Protein translation processes during long-term memory formation in the honeybee (*Apis mellifera* L.)

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And the beasts, too, are able both to perceive things corporeal from without, through the senses of the body, and to fix them in the memory, and remember them, and in them to seek after things suitable, and shun things inconvenient.

St. Augustine of Hippo “On the Trinity”

Hence not only human beings and the beings which possess opinion or intelligence, but also certain other animals, possess memory.

Aristotle “On Memory and Reminiscence”

Table of contents

1 Introduction

Memory is a property of our minds that let us store past events or acquired abilities, and transfer them into the present time and future. It is a kind of time machine that let us mentally travel to langsyne, visit places seen long ago, meet people encountered in the past. Memory is central to the perpetuation of the self, to our identity as individuals, as separate human beings. It is also an indispensable part of human reasoning, source of knowledge, moral life, culture and civilization. We have to study memory to understand the mind-body relation, comprehend our experience of time, fight psychiatric and developmental diseases, alleviate emotional traumas and improve educational systems.

1.1 Learning and memory

1.1.1 Memory systems

One of the most popular models conceptualizing memory in cognitive psychology was created by Atkinson and Shiffrin and is depicted on Figure 1-1 (Atkinson and Shiffrin, 1968). This model is based on time phases of memory formation. It assumes existence of three memory stores: sensory store capable of storing limited amounts of information for very brief periods (milliseconds); short-term memory (STM) storing information for somewhat longer times (seconds), but with very limited capacity; long-term memory (LTM) having very large capacity and capable of storing information for very long periods (Sternberg, 2006).
This model become very popular in cognitive and molecular neurobiology and with minor modification will be used in this thesis. There are however many other models which can be used to conceptualize memory system (Murdock, 2003).

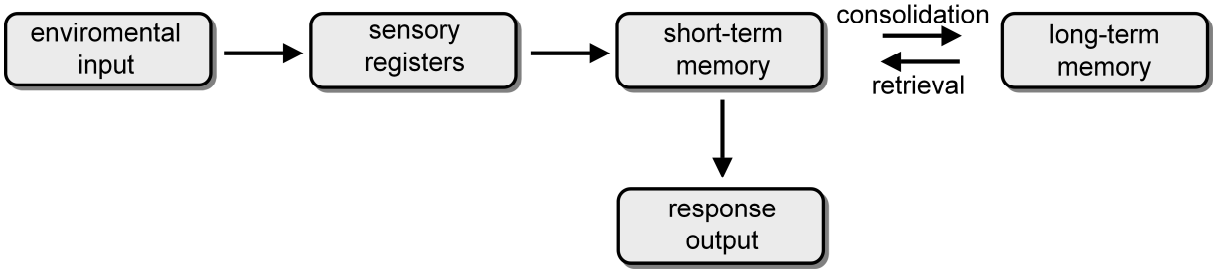


Figure 1-1 Atkinson-Shiffrin model of memory.

Information from environment is stored in sensory registers and later transferred to STM from which it may be used to generate behavioural output or transmitted (consolidated) to LTM for permanent store. Memories stored in LTM may be retrieved to STM and used to create behavioural responses. Adapted from Sternberg, 2006.

Long-term memory can be divided into declarative and procedural one. Declarative (or explicit) memory requires conscious recall and stores information about facts (semantic memory) and events (episodic memory). The other form of long-term memory is procedural (implicit) memory. It is not based on conscious recall and can be studied in invertebrate models as well. Procedural memory can be further subdivided, *inter alia*, into: nonassociative (habituation and sensitisation) and associative (classical and operant conditioning) (Kandel et al., 2000).
During classical conditioning paradigm, animals learn about the temporal dependence of two stimuli and try to detect the most salient environmental stimulus preceding unexpected

appetitive or aversive stimulus (Menzel et al., 2007). In laboratory conditions, the animal is presented with a neutral stimulus (conditioned stimulus, CS) and subsequently with an unconditioned stimulus (US), which elicits an unconditioned response (UR). After a few (sometimes even one) trials the animal associates CS with US, and as a result, CS is now by itself able to evoke UR, which becomes the conditioned response (CR).

1.1.2 Cellular and molecular basis of memory formation

For many years scientist were trying to relate behaviour and psychological phenomena with brain structures and functions, but only in the second half of the 20th century it became possible to research the issue with full scientific scrutiny.

The most important ideas regarding possible cellular mechanisms of memory formation came from Konorski (Konorski, 1948) and Hebb (Hebb, 1949) who suggested, that strengthening of synapses between neurons that are active at the same time, is the cellular mechanism of memory formation. Subsequent decades of research dealing with neural basis of learning and memory in systematically diverse range of organisms led to corroboration of the abovementioned idea that the cellular analogue of learning and memory is experience-dependent modulation of synaptic strength and structure (Kandel, 2001;Lamprecht and LeDoux, 2004).

It is widely believed that cellular models of long-term memory formation are LTP, LTD and LTF. These phenomena are based on strengthening, in long-term potentiation (LTP), or weakening, in long-term depression (LTD), of synaptic connections in mammalian hippocampus (Lynch, 2004;Pastalkova et al., 2006;Whitlock et al., 2006). Long-term facilitation (LTF) is modulation of sensory neuron – motor neuron synapses in a mollusc *Aplysia* (Hawkins et al., 2006).

Recent years brought also enormous progress in understanding molecular basis of memory formation and pointed out that general strategies used by neurons to process and store information are conserved among species evolutionally as different as molluscs (*Aplysia*) (Hawkins et al., 2006), insects (honeybee, *Drosophila*) (Schwarzel and Muller, 2006) and mammals (Alberini et al., 2006).

In general, STM is said to depend on covalent modification of pre-existing proteins while LTM formation requires protein translation and gene transcription that lead to long-lasting morphological changes in neuronal connections (Kandel, 2001).

