a small part failed the test, moving quickly toward the molasses-containing gruel. In fact, they showed a P.I. of about -0,1 (Figure 29 A).

Moreover, the climbing test was performed to investigate flies behavior in locomotion. Flies overexpressing RACK1-HA reached the top of the tube faster than the control. In addition, once reached the top of the tube, they were not able to maintain their position, jumping from one side to the other. Flies downregulating eIF3d presented the same behavior.

For double transgenic flies, the climbing test revealed that there are no abnormal behaviors in locomotion. Indeed, they quickly reached the top of the tube and just a small part of the flies presented irregular movements, jumping from one side to the other of the tube (Figure 29 B).

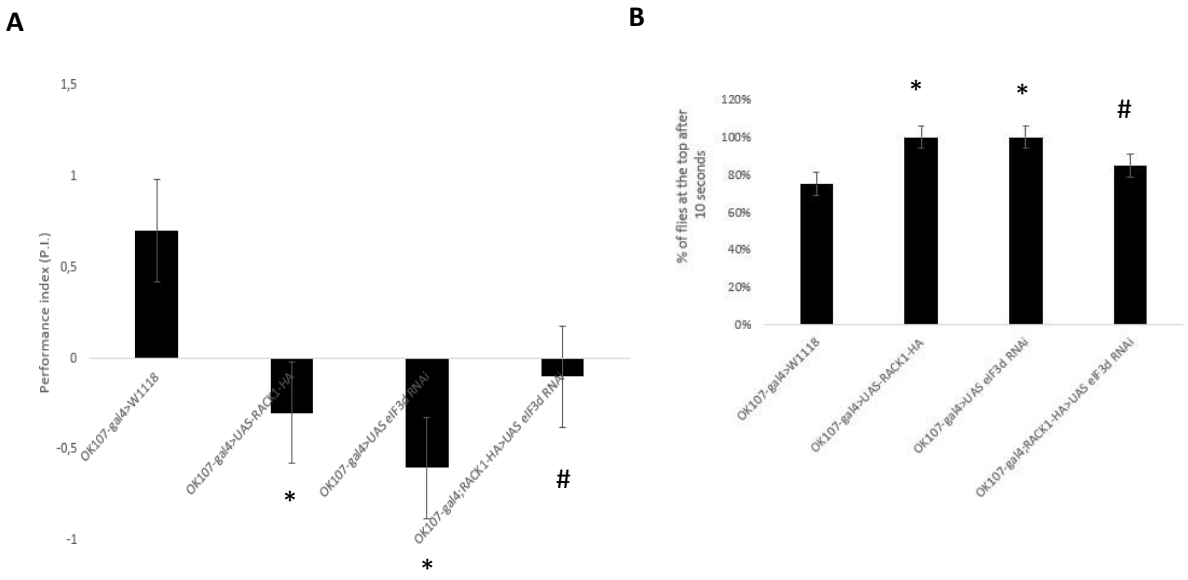
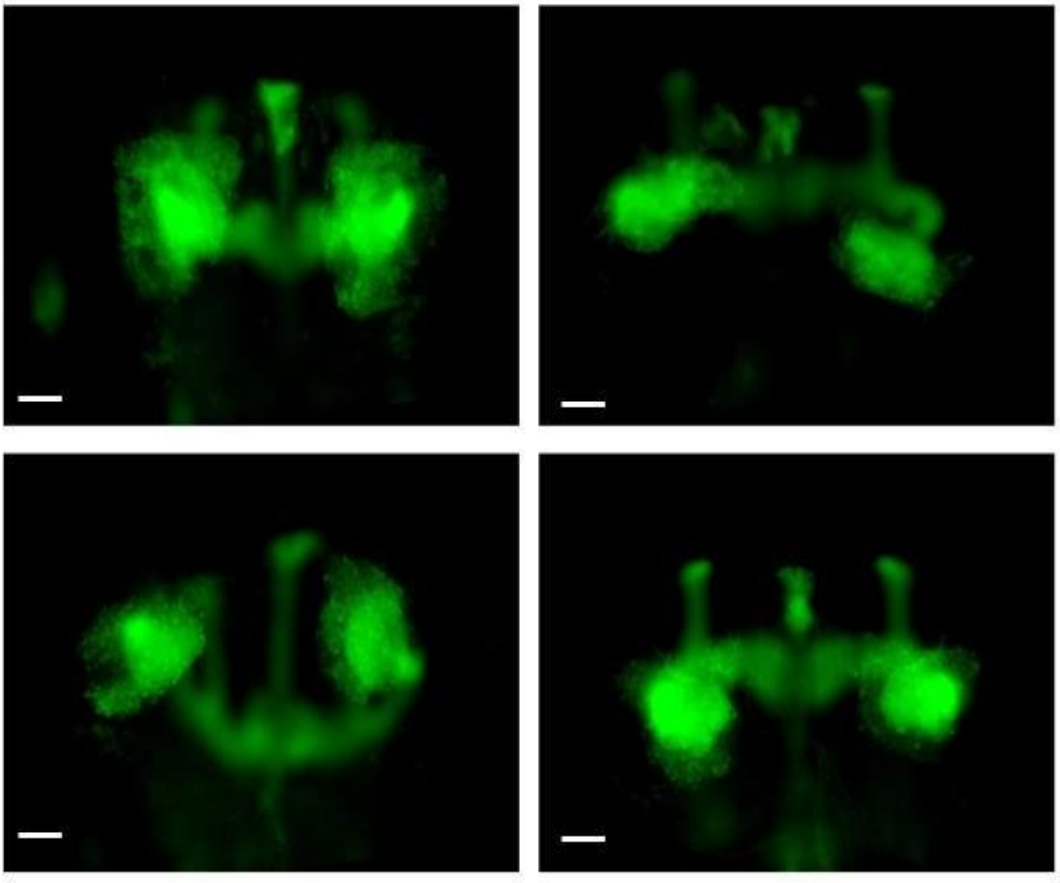


Figure 29. A The learning test reveals that the double transgenic flies have a recovery in learning abilities compared to the adult flies overexpressing RACK1 and downregulating eIF3d. In fact, the P.I. is about -0,2 compared to -0,3 and -0,6 for RACK1 and eIF3d RNAi respectively. **B** Also for the behavior in locomotion, double transgenic flies show a more normal behavior, not jumping from one side to the other, but moving with regular movements. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi.

Transgenic flies for eIF3d and RACK1 showed a recovery in the MBs structure

Then, the MBs structure has been analyzed. Previously, it was seen that flies downregulating eIF3d, showed a strong reduction of about 50% of the Kenyon cells area, while the α'/ α and the β'/ β lobes were thinner, more disorganized, and elongated compared to those observed in control flies. Flies overexpressing RACK1 presented the same impairments in the MBs structure.