1.1.3 Learning and memory in *Aplysia californica*

Aplysia californica, (California sea hare) was introduced to neurobiology by Eric Kandel as a radically simple learning and memory model. Upon gentle touching of the siphon, the mollusc contracts and withdraws the gill. This gill-withdrawal reflex may be habituated, sensitised and classically conditioned (Hawkins et al., 2006). Sensitisation is the main experimental procedure in *Aplysia* and it has at least two phases: transient one lasting minutes which can be converted to an enduring one (long-term sensitisation, LTS) by repeated, spaced trials (Castellucci et al., 1986). Neuronal process responsible for sensitisation is the heterosynaptic facilitation (Kandel, 2001), and this phenomenon was extensively studied at the molecular level. The most important factors of LTM formation in the species are serotonin induced PKA (cAMP-dependent protein kinase A), MAPK (mitogen-activated protein kinase) and CREB (cyclic AMP response element binding protein) activation as well as local and dendritic protein synthesis (Casadio et al., 1999;Hawkins et al., 2006).

1.1.4 Learning and memory in the fruit fly (*Drosophila melanogaster)*

Since its introduction three decades ago, the classical conditioning paradigm in *Drosophila* brought substantial knowledge about genetics of learning and memory (Keene and Waddell, 2007;Tully and Quinn, 1985). The most popular paradigm in *Drosophila* is the aversive odour conditioning. This paradigm is based on learning to differentiate between two odours: one odour (CS+) is paired with an aversive electroshock stimulus (US), while the other odour (CS-) remains "unpunished" (McGuire et al., 2005).

Basing on studies of mutants and transgenic organisms, memory in the fruit fly can be divided into 4 phases: STM appearing immediately after learning and lasting to 1h; MTM lasting a few hours; ARM (anaesthesia resistant memory) being formed in the range of hours and decaying after 1 day; and LTM lasting for days (Margulies et al., 2005). ARM is formed after massed conditioning and is not dependent on protein synthesis, whereas LTM is created after multiple spaced conditioning and depends on translation processes (Mery and Kawecki, 2005;Tully et al., 1994).

Genetic studies of memory formation in the fruit fly lead to discrimination of several genes involved in this process: *dunce* (encoding cAMP phosphodiesterase) and *rutabaga* (encoding adenylyl cyclase) engaged in STM; *amnesiac* (encoding a putative neuropeptide) involved in MTM and *radish* partaking in ARM. There are also several genes encoding transcription and translation factors involved in LTM: *dCREB2* (repressor of transcription factor), *staufen* (mRNA localisation), *pumilio* (mRNA binding), *oskar* (translational control) (Keene and

Waddell, 2007;Margulies et al., 2005). Most of these mutations are localised in the mushroom bodies pointing to the foremost function of this structure in memory formation (Keene and Waddell, 2007). These mutants suggest the central role of PKA, protein translation and transcription in memory formation processes in the fly.

1.1.5 Learning and memory in the honeybee (*Apis mellifera*)

Honeybee is a perfect model animal for studying learning and memory in a relatively uncomplicated nervous system. In this insect, one can address the problem at all levels of memory organisation: behaviour, circuits, neurons and molecules. Bees exercise diverse cognitive functions with a very limited brain capacity consisting only of about one million of neurons. Honeybees use elementary forms of learning (classical, operant) as well as many nonelementary forms of learning such as rule, contextual or observatory learning (Menzel et al., 2006;Menzel et al., 2007). In natural conditions they learn in a wide range of situations e.g. foraging, when animals associate flower parameters such as smell, colour, shape and location with food rewards (Menzel and Muller, 1996a).

In the laboratory, memory can be studied using classical conditioning of restrained animals (Bitterman et al., 1983;Menzel et al., 1974) utilising observation that the bee extends its proboscis as reaction to the application of sucrose solution to the antennae, proboscis or the tarsi of the front legs. This phenomenon is called proboscis extension response (PER) (Menzel, 1990). PER may be conditioned classically (Kuwabara, 1956;Takeda, 1961) and during this procedure a neutral odour (CS) is applied shortly before the sucrose presentation (US) to the antennae or proboscis. As the result, the animal associates both stimuli and reacts to an odour with proboscis extension (CR) (Bitterman et al., 1983). A single conditioning trial consisting of an odour presented 1 to 3 seconds before sucrose, typically leads to 50% PER probability and decays after 1 day. When bees are presented with multiple conditioning trials, the response reaches 80-90% PER probability and lasts over a few days (Menzel and Muller, 1996b).

In the honeybee, the olfactory information (CS) is perceived through the chemoreceptors on antennae and mouthparts and is transmitted to the glomeruli of the antennal lobe (AL). Then signals are delivered through antenno-glomerularis tracts (AGTs) to the calyces of the mushroom bodies (MB) and the lateral protocerebral lobe (LPL) (Menzel and Muller, 1996b). Sucrose (US) is perceived by the sensory neurons on the antennae and proboscis and this information is transferred to the subesophagal ganglion (Hammer and Menzel, 1995). One of the neurons localised there, the VUM_{mx1} (ventral unpaired median mx1 neuron), sends

arborisations that converge with the olfactory (CS) pathway in mushroom body calyces, antennal lobes and lateral protocerebral lobe (Menzel and Muller, 1996b). This neuron seems to be critically involved in memory processes as it substitutes the sucrose input in classical conditioning: its depolarisation (Hammer, 1993) or the application of putative transmitter (octopamine) (Hammer and Menzel, 1998) is sufficient to replace the US in the CS-US conditioning protocol.

The short-term memory trace can be localized to the ALs and α-lobes of the MBs within minutes after conditioning (Menzel et al., 1974), as well to the calyces of the MBs (Erber et al., 1980). It seems that honeybee mushroom bodies are the most important place of associative memory trace development in the species (Menzel and Muller, 1996a). Other neurons, such as an MB-extrinsic neuron, the PE1, are also involved in associative learning (Mauelshagen, 1993;Menzel and Manz, 2005;Okada et al., 2007).

Memory in the honeybee can be divided into 3 phases: STM (short-term memory) lasting in the range of minutes, MTM (mid-term memory) lasting for hours and the stable LTM (long-term memory) lasting for days and weeks. The last phase may be dissected into two subphases: eLTM (early LTM) lasting from 1 to 2 days, which depends on translation (new protein synthesis) and lLTM (late LTM) lasting from 3 days on and depending on transcription (new mRNA synthesis) (Schwarzel and Muller, 2006).

On the molecular level, memory in the bee is mediated by factors such as PKA, PKC (protein kinase C), glutamate, MAPK or CREB, that are known from other model systems as well (Muller, 2002;Schwarzel and Muller, 2006).