A recovery in the MBs structure has been, instead, observed for double transgenic flies for eIF3d and RACK1. Indeed, the area of Kenyon cells, although it is smaller than the control one, is enhanced compared to that observed in single crosses (Figure 30). Moreover, the α' and α lobes appear shorter, even though thinner, and more elongated than those observed in the control, while the β' and β lobes were more organized (Figure 31).



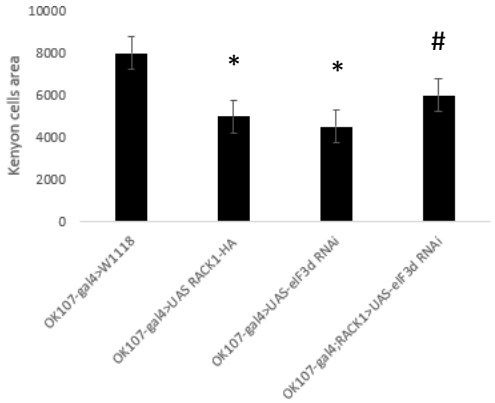


Figure 30. Double transgenic flies showed a recovery in the Kenyon Cells area compared to the single crosses**.** OK107-gal4>W1118 (top left), OK107-gal4>UAS-RACK1-HA (top right), OK107- gal4>Uas-eIF3d RNAi (bottom left) and OK107-gal4; RACK1-HA>UAS-eIF3d RNAi (bottom right).The graph showed the size of Kenyon Cells in the different samples. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi

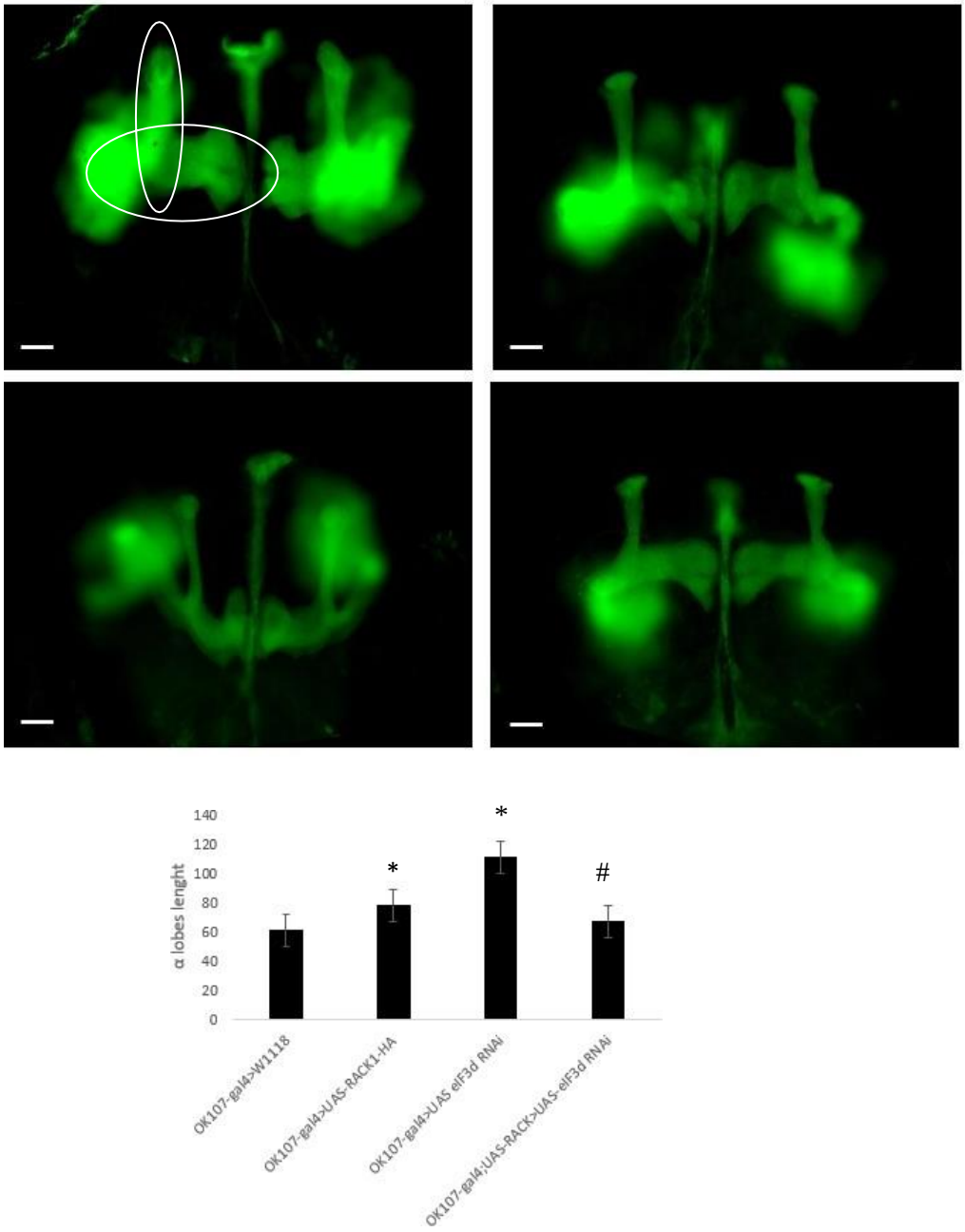


Figure 31. The downregulation of eIF3d and the overexpression of RACK1 causes thinning and elongation of α'/ α lobes and disorganization of β'/ β lobes. The double transgenic flies, instead, showed a recovery in MBs structure. OK107-gal4>W1118 (top left), OK107-gal4>UAS-RACK1-HA (top right), OK107-gal4>UAS-eIF3d RNAi (bottom left) and OK107-gal4; RACK1-HA>UAS-eIF3d RNAi (bottom right). In graph, the α lobes lengths of the different samples are reported. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107- gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi.

eIF3d and RACK1 determine structural defects on single neurons

From the data obtained, it appears that the eIF3d-RACK1 axis probably plays a role in the MBs development. In fact, the modulation in the expression of eIF3d and RACK1 determines alterations in the MBs structure, which reflect modifications in learning abilities and locomotion behavior. To deeply analyze the structure of the single neurons, the expression of eIF3d and RACK1 has been modulated in the sensory class IV dendritic arborization neurons (c4da), which are peripheral sensory neurons, very useful for this purpose. Indeed, they are characterized by high dendritic arborization (Grueber et al., 2007).
For this purpose, flies from the UAS-eIF3d RNAi and the UAS-RACK1-HA strains have been crossed with the Uas-5'Utr Mical::GFP/CyO weeP; ppk-Gal4, Uas-Td::tomato/TM6b driver strain. This cross allows downregulating the endogenous expression of eIF3d and overexpressing RACK1 specifically in c4da neurons, through the *ppk* promoter.
Single neuron structure has been analyzed in pupae 18 hours after puparium formation (APF), which is the time necessary to c4da neurons to complete dendritic pruning. For this purpose, the GFP protein carried by the driver strain has been used to examine the neuron structure.
From the images obtained, it seems that c4da neurons of pupae downregulating eIF3d have smaller cellular bodies with a minus number of dendrites and they appear less branched compared to c4da neurons of control.
Also, for RACK1, the phenotype obtained was like that observed for the downregulation of eIF3d. In fact, c4da neurons of pupae overexpressing RACK1-HA have a smaller number of dendrites and they appear less branched compared to c4da neurons of control (Figure 32).

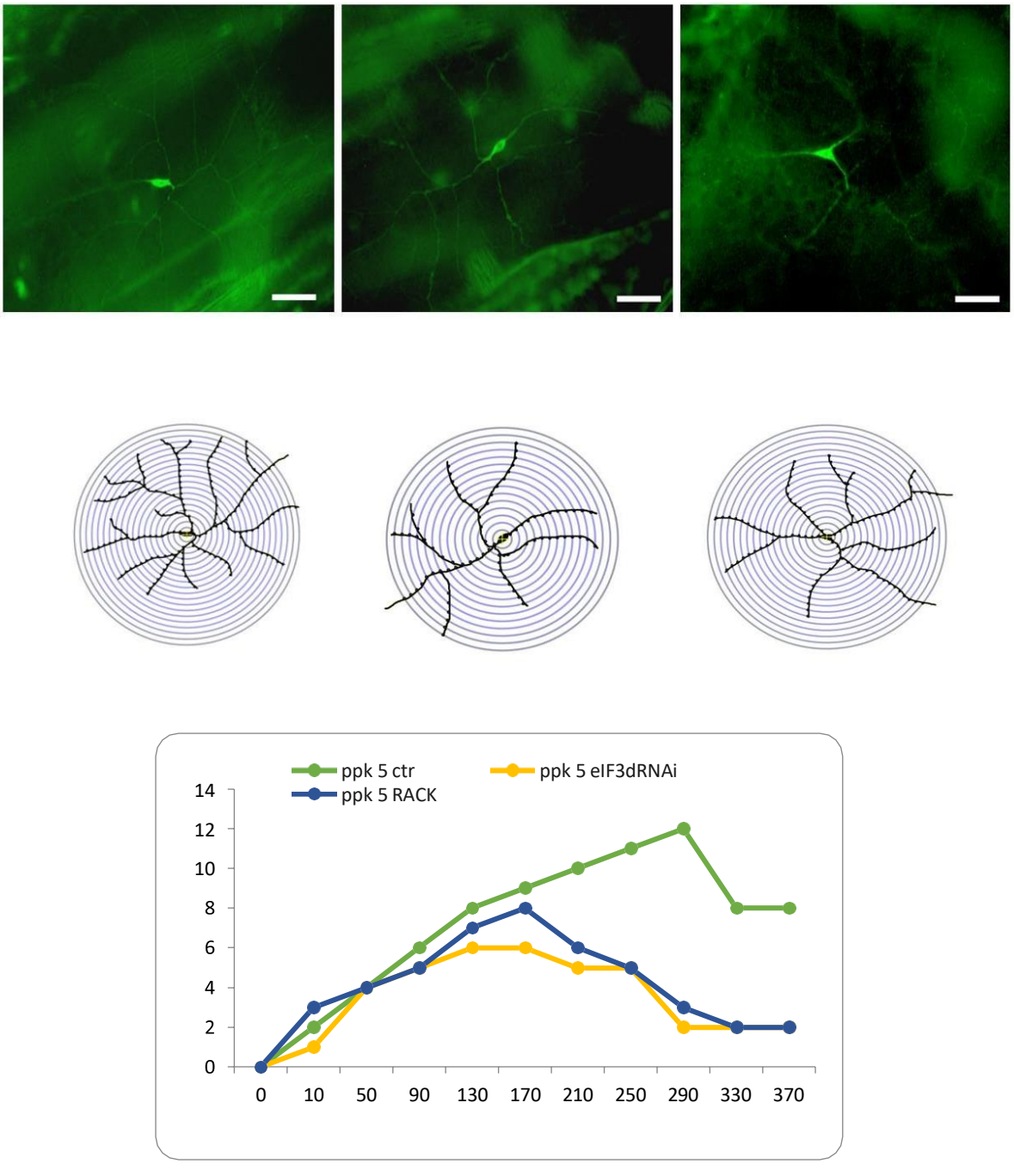


Figure 32. ppk-gal4; GFP>W1118 (top left), ppk-gal4; GFP>UAS-eIF3d RNAi (high in the center), ppk-gal4; GFP>UAS-RACK1-HA (top right). Scholl analysis for ppk-gal4>W1118 (on left), ppk-gal4>UAS-eIF3d RNAi (in the middle) and ppk-gal4>UAS-RACK1-HA (on right), respectively. The scholl analysis analyzes the number of intersections for each sample, which are reported in the graph.

eIF3d and RACK1 modulate the protein synthesis rate and the expression of Dlg1

The downregulation of eIF3d and the overexpression of RACK1-HA respectively determine an alteration in the MBs structure and in learning and locomotion abilities. But, when these proteins are expressed at the same time, determine a recovery in the structure of the Mushroom Bodies, which are very similar to the control. Also, the learning and locomotion behavior is recovered.

Moreover, the data obtained using the ppk-gal4 revealed that the expression of both these proteins determines smaller neurons and less branched and these defects could explain the impairments obtained.

To better understand what the observed phenotypes depend on for both individual and double-crosses, a molecular investigation has been performed.

Considering that both eIF3d and RACK1 are involved in the eIF4F-independent translation initiation, the protein synthesis rate has been investigated through a SUnSET assay conducted on adult flies. This assay allows estimating the protein synthesis level using puromycin, an antibiotic aminonucleoside that has a structure like the tRNA initiator. Binding to the nascent polypeptide chain, puromycin can block its elongation.

Western blotting analysis, performed using the antibody anti-puromycin, showed that flies overexpressing RACK1 and downregulating eIF3d respectively incorporate more puromycin compared to the control, revealing an increase in protein synthesis rate. In the double transgenic flies, instead, the incorporation is reduced compared to the single crosses, but it remains still higher than the control. (Figure 33 A).