In the honeybee, as in other model systems, multiple training trials render a prolonged PKA activation (in the range of 2-3 min) and this is required for LTM induction (Muller, 2000). This prolonged activation is mediated by NO/cGMP system in the ALs (Muller and Hildebrandt, 2002). Additionally, downregulation of the amount of catalytic subunit of the PKA amount in the brain also deteriorates long-term memory performance (Fiala et al., 1999). The other second messenger system used in LTM formation across species is PKC system (Selcher et al., 2002) and in the honeybee, inhibition of PKC leads to an LTM deficit (Grunbaum and Muller, 1998). MAPK pathway is implicated in long-term memory formation in mammals (Kelleher, III et al., 2004a) as well as in the bee (Plekhanova, 2005). Glutamate was shown to be involved in LTM formation in the honeybee as local release of glutamate in the mushroom bodies increases long-term memory performance (Locatelli et al., 2005). Moreover, blocking of NMDA receptor or glutamate transporter during training leads to impairment of LTM (Si et al., 2004). Honeybee homolog of the CREB was found in the brain

of the insect (Eisenhardt et al., 2003) and this transcription factor is involved in LTM of the insect (Eisenhardt, personal communication).

1.2 Protein translation processes

1.2.1 Introduction

As written above, prolonged and stable forms of memory and synaptic plasticity require synthesis of new proteins. In eukaryotic cells, translation is a very complex process requiring precise and not yet fully understood control system as well as substantial amount of cellular energy. Translation is conventionally divided into 3 main phases: initiation, elongation and termination. Each of the phases requires its own set of highly specialized proteins called eukaryotic initiation (eIF), elongation (eEF) and release (eRF) factors, respectively (Merrick and Nyborg, 2000).

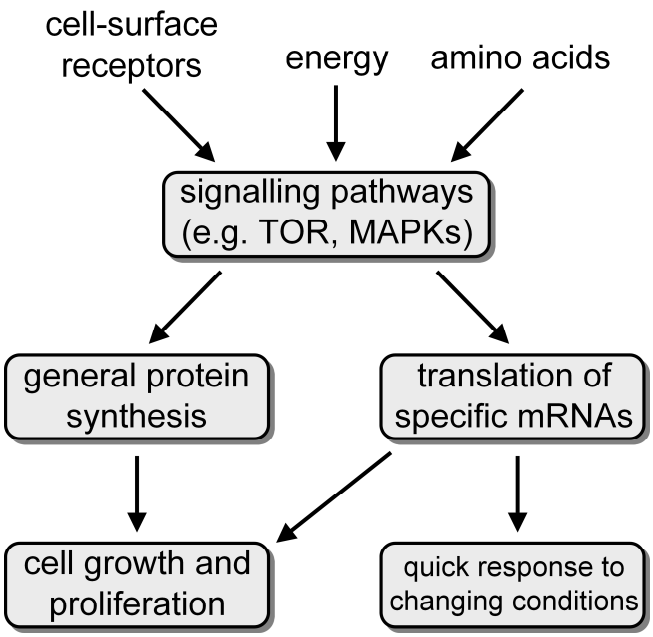


Figure 1-2 Overview of the translation control system in eukaryotes.

Information from extra- and intra- cellular stimuli converges on signalling pathways (e.g. MAPKs, TOR) controlling translation factors and RNA-binding proteins. General translation is responsible for cell growth and proliferation, whereas specific mRNAs are translated as a quick response to changing conditions. Adapted from Proud, 2007.

Translational processes may be regulated at the level of translational factors and at the level of signalling pathways modulating these factors. Signalling pathways integrate extracellular (e.g. information from surface receptors) and intracellular (e.g. energy level, amino acids availability) signals and thus provide central and integrative targets for translational control. Regarding specificity of translation, this process may be divided into two types. First type is general protein synthesis, being controlled by general translation factors. Second type is

specific mRNAs translation being controlled by subtle changes in general translation factors activity as well as specific RNA-binding proteins. General protein synthesis is responsible for cellular growth and proliferation, whereas translation of specific mRNAs has critical role in rapid synthesis of specific proteins as a quick answer to changing conditions. Overview of eukaryotic translation system is presented on Figure 1-2 (Proud, 2007).

1.2.2 Control of protein synthesis by translation factors

Protein synthesis processes may be controlled at the level of translation factors. Each of the translation phases (initiation, elongation and termination) requires its own set of factors. Most control is exerted on initiation phase, but there are also many examples of modulating elongation. Termination is usually thought to be unregulated and will not be discussed here. There are a few mechanisms by which initiation phase can be started, and two of them seem to be particularly important in neurons: cap-dependent initiation, which is the classic, "handbook" form of initiation and cap-independent initiation that is an unorthodox way of initiating translation.

1.2.2.1 Cap-dependent initiation

Cap-dependent initiation of protein synthesis is the typical way of starting translation and mechanism on which most of translational control is exerted (Hershey and Merrick, 2000). One of the most important events during this process is activating of the 5'-cap structure (7-methylguanosine attached to the 5'-end of primary transcript) of the mRNA by binding of eIF4E (eukaryotic initiation factor 4E) and other initiation factors. This enables binding of the large ribosomal subunit to the mRNA and scanning for the first start codon (Lopez-Lastra et al., 2005;Merrick, 2004).

1.2.2.2 Cap-independent initiation through IRES

One of the unorthodox ways of initiating translation is the cap-independent initiation. It was discovered 20 years ago, that translation machinery could be assembled independently of 5'-cap structure, at positions very close to the start codon of mRNA. These positions are called internal ribosome entry sites (IRES) and contain specific, although unrelated, secondary structures. IRES seems to mimic eIF4E and renders initiation independent of eIF4E and cap (Lopez-Lastra et al., 2005). These type of translation initiation is enhanced when general, cap-dependent initiation is impaired, and may be used to provide protein synthesis from specific mRNAs during general protein synthesis impairment (Baird et al., 2006). IRES sequences are

found in many vertebrate (Mokrejs et al., 2006) and invertebrate (e.g. *Aplysia* neurons) (Ross et al., 2006) mRNAs.