Later, also the expression of p-eIF2α has been investigated. Indeed, eIF2α in its phosphorylated form is one of the major canonical initiation translation regulators. Precisely for this role, p-eIF2α is fundamental in synaptic plasticity and memory formation (Buffington et al., 2014). Therefore, it was wondered if the eIF3d-RACK1 axis could act on the p-eIF2α activity, determining impairment in the MBs structure. To understand if the expression of p-eIF2α is modulated by eIF3d and RACK1, Western Blotting analysis has been done. From data obtained using an antibody anti-peIF2α, it seemed that eIF2α

phosphorylation levels remained unchanged in all the samples, suggesting also that eIF3d and RACK1 do not alter the p-eIF2α expression (Figure 33 B).

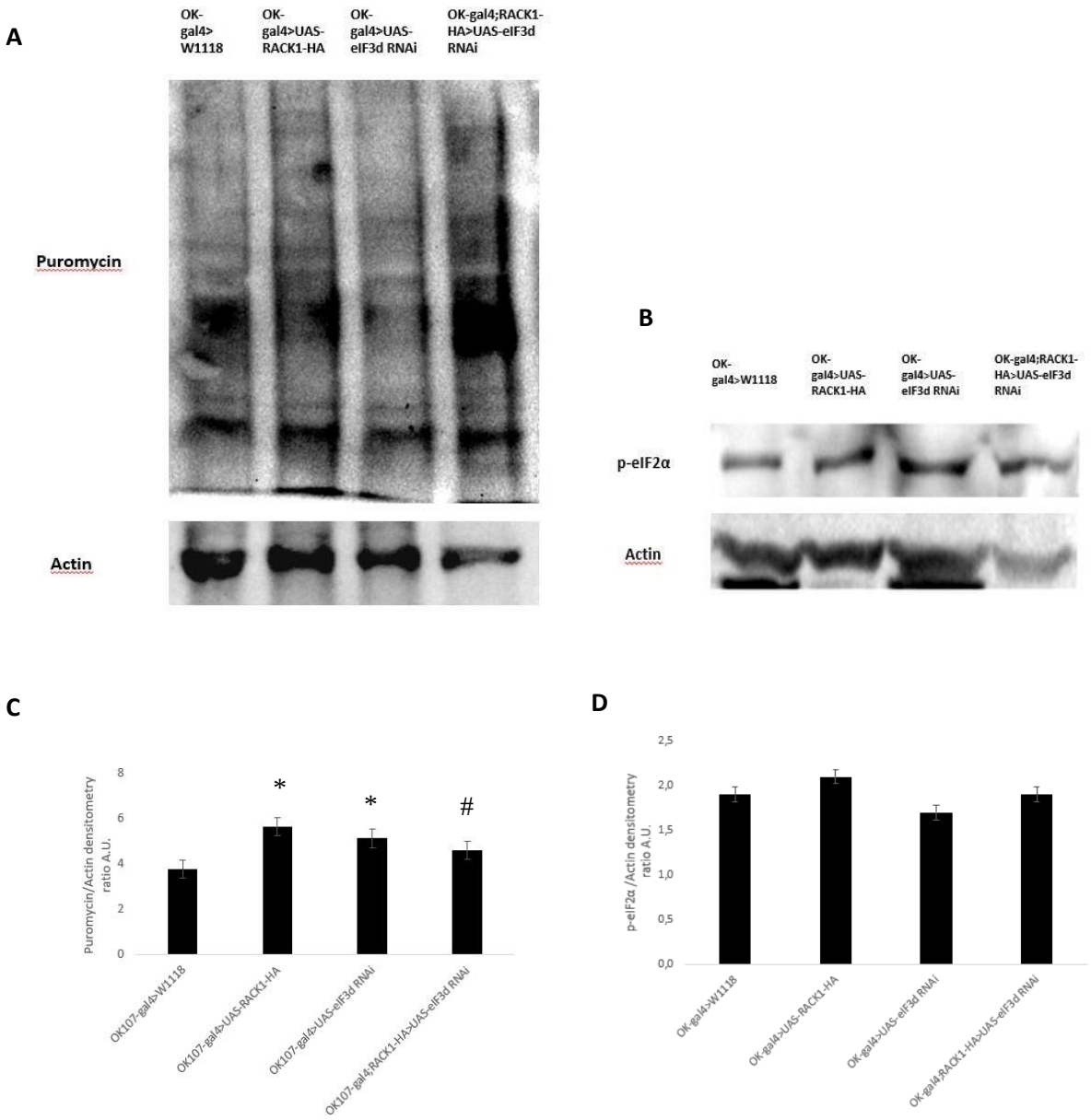


Figure 33. RACK1 overexpression and eIF3d downregulation determine an increase in protein synthesis rate, which is recovered and reported to wild-type levels in the double transgenic. Despite the increase in protein synthesis, flies do not show stress, as demonstrated in Western Blotting analysis for p-eIF2α. The expression of p-eIF2α is unvaried in all samples. Bar graphs represent

means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi.

Moreover, it is known from the literature that RACK1, as a ribosomal protein, interacts with the RNA-binding protein FMRP. The depletion of FMRP leads to dendritic and axonal aberrant development, which are two main features of Fragile X syndrome patients. The binding of FMRP with RACK1 removes the translational repressive activity of FMRP and promotes the translation of specific mRNAs, e.g PSD-95(Post-synaptic Density) mRNA (Romano et al., 2022).

Considering also the reduced neuronal arborization observed on single neurons, the expression of Dlg1, which is the PSD-95 ortholog, has been examined. Dlg1, in fact, is a scaffolding protein, which plays an important role in synaptic physiology and has been also associated with calcium regulation, which is important for synaptic plasticity (Bertin et al.,2022). Considering the importance of Dlg1 in synaptic plasticity and the role of RACK1 in modulating the PSD-95 translation in neurons, the expression of Dlg1 has been investigated both in individual and double-crosses.

In Western blotting analysis, the antibody anti-Dlg1 recognizes two different isoforms of the protein, one to 97 KDa and the other to 110 KDa.

From results obtained by Western Blotting, it was found that the expression of Dlg1 is increased in flies overexpressing RACK1, while it is reduced in flies downregulating eIF3d. In double transgenic flies, instead, Dlg1 appeared to increase compared to the control, but not what was observed for the individual crossing with RACK1-HA (Figure 34).