1.2.2.3 Elongation phase

After localisation of the start codon during initiation phase, the main part of protein synthesis cycle, elongation, begins. Elongation consumes over 99% of energy used during translation (Proud, 2007) and one amino acid is added to the growing polypeptide at a time. Ribosome selects aa-tRNA (amino acid – tRNA complex) according to the sequence of the triplet codon on the mRNA strand and forms a peptide bond between the growing peptide and the incoming amino acid. Three elongation factors are involved in catalysis of this process: e eEF1α (EF1A), eEF1β (eEF1B) and eEF2 – they secure speed and accuracy of the process. eEF2 is the main elongation factor and catalyses translocation of the growing peptide chain on the ribosome – it physically forces the peptidyl-tRNA (newly formed peptide – tRNA complex) out of the A (amino acid) site to the P (peptide) site of the ribosome (Merrick and Nyborg, 2000).

1.2.3 Signalling pathways controlling translation

1.2.3.1 Control of the initiation phase (eIF4E)

Initiation phase is influenced by many factors and is the last control point for synthesis of a protein from given mRNA (Mathews et al., 2000).

One of the most important players controlling cap-dependent initiation of translation is eIF4E. This factor is sequestrated and inactivated by 4E-BP (eIF4E binding protein), which in turn is deactivated by TOR (Proud, 2007). Phosphorylation of eIF4E plays an important role in controlling binding of the protein to mRNA cap structure, but the overall effect of phosphorylation is not clear as data are contradictory (Klann et al., 2004). Deactivation of the factor was reported to function as a switch from cap-dependent to cap-independent (IRES-dependent) initiation of translation in *Aplysia* neurons (Dyer et al., 2003). Figure 1-3 depicts a scheme presenting control over eIF4E.

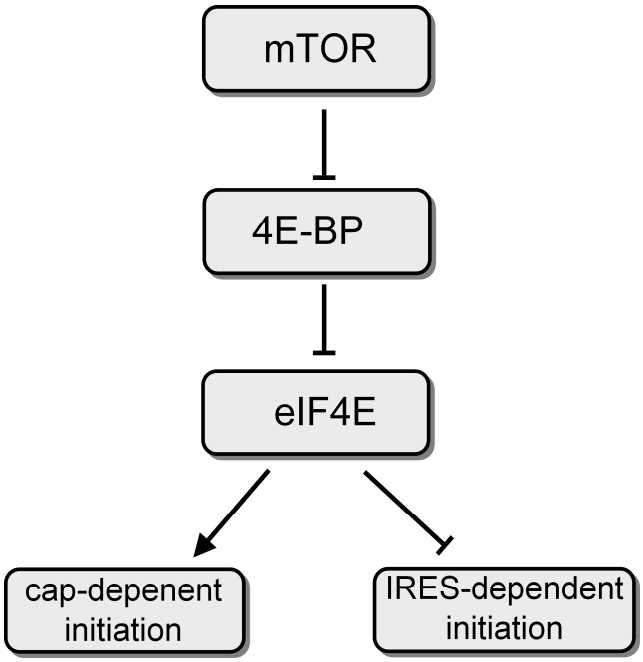


Figure 1-3 Function and regulation of eIF4E.

eIF4E is a positive regulator of cap-dependent initiation of translation and negative regulator of cap-independent (IRES-dependent) one. This factor can be sequestered by 4E-BP, which in turn is inhibited by TOR.

1.2.3.2 Control of the elongation phase (eEF2)

Control of the elongation phase of translation is concentrated on eEF2 phosphorylation at Thr56 (Browne and Proud, 2002). Phosphorylated eEF2 is unable to catalyze translocation (Ryazanov and Davydova, 1989) and thus substantially decreases the rate of elongation and handicaps protein synthesis (Ryazanov et al., 1988). In mammalian tissues eEF2 is phosphorylated by a specific kinase - eEF2 kinase (eEF2K) (Ryazanov et al., 1988), but this enzyme was not found in insects. Moreover, insects seem to lack the whole family of non-orthodox α-kinases (which comprises of eEF2K and a few other enzymes) (Ryazanov, 2002). In mammalian tissues (Figure 1-4) eEF2K is activated by Ca^{2+}/calmodulin and cAMP/PKA (Ryazanov et al., 1988), as well as S6K (S6 kinase) and AMPK (5'AMP-dependent protein kinase) (Browne and Proud, 2002).

On the other hand, eEF2 is activated by dephosphorylation catalysed by PP2A (protein phosphatase 2A) (Browne and Proud, 2002). PP2A is triggered *inter alia* by PKA (Ahn et al., 2007). It seems thus that PKA may also activate eEF2, but this was not so far shown experimentally. Surprisingly, cAMP was reported to activate eEF2 in a non-PKA way (Feschenko et al., 2002). This complicates the overall picture of cAMP-PKA-PP2A-eEF2 pathway, but constituents of such pathway do exist.

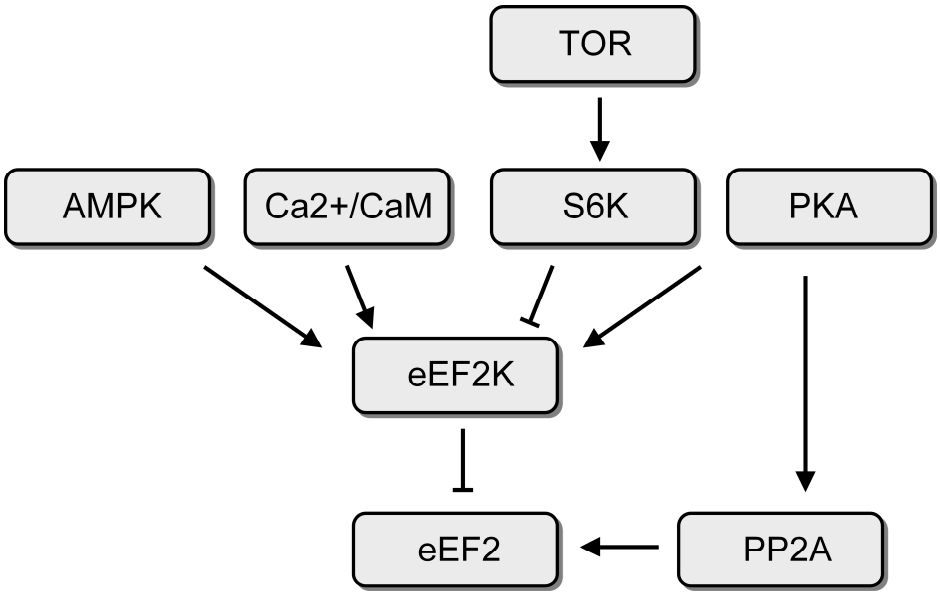


Figure 1-4 Factors controlling eEF2 activity in mammalian cells.

The protein is inactivated by phosphorylation through eEF2K, and activated by dephosphorylation catalysed through PP2A. AMPK, Ca^{2+}/calmodulin and PKA activate eEF2K what blocks eEF2. TOR-S6K pathway inhibit eEF2K what activates eEF2. On the other hand, PKA is able to enhance PP2A function and thus probably activate eEF2 (but see remarks in the text).

1.3 Protein translation in memory formation and synaptic plasticity

Involvement of protein synthesis in memory formation was suggested over four decades ago (Flexner et al., 1963). In this pioneering study, intracerebral injections of puromycin, a protein synthesis inhibitor (PSI), abolished memory formation in rodents. Subsequent wave of experiments lead to general conclusion, that protein synthesis inhibitors injected to the brain shortly before training do not inhibit acquisition phase, and animals exhibit normal memory performance for a short time. However, in the range of days memory performance gradually deteriorates to the baseline. Degree of this deterioration depends on species, training protocol, blocking agent etc, but usually can be observed in the range of days. PSIs are most effective when injected shortly before training, and as the interval between training and injection increases, memory performance becomes resistant to the translation blockers. This suggests the existence of a short, well-defined time window around training, during which protein synthesis processes are specifically involved in memory formation. This concept was expressed as “brain protein synthesis during or shortly after training is required for the establishment of long-term memory”(Davis and Squire, 1984). In spite of some criticism (Gold, 2008), this idea is still a tenet of molecular neurobiology, and has been reformulated recently (Klann and Sweatt, 2008).

1.3.1 Translation in neurons: importance of dendritic protein synthesis

Ramon y Cajal discovered that survival of dendrites and neuritis depends on the cell body as the outgrowths degenerate upon dissection from the cell body. Subsequently he formulated a thesis that the cell body is the “trophic centre of the neuron”. This idea was supported by the discovery of RNA and protein synthesis apparatus in the cell bodies of neurons and macromolecular transport mechanisms in axons and dendrites. These findings led to the tenet saying that proteins are build exclusively in the soma and are subsequently transported to the outgrowths (Steward and Schuman, 2003). This concept was not refuted by discovery of ribosomes in dendrites (Bunge et al., 1965). Acceptance of dendritic protein synthesis was brought by experiments demonstrating perikaryon-independent translation in dendritic spines (Steward and Fass, 1983) and existence of polyribosome complexes in the same structures in rat hippocampus (Steward and Levy, 1982). The conception was corroborated by direct proof showing that fractionated synapses incorporate radiolabeled amino acids into proteins (Rao and Steward, 1991).

1.3.2 Dendritic protein synthesis and neural activity

Research over the last 10 years showed the importance of protein synthesis processes in neural activity. BDNF-induced enhancement of synaptic transmission requires dendritic protein synthesis (Kang and Schuman, 1996), and this discovery initiated a wave of experiments based on an idea that activity-induced protein synthesis occurs at the stimulated synapses rather then in cell bodies (Kelleher, III et al., 2004b).

Modern assumptions view local, dendritic protein translation as an important factor in neuronal activity during synaptic plasticity and memory formation (Steward and Schuman, 2003). They point to its central role in stabilising activity-evoked changes in synaptic strength, in a single synapse-restricted way (Kelleher, III et al., 2004b). Local regulation of translation in dendrites is thought to provide the local sites with the possibility to control its function in an autonomous, nucleus-independent way as well as facilitate biochemical economy (Kindler et al., 2005).

1.3.3 Protein translation during LTP and LTD

Based on the dependence of LTP and LTD on new protein synthesis, these phenomena are differentiated into two phases. The first stages are called early-LTP and early-LTD (E-LTP, E-LTD) that last usually from 1 to 3 hours and do not depend on translation. The next phases are called late-LTP or late-LTD (L-LTP, L-LTD). They are quite persistent, last over 8 h, and require new protein synthesis (Abraham and Williams, 2008;Sutton and Schuman, 2006). L-LTP and L-LTD are considered neural substrates of LTM and much of our knowledge about translation during memory-related processes comes from studying these phenomena in mammalian hippocampal slices (Govindarajan et al., 2006). Although LTP and LTD change the synaptic strength in opposite directions, they presumably rely on common biochemical pathways such as MAPK and mTOR dependent translation (Kelleher, III et al., 2004b).

Although it is very often supposed that LTP induces general protein synthesis enhancement (Kelleher, III et al., 2004b), direct evidence is very limited (Feig and Lipton, 1993;Kelleher, III et al., 2004a) and most of such assumptions are based only on observed activation of translation control pathways (Kelleher, III et al., 2004b). There are also results that contradict this idea: some researchers report a decrease in *de novo* protein synthesis after LTP induction (Chotiner et al., 2003). In my opinion, the issue whether LTP induction augments or retards general levels of protein synthesis remains unresolved and a final conclusion cannot yet be made.

Amount of translation machineries available at the synapse is very limited (Ostroff et al., 2002) and this suggests that there is a competition between mRNAs for ribosomes. Consequently, there is a postulate for an mRNAs selection mechanism that decides which mRNAs are bound to be translated into proteins upon synaptic stimulation (Schuman et al., 2006). According to this idea, the overall rate of protein synthesis is fairly stable, and does not change significantly after stimulation. What changes is the type of mRNAs from which proteins are translated and the general protein synthesis is not significantly upregulated.