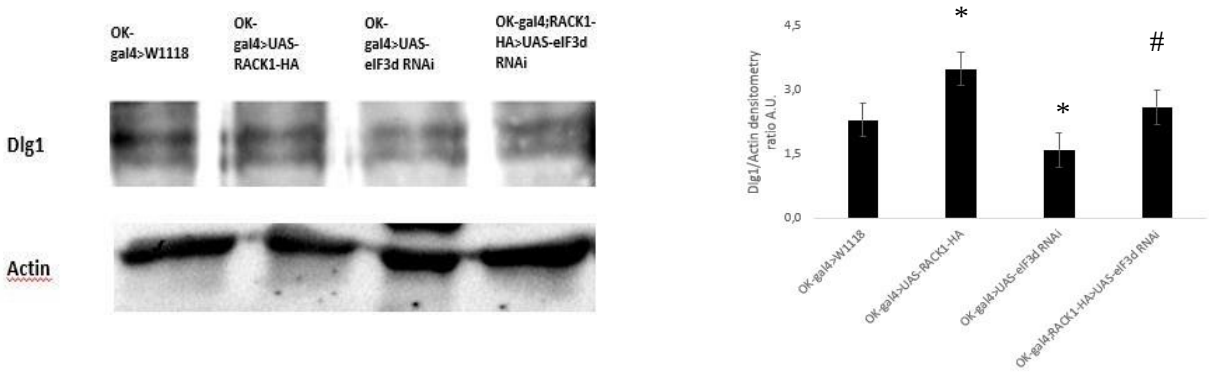


Figure 34. The eIF3d-RACK1 complex modulates the Dlg1 expression. In fact, the overexpression of RACK1 determines an increase in Dlg1 expression, while the eIF3d downregulation causes a decrease in Dlg1 expression. In double transgenic flies, the Dlg1 expression is recovered. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs OK107-gal4>W1118 and # $p \leq 0.05$ vs OK107-gal4>UAS-RACK1-HA and OK107-gal4>UAS-eIF3d RNAi.

Data obtained suggest that the modulation in the expression of both eIF3d and RACK1 causes an increase in the protein synthesis rate, which is partially restored when these proteins are expressed at the same time in Mushroom Bodies. Moreover, the eIF3d-RACK1 axis is probably involved in learning and memory formation.

eIF3d and RACK1 cause defects in MBs also using the *elav* promoter

To confirm what was observed using OK107-gal4, the phenotypes obtained modulating the eIF3d and RACK1 expression have been investigated using another promoter.

For this purpose, the expression of eIF3d and RACK1 has been modulated directly in the central nervous system, using the elav-gal4/Fm7; UAS-GFP/cyo driver strain. This strain not only allows to investigate the effects of the expression of interest in the nervous system, but, carrying the GFP, allows also to simply investigate of the structure of the central nervous system. Also, the double transgenic flies have been investigated.

Unlike the OK107 promoter, which is specific for MBs, the *elav* promoter allows the expression of the protein of interest in the central nervous system.

Elav is a gene that encodes an RNA-binding protein that is specifically expressed in all post-mitotic neurons from the earliest stages of their differentiation (Yao et al., 1993). It has also a role in axon guidance, synapse formation, and photoreceptor differentiation.

Flies from the UAS-eIF3d RNAi and the UAS-RACK1-HA strains, respectively, have been crossed with the elav-gal4/Fm7; UAS-GFP/cyo flies and the offspring obtained have been analyzed. The W1118 strain crossed with the elav-gal4/Fm7; UAS-GFP/cyo driver strain has been used as a control. For the double strains, the elav-gal4/Fm7; UAS-GFP/cyo flies were crossed with the UAS-eIF3d RNAi flies.

A larval development delay was first observed. Larvae downregulating eIF3d and overexpressing RACK1, in fact, reach both the third larval stage with a delay of about 18-24 hours compared to the larvae control, which reached the third larval stage in about 3-4 days. Double transgenic larvae showed a recovery in the timing of larval development. Indeed, they reached the third larval stage at the same time as the control larvae (Figure 35).

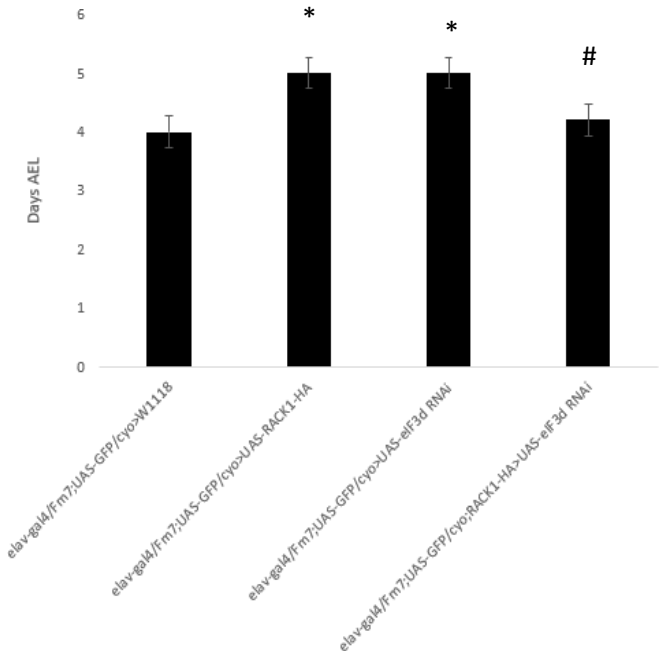


Figure 35. The downregulation of eIF3d and the overexpression of RACK1 in the central nervous system causes a delay of about 18-24 hours compared to the control. The double transgenic flies, instead, showed a recovery in the time necessary to reach the third larval stage, which is of about 3-4 days. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs elav-gal4/Fm7; UAS-GFP/cyo>W1118 and # $p \leq 0.05$ vs elav-gal4/Fm7; UAS-GFP/cyo >UAS- RACK1-HA and elav-gal4/Fm7; UAS-GFP/cyo >UAS-eIF3d RNAi.

Then, the climbing test have been performed to investigate the locomotor behavior.

Flies downregulating eIF3d and overexpressing RACK1 showed an anomalous behavior since flies quickly reached the top of the tube and appeared more reactive in locomotion thanthe control flies, jumping from one side to another of the tube.

Double transgenic flies showed, instead, a recovery in the climbing test, reaching the top of the tube at the same time as flies control linearly (Figure 36).