There are many indications that local, dendritic translation is the most important form of LTP-related protein synthesis in neurons, while perikaryal one plays merely a permissive role. For example LTP may be induced in hippocampal slices from which cell bodies are removed and this process still requires new protein synthesis (Kang and Schuman, 1996). The similar effect is observed after LTD induction (Huber et al., 2000). Additionally, focal application of PSIs to dendritic regions inhibits LTP in intact slices (Bradshaw et al., 2003). Importance of dendritic translation was directly proved in an elegant experiment, in which dendritic targeting of CaMKIIα was impaired. There was no change in E-LTP, but L-LTP, as well as *in vivo* memory performance, were strongly reduced (Miller et al., 2002). Another example shows that following tetanisation, polyribosomes translocate from dendritic shafts to spines (Ostroff et al., 2002). Protein translation was also shown to contribute integrally to the enlargement of dendritic spines after LTP induction (Tanaka et al., 2008). All these experiments suggest that local, dendritic, rather than perikaryal, protein synthesis is indispensable for L-LTP.

If protein translation is a crucial process during synaptic plasticity one has to ask, which proteins are specifically translated as the result of LTP. A few proteins are very quickly synthesised following stimulation leading to LTP. First described protein was CaMKII, whose amount in dendrites increases as fast as 3 min after end of LTP stimulation (Ouyang et al., 1999). Other quickly translated mRNAs include, *inter alia*, fragile X mental retardation protein (FMRP) (Hou et al., 2006) and Arc (Chotiner et al., 2003;Messaoudi et al., 2007). The other group of such mRNAs are 5'TOP mRNAs that code elements of translation machinery: elongation factors and ribosomal proteins such as eEF1α (Tsokas et al., 2005) or eEF2, rpS6 (ribosomal protein S6) or PABP (poly(A) binding protein) (Tsokas et al., 2007).

Protein translation is usually thought to merely provide new proteins for building synapses during memory formation and synaptic plasticity. A new set of experiments suggests that translation and its control system may also play an integrative and control function during neuronal activity. Such integrative role is based on following phenomenon: major pathways

controlling translation (e.g. MAPK and TOR) once activated, can maintain this state over minutes. L-LTP and L-LTD evoked translation depends on activation of this kinases, thus the pathways enable integration of inputs in the range of minutes. This long-range, kinase-dependent integration is referred to as synaptic integration in translational activation (SITA) (Govindarajan et al., 2006). Translation machinery can also decode electrical activity of postsynaptic neurons and transform them into biochemical signals: one of the elongation factors (eEF2) is activated by action potentials, while miniature neurotransmission turns the factor off (Sutton et al., 2007). Thus, protein synthesis system can react in opposite directions to small changes in electrical activity, what presumably is of great importance during memory formation.

One of the initiation factors (eIF2α) bidirectionally regulates synaptic plasticity and memory and functions as the "master switch" from short- to long-term synaptic plasticity and memory. Moreover, this protein controls an important transcription factor (ATF4/CREB2) (Costa-Mattioli et al., 2007). This experiment suggests that in neurons, protein synthesis system controls mRNA synthesis and proposes a new paradigm assuming that translation governs transcription (Hoeffer and Klann, 2007).

1.3.4 Protein synthesis system during spatial learning and contextual conditioning in mammals

In contrast to synaptic plasticity, detailed engagement of the translation system in memory formation is much less studied. There are however reports describing memory deficits in transgenic rodents with an impaired protein translation system. For example interfering with regulation of MAPK in mice, deteriorates spatial learning and contextual fear conditioning (Kelleher, III et al., 2004a). Downregulation of mice GCN2 kinase (inhibitor of translation initiation factor eIF2α) renders very peculiar properties of spatial memory: LTM is enhanced after training in weak procedures while memory is deteriorated after strong conditioning. Similar phenomenon is observed in LTP (Costa-Mattioli et al., 2005). Deletion of 4E-BP reduces spatial and conditioned fear memory in mice (Banko et al., 2005). Another example is that extinction is reduced in mice lacking CPEB (cytoplasmic polyadenylation element binding protein - a protein activating dormant mRNA) (Berger-Sweeney et al., 2006). Interestingly, one of initiation factors (eIF2α) act as switch from short- to long-term memory (Costa-Mattioli et al., 2007). There are also experiments directly addressing local, dendritic protein translation during memory formation. Impairment of CaMKIIα mRNA targeting to dendrites result in deficits in spatial memory and associative fear conditioning (Miller et al.,

2002). Another interesting experiment shows that after fear conditioning in mice, newly synthesised AMPA receptors are recruited to the specific type of dendritic spines in hippocampus (Matsuo et al., 2008).

These experiments provide a sound basis for the notion that protein translation is a crucial factor in memory formation processes in rodents.

1.3.5 Protein translation during synaptic plasticity and memory formation in *Aplysia*

Protein translation processes play very important role in memory formation and synaptic plasticity in the mollusc *Aplysia* (Hawkins et al., 2006). In the species, it was shown that individual synapses can be modulated independently, giving rise to the synaptic specificity during formation of cellular analogues (long-term facilitation) of memory. This synapse-specific LTF depends on the local protein synthesis in the presynaptic cell and is blocked by rapamycin (Casadio et al., 1999;Martin et al., 1997). However, somatic protein synthesis takes also part in LTF formation (Guan and Clark, 2006).

Long-term sensitisation (LTS) and LTF in *Aplysia* depend on changes in synaptic structure, formation of new synapses and activation of silent ones (Hawkins et al., 2006). After LTS induction there is an increase in number, size of vesicles in active zones of the synapses (Bailey and Chen, 1983), as well as a strong increase in the number of varicosities per single sensory neuron (Bailey and Chen, 1988). Rapid activation of silent synapses can also be observed during LTF and this process is dependent on translation but not on transcription. Generation of new varicosities depends however on both transcription and translation (Kim et al., 2003). During synaptic plasticity mRNAs are not only simply translated, but also rapidly translocated to the distal parts of neurites and this process is indispensable for maintenance of the newly formed connections (Lyles et al., 2006).

Very interesting question is how synaptic enhancement can be stable during intensive protein turnover. One of such mechanisms could be a model based on CPEB. When *Aplysia* CPEB is activated by serotonin it acquires prion-like, self-perpetuating properties that can enhance protein translation at activated synapses in a self-sustaining, synapse-specific and long-term manner (Si et al., 2003a;Si et al., 2003b).

All these experiments point to the central role of protein translation processes in memory and synaptic plasticity in *Aplysia*.

1.3.6 Protein translation during memory formation in *Drosophila*

Surprisingly, translational processes during memory formation in *Drosophila* were not studied intensively, but also in this species protein translation is an indispensable part of long-term memory formation processes.