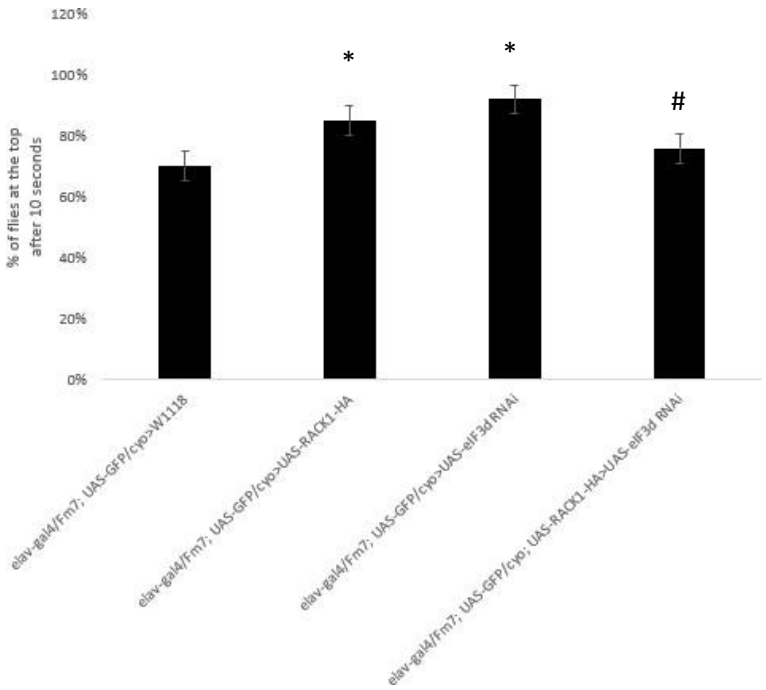


Figure 36. The climbing test revealed that flies overexpressing RACK1-HA and downregulating eIF3d showed an anomalous behavior, unlike what was observed for the double transgenic flies. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs elav- gal4/Fm7; UAS-GFP/cyo>W1118 and # $p \leq 0.05$ vs elav-gal4/Fm7; UAS-GFP/cyo >UAS-RACK1-HAand elav-gal4/Fm7; UAS-GFP/cyo >UAS-eIF3d RNAi.

Later, the structure of the MBs was simply investigated, using the GFP conjugated to the driver strain. Also, in this case, the MBs structure seems to be altered. In fact, Kenyon cells area appeared reduced by about 50% in larvae overexpressing RACK1 and downregulating eIF3d compared to the control. The double transgenic larvae show again a recovery in the Kenyon cells area (Figure 37). Moreover, also the α'/ α and the β'/ β lobes structure is altered with thinner α' and α' lobes and disorganized β' and β lobes. The double transgenic larvae show again a recovery in the Kenyon cells area and in the α/β lobes structure (Figure 38).

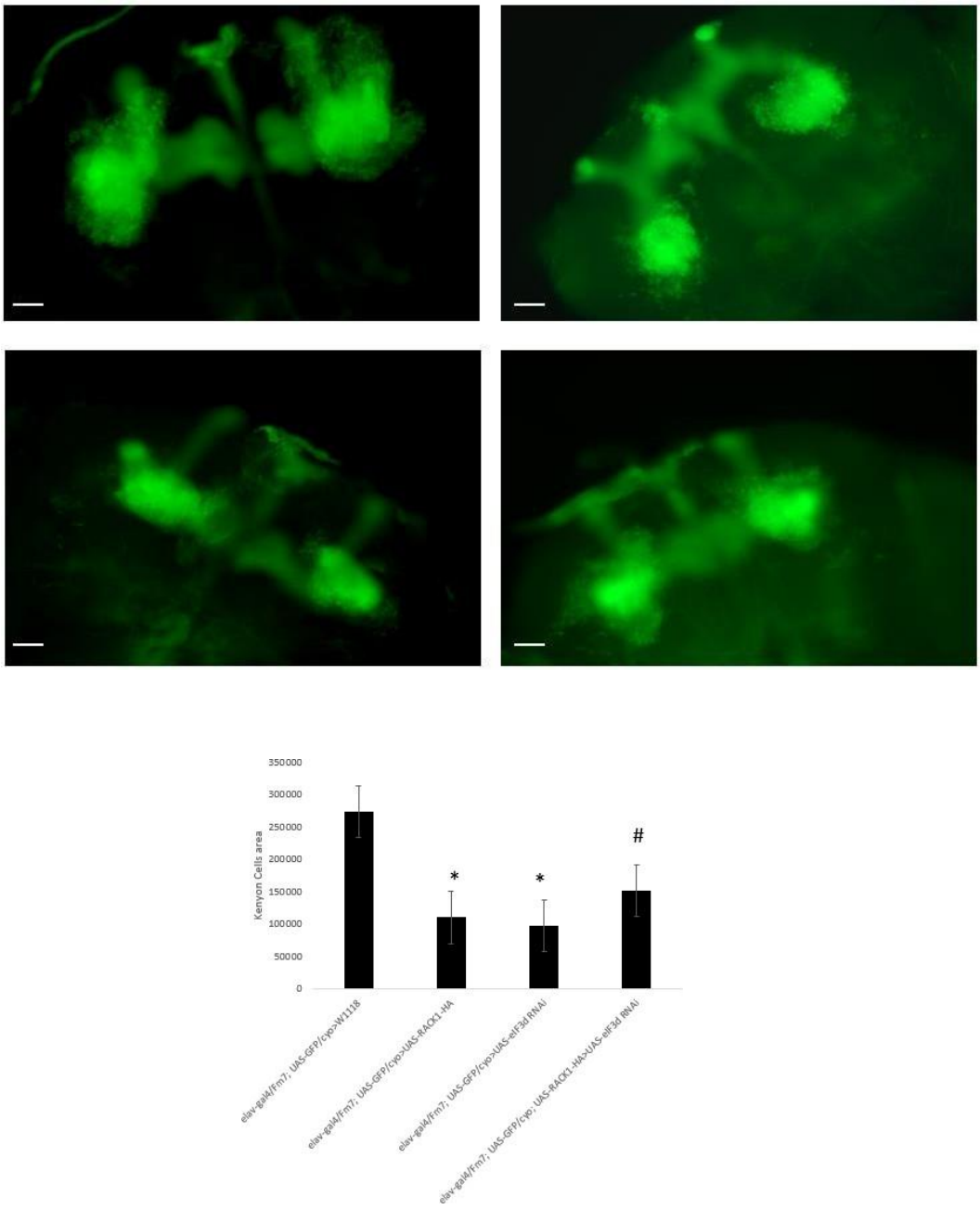


Figure 37. elav-gal4/Fm7; UAS-GFP/cyo>W1118 (top left), elav-gal4/Fm7; UAS-GFP/cyo>UAS-RACK1-HA (top right)**,** elav-gal4/Fm7; UAS-GFP/cyo>UAS-eIF3d RNAi (bottom left), elav-gal4/Fm7; UAS-GFP/cyo; UAS-RACK1-HA>UAS-eIF3d RNAi (bottom right). The downregulation of eIF3d and the overexpression of RACK1-HA determines a strong reduction in Kenyon cells area, which is recovered in the double transgenic flies. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs elav-gal4/Fm7; UAS-GFP/cyo>W1118 and # $p \leq 0.05$ vs elav-gal4/Fm7; UAS-GFP/cyo >UAS-RACK1-HA and elav-gal4/Fm7; UAS-GFP/cyo >UAS-eIF3d RNAi.

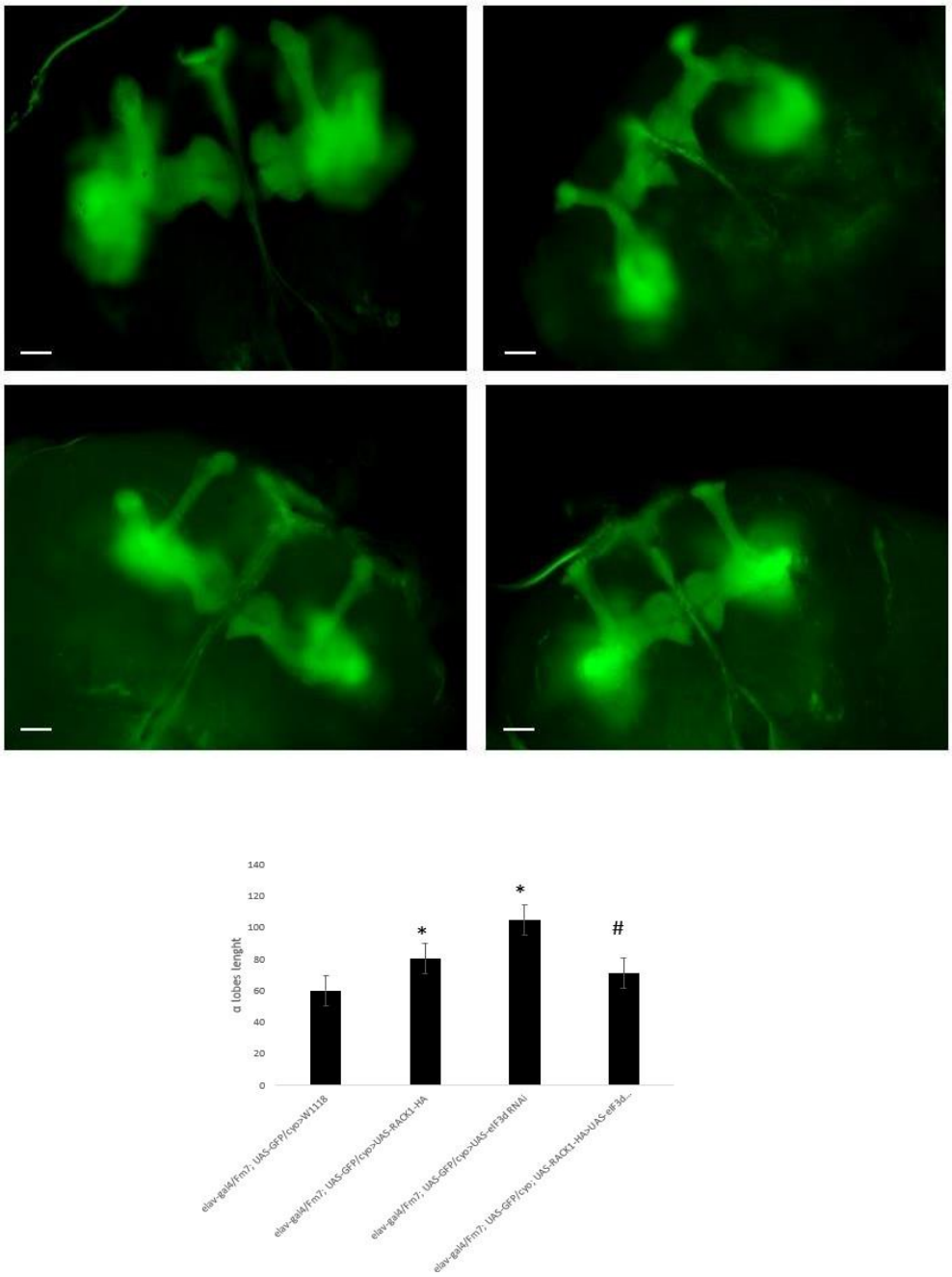


Figure 38. elav-gal4/Fm7; UAS-GFP/cyo>W1118 (top left), elav-gal4/Fm7; UAS-GFP/cyo>UAS-RACK1-HA (top right)**,** elav-gal4/Fm7; UAS-GFP/cyo>UAS-eIF3d RNAi (below left), elav-gal4/Fm7; UAS-GFP/cyo; UAS-RACK1-HA>UAS-eIF3d RNAi (below right). The downregulationof eIF3d and the overexpression of RACK1-HA determine impairments in α lobes structure, which appeared more elongated and thinner. These defects are recovered in the double transgenic flies. Bar graphs represent means ± S.D. One-way ANOVA with Tukey's Post Hoc test. * $p \leq 0.05$, vs elav-gal4/Fm7; UAS-GFP/cyo>W1118 and # $p \leq 0.05$ vs elav-gal4/Fm7; UAS-GFP/cyo >UAS-RACK1-HA and elav-gal4/Fm7; UAS-GFP/cyo >UAS-eIF3d RNAi.

Therefore, the data obtained modulating the expression of eIF3d and RACK1 in MBs are confirmed from the data obtained by modulating the expression of these proteins directly in the nervous system and using another promoter.

Discussion

In this study, we have investigated the role of the eIF4F-independent eIF3d-RACK1 translational axis in the development of Mushroom Bodies and in learning and memory formation in *Drosophila melanogaster*. It is known that eIF4F-dependent protein translation plays a fundamental role in synaptic plasticity, learning, and memory formation. In particular, the eIF4E protein is one of the main targets in the translational control. At the molecular level, eIF4E is critical for translation initiation. Since eIF4E binds the 5'UTR mRNA cap, it allows the anchoring of the 43S preinitiation complex to the mRNA. This step makes possible the 48S preinitiation complex formation and, subsequently, once the initial AUG codon has been identified, the pairing of the ribosomal subunits occurs (Buffington et al., 2014).

Given its role, the eIF4E activity must be finely regulated. In fact, dysfunction of eIF4E leads to impairment in learning and learning-related synaptic plasticity (Gkogkas et al., 2010; Buffington et al., 2014; Santini et al., 2014). Among the eIF4E regulators, there is alsothe Fragile X Mental Retardation protein (FMRP) and its binding partner CYFIP1 (cytoplasmic FMRP interacting protein), which regulates eIF4E, repressing its activity. Mice overexpressing eIF4E or lacking FMRP have impairments in synaptic plasticity and learning,also showing an abnormal spine morphology like that observed in Fragile X disorders.

As further confirmation of this, we have conducted experiments on *Drosophila* Mushroom Bodies revealing that the eIF4E downregulation causes severe structural defects, both in Kenyon cells and in α/β/γ lobes structure. These structural defects correspond to impairments in learning abilities and locomotion behavior.

An interesting aspect is that also the downregulation of eIF3d in Mushroom Bodies determines a similar phenotype. In fact, microscopy images reveal that the Kenyon cells areais strongly reduced while the lobes structure is completely altered. The α'/ α lobes are thinner and more elongated, while the β'/ β lobes appeared more disorganized. These defects cause impairments in learning abilities and in behavior, although compared to eIF4E, eIF3d downregulation makes flies more responsive in movements.