The first three phases of memory in *Drosophila*: STM, MTM and ARM do not depend on protein translation, whereas LTM does (Keene and Waddell, 2007). For example, feeding flies for a long time with a protein synthesis inhibitor (cycloheximide) leads to LTM impairment while ARM remains insensitive to the treatment (Mery and Kawecki, 2005;Tully et al., 1994). Also the pathways controlling translation (*Staufen/pumilio*) were shown to be indispensable for LTM formation (Dubnau et al., 2003). One of the proteins critically involved in memory in many species is CaMKII. After LTM induction, its mRNAs in specific glomeruli of antennal lobes rapidly translocate to postsynaptic sites and the protein is quickly translated there. Proteasome and RNA interference (RISC) pathway also partake in this process (Ashraf et al., 2006).

1.3.7 Protein translation during memory formation in the honeybee

First experiments regarding translation during memory formation in the honeybee led to a surprising notion that the honeybee is quite an exception, as it seemed that new protein synthesis is not necessary for memory creation. Cycloheximide, a protein synthesis inhibitor, failed to reduce LTM performance in a series of experiments (Menzel et al., 1993;Wittstock et al., 1993;Wittstock and Menzel, 1994). Subsequent results contradicted this hypothesis: another PSI, anisomycin, did impair memory 4 days after conditioning (Wustenberg et al., 1998). Further results corroborated the idea that protein translation is involved in LTM in this insect. Translation blocker emetine injected before 3 trials olfactory conditioning decreases memory performance from 1st day on, leaving acquisition and STM intact. Injecting emetine before 1 trial conditioning does not influence memory. These experiments were done on hungry animals - when bees receive additional food before conditioning they do not form typical LTM. Moreover, this memory is independent of protein synthesis as one of the PSIs (emetine) does not deteriorate memory scores in hungry animals (Friedrich et al., 2004). Another example of protein synthesis processes during memory in the bee shows that emetine injected before extinction trials leads to decrease of the spontaneous recovery (Stollhoff et al., 2005).

While one can be sure that memory processes in the honeybee depend on protein synthesis, virtually nothing is known about specific involvement of translation factors and signalling pathways controlling translation in this process.

1.4 Aims of this work

In the previous chapter, translation processes during memory formation and synaptic plasticity were presented. Although it is well known that memory formation critically depends on new protein synthesis, the specific role of translation factors in this process is poorly investigated. Much attention was given to the initiation phase of translation while elongation remains unsatisfactorily researched. Protein synthesis processes during memory formation were mainly studied in vertebrates, and this field is not well understood in insects like honeybee and *Drosophila*. This work will try to use bottom-up as well as top-down approach to tackle this issue. Most of research aiming at elucidation how biochemical pathways work during memory processes tries to manipulate these pathways and search for behavioural changes. The opposite approach: manipulation of behaviour and searching for biochemical changes is much less common. In this thesis, both strategies will be used. On one hand it will be asked how blocking of translation or blocking of processes that modulate translation influence memory and on the other hand how learning regulates translational machinery. The major aim will be to identify the translation factors and translation regulatory pathways that are modulated by olfactory learning in the brain of the honeybee. Subsequent manipulation of the translation machinery and its control system will help to corroborate the involvement of specific proteins in memory formation. Moreover, experiments are planned to address the problem of how the feeding status affects learning and how these processes interrelate with translation. Finally, the role of protein degradation in memory formation will be addressed.

2 Materials and methods

2.1 Materials

2.1.1 Antibodies and enzymes

- pheEF2 (phospho-eEF2 (Thr56)) Cell Signaling Technology
- eEF2 Cell Signaling Technology
- phS6K (phospho-p70 S6 kinase) Cell Signaling Technology
- anti-rabbit IgG, biotin conjugated Sigma-Aldrich
- anti-rabbit IgG, Cy3 conjugated Sigma-Aldrich
- anti-rabbit IgG, peroxidase antibody Sigma-Aldrich
- ExtrAvidin–Alkaline Phosphatase Sigma-Aldrich

2.1.2 Chemicals

- ATP Sigma-Aldrich
- bromo-cAMP Sigma-Aldrich
- carnation oil local pharmacy
- CNB Invitrogen
- DMNB Invitrogen
- fostriecin Sigma-Aldrich
- LY294002 Cell Signaling Technology
- MG132 Tocris
- okadaic acid Calbiochem
- rapamycin Sigma-Aldrich
- SB 203580 Tocris
- Western Lightning (Enhanced Luminol) PerkinElmer
- ECL Plus Amersham
- The rest of chemicals was bought from
    - Applichem ($MgCl_2$, NaCl, Na_2HPO_4, Tris, SDS)
    - Roth (KH_2PO_4, BSA, glycin, HCl, KCl, Roti-Histol, Roti-Histokitt, glycerol)
    - Sigma-Aldrich (TWEEN, EGTA, UREA, EDTA, natrium azide, pNPP, acrylamide, bis-acrylamide APS, mercaptoethanol, NBT, Triton X-100)
    - Fluka (paraffin)

- Z-Chem (paraformaldehyde, isopropanol)

2.1.3 Equipment

Bee tubes (copper, aluminium): self-made

Binocular: SZ40 (Olympus)

Blotting apparatus: Trans-BlotSD (BioRad)

Digital camera: ProgRes C10 (Jenoptik)

ELISA-reader: SLT 400 ATX and safire^2 (Tecan)

Fluorescence microscope: Leitz DM RB (Leica Microsystems)

Gel electrophoresis apparatus: self-made

Homogenisator: glass-teflon Duall 1 ml (Kimble Kontes)

Microinjector: Picospritzer II (General Valve Corporation)

Micropipette puller: P-97 (Sutter Instrument Co.)