Therefore, eIF4E initiation translation is critical for the normal function of neuronal cells, and it is known that impairments in its activity have dramatic effects on synaptic functionand behavior, affecting the translation of neuronal critical mRNAs (Kats and Klan, 2019).

In the same way, eIF3d probably modulates the translation of other specific mRNAs, important for learning and memory formation. In this case, eIF3d would stimulate the initiation of translation directly recruiting the 43S pre-initiation complex in the mRNA 5'UTR region independently from the eIF4F complex formation (Lee et al., 2016).

This has led to the assumption that beyond eIF4E-dependent translation initiation, the eIF3d specialized translation could play an important role in synaptic plasticity, learning, and memory.

As previously demonstrated, eIF3d interacts with RACK1 and more specifically RACK1 recruits eIF3d on the ribosome, making it available for specific mRNAs translation.

From the literature, it is already known that RACK1 plays a fundamental role in neuronal development since mice lacking RACK1 are embryonic lethal. In addition, RACK1 regulates axon growth (Kershner and Welshhans, 2017) and participates in the dendritic local translation of specific mRNAs to promote neuronal development (Ceci et al., 2012; Russo et al., 2017; Romano et al., 2022).

Therefore, the role of the eIF3d-RACK1 specialized translation has been investigated in the Mushroom Bodies development and in memory formation.

Data obtained downregulating eIF3d have already been discussed. Not surprisingly, the RACK1 overexpression determines defects in the MBs structure, very similar to those observed for eIF3d downregulation. Therefore, adult flies show impairments in Kenyon cells and α/β lobes structure, which determine defects in the learning abilities and in behavior. Moreover, a larval developmental delay has been observed both for eIF3d downregulation and RACK1 overexpression. Our hypothesis is that larvae with an altered expression of eIF3d or RACK1 try to recover the structural defects caused by the eIF3d and RACK1 misexpression, delaying the metamorphosis from larva to pupa. To note that, despite the pupae performing the dendritic pruning, which is necessary to eliminate all those malformed neurites (Rode et al., 2018), the defects caused by eIF3d downregulation and RACK1overexpression are not recovered and then, remain in the adult flies. However, it appears clear that the overexpression of RACK1 determines a less severe phenotype compared to that observed for eIF3d downregulation, probably due to the direct role of eIF3d in the translationinitiation.

Interestingly, when these proteins are expressed together in the MBs, a recovery in MBs structure and then, also in learning abilities and behavior have been observed. Not even a delay in larval development has been observed, as, instead, previously seen. These data would seem to confirm what was previously hypothesized, i.e., the larvae delay their development to try to recover the structural defects determined by the altered expression of eIF3d and RACK1.

To explain these data, we speculate that the defects caused by eIF3d downregulation and RACK1 overexpression could be linked to changes in protein synthesis rate, considering the role of eIF3d and RACK11.

Data obtained revealed that in MBs of single transgenic flies for eIF3d and RACK1 the protein synthesis rate is increased. Also, in the double transgenic flies protein synthesis is slightly increased.

It was assumed that the increase in protein synthesis could be due to both the role of RACK1 in regulating protein synthesis when up-regulated (Romano et al., 2019) and to the competition between eIF3d and eIF4E in binding the 5'UTR mRNA cap (Pestova et al., 2009). In fact, being reduced the endogenous expression of eIF3d, the 5'UTR mRNA cap is more available to the binding with eIF4E.

However, the increase in translational levels, with a consequent increase in proteins produced, could lead to alterations in the MBs, which cause impairments in learning and behavior. In support of this, the double transgenic flies show a protein synthesis less increased compared to the single transgenic flies. In fact, the MBs structure is recovered, together with learning skills and behavior.

Behavioral defects seen previously together with an increased rate of protein synthesis have induced the hypothesis that eIF3d-RACK1 specialized translation may be linked in some way to diseases with autism spectrum. In fact, it is known from the literature that increased synthesis of synaptic proteins and consequently augmented connectivity, which determines dysregulation of the molecular machinery controlling synaptic messenger RNA translation, can cause autism spectrum disorders (ASD) (Gkogkas et al., 2013).

Analyzing, moreover, the morphology of single neurons, it has emerged that in flies downregulating eIF3d and overexpressing RACK1, neurons appear smaller and with fewer arborizations. We have hypothesized that these defects could be determined by alterations in Dlg1 expression, the PSD-95 ortholog. In fact, the overexpression of RACK1 causes an increase in the Dlg1 expression, while the downregulation of eIF3d determines a reduction in Dlg1 expression. Whereas Dlg1 plays an important role in synaptic plasticity (Bertin et al.,

2022), the variations in Dlg1 expression caused by RACK1 overexpression and eIF3d downregulation individually might be another cause of the defects observed in MBs structure and consequently in learning and behavior. Indeed, in the double transgenic flies, in which the Dlg1 expression is reported to wild-type levels, MBs impairments are recovered, and flies show normal behavior and learning capabilities.

In addition, besides eIF4E, also the expression of p-eIF2α has been investigated. In fact, eIF2α is the second major target of translational control mechanisms, whose regulation has been implicated in amygdala-based memory (Trinh and Klann, 2013; Jian et al., 2014). eIF2α phosphorylation plays an important role in synaptic plasticity and long-term memory. In fact, it is reported that increased levels of p-eIF2α determine impairments in synaptic formation, learning, and memory (Costa-Mattioli et al., 2007).

From the data obtained, it appears clear that in flies downregulating eIF3d, and overexpressing RACK1 both individually and together, the expression of p-eIF2α remains unchanged.

These data further confirm that the canonical protein translation and the eIF3d-RACK1 specialized translation act independently of each other, although both play an important role in the development of Mushroom Bodies, learning, and memory formation.

Conclusions and future perspectives

In conclusion, data obtained suggest that the eIF3d-RACK1 specialized translation plays a fundamental role in learning and behavior formation, as demonstrated by the phenotypes obtained modulating the expression of these two proteins. The most interesting fact is that from the data obtained, the eIF3d-RACK1 axis has a role in learning and memory formation comparable to that played by the eIF4E-dependent translation. Moreover, alterations in the eIF3d-RACK1 complex cause impairments in learning and behavior, and at the molecular level comparable to those observed in ASD. This aspect could suggest a role of the eIF3d-RACK1 specialized translation in diseases with autism spectrum.

However, further experiments will be required to better understand the molecular mechanisms underlying the strong defects obtaining and modulating the expression of eIF3d and RACK1 and the structural and behavioral recovery in the double transgenic flies.

Other behavioral tests should be performed to better define memory and behavioral impairments.

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