Microtome: Autocut 2040 (Reichert-Jung)

Photo-adapter: SZ-CTV (Olypmus)

Sonicator : HTU Soni 130 (G. Heinemann)

UV lamp: UV Flash (T.I.L.L. Photonics)

2.1.4 Consumables

autoradiography film developer: G150 (Agfa)

autoradiography film fixer: G354 (Agfa)

autoradiography film: X-OMAT AR (Kodak)

dental wax: medium (Ubert)

ELISA 96-well microplates: Falcon 353915 (BD Biosciences)
Immulon 1B (Thermo Labsystems)

glass capillary piston: Capilettor Stick 1 – 5 µl (Selzer)

glass capillary: Capilettor 1 – 5 µl (Selzer)

microscope slide: SuperFrost white (Roth)

nitrocellulose membrane: Optitran BA-S 83 (Schleicher & Schuell)

silicon: Baysilone medium viscosity (Bayer)

sucrose: sugar from a local shop

syringe for odour application: 20 ml (Roth)

test tubes: 1,5 ml; 0,3 ml (Eppendorf)

toothpicks: local shop

tweezers: Dumont precision tweezers 5 and 2 (Dumont)

Whatman filter paper: (Schleicher und Schuell)

2.1.5 Buffers

Western Blotting buffers:

- all buffers were prepared with MiliQ water
- PBS (Phosphate Buffered Saline): NaCl 137 mM; KCl 2,7 mM; Na_2HPO_4 10 mM; KH_2PO_4 1,7 mM
- Blocking buffer: BSA 1%; TWEEN 0,3%; in PBS
- Homogenisation buffer: EGTA 5 mM; in PBS
- Blotting buffer: methanol 20%; Tris 50 mM; glycin 20 mM; SDS 140 µM
- Resolving buffer: Tris 1,5 M; SDS 0,4% (w/v); pH 8,8 (HCl adjusted)
- Stacking buffer: Tris 0,5 M; SDS 0,4% (w/v); pH 6,8 (HCl adjusted)
- Running buffer: Tris 25 mM; glycin 192 mM; SDS 1% (w/v)
- Acrylamid 30%: acrylamid 30% (w/v); bisacrylamid 0,8% (w/v)
- APS: ammonium persulfate 10% (w/v)
- Loading (sample) buffer: Tris-HCl 0,5 M; SDS 5%; 2-mercaptoethanol 5%; glycerol 20%; pH 6,8; bromophenol blue

ELISA buffers:

- all buffers were prepared with MiliQ water
- PBS (Phosphate Buffered Saline): NaCl 137 mM; KCl 2,7 mM; Na_2HPO_4 10 mM;
- Homogenisation buffer: EGTA 1 mM; EDTA 1 mM; UREA 1 M; in PBS
- Blocking buffer: 0,5% BSA in PBS
- RxN: 0,1 M Tris-HCl; pH 8,7; 1 mM $MgCl_2$
- Staining solution for ELISA: 1 mM pNPP in RxN buffer

Immunohistochemistry buffers:

- PBST: PBS; 1% BSA; 0,1% Triton X100
- Blocking buffer: 0,5 % BSA; 0,1 % Triton X-100 in PBS
- paraformaldehyd solution: 50 ml 8 % paraformaldehyd; 50 ml 0,2 M phosphate buffer; dissolved with 1 M NaOH

- Phosphate buffer:
  - 2 parts of base solution A + 8 parts of base solution B
  - base solution A: 0,2 M KH_2PO_4
  - base solution B: 0,2 M Na_2HPO_4 $2H_2O$

2.1.6 Injection solutions

- following concentrations express concentrations of injections solutions and not the final concentrations in the honeybee tissues
- fostriecin 100 µM or 500 µM in 1 µl of PBS; contrd: 1 µl of PBS
- MG132 10 mM in 1 µl of 50% DMSO in water; control: 1 µl of 50% DMSO in water
- okadaic acid 100 µM in 0,2 µl of 10% DMSO in PBS; control: 0,2 µl of 10% DMSO in PBS (experiments with memory blocking, head injected)
- okadaic acid 100 µM in 1 µl of 10% ethanol in PBS; control: 1 µl of 10% ethanol in PBS (experiments with learning induced eEF2 dephosphorylation, thorax injected)
- rapamycin 100 µM in 1 µl of 4% ethanol in PBS; control: 1 µl of 4% ethanol in PBS (experiments with memory blocking)
- SB 203580 10 mM in 1 µl H_2O; control: 1 µl H_2O

2.2 Experimental animals

Honeybee foragers *(Apis mellifera carnica)* were caught from spring to autumn with transparent, pyramid boxes while they were flying out of their hives for foraging. To maximize caching of foragers, the box was kept at a 10 cm distance from the hive entrance. The bees were transferred to small glass containers, shortly anesthetised on ice and restrained in metal tubes. Their heads and abdomens were fixed with an adhesive tape and heads were additionally fastened with warm dental wax. The animals could not move their heads, but were permitted to freely move the antennae, mandibles and proboscis. Bees were kept in plastic, humid and dark boxes at room temperature throughout the experimental period, except for the experimental procedure itself. In periods of very hot weather, boxes with bees were placed in an air-conditioned room. The animals were fed with 4-6 drops of 1 M sucrose solution at about 5 - 7 p.m. Those animals that did not extend the proboscis and accept food were eliminated from further procedures. After catching and harnessing, bees were let to adapt to the new environment through the night and they were taken to the experiment on the subsequent day.

During winter bees were caught to biochemical experiments from a glasshouse with an artificial illumination and constant temperature. The hives stood in flight chambers and bees were allowed to flight freely to the feeders placed outside of the hives. They were caught while flying and treated further like the summer bees.

Bees were fed with 4-6 drops with 1 M sucrose solution at about 5 - 7 p.m. throughout experimental period.

In all ELISA, Western blotting and glutamate uncaging experiments honeybees, directly after harnessing in tubes, had a small part of the head capsule removed. The dissected area was limited by compound eyes, ocelli and antennae. The procedure was done to enable ethanol penetration during fixing or to permit visual access to the brain during UV flashing.

2.3 Methods

2.3.1 Olfactory (PER) conditioning

Bees were classically conditioned for proboscis extension response (PER) using carnation (clove oil derivate) odour as the conditioned stimulus (CS) and 1M sucrose solution in tap water as the unconditioned stimulus (US).

The insects were kept in covered boxes and the cover was removed 30 min before conditioning to allow adjustment to the environment. Honeybees in tubes were taken from the box and placed in front of the exhaust pipe. This equipment provided an air stream for odour application and prohibited spreading of the odour to the ambient environment. Animals were left for accommodation in front of the exhaust pipe for 10-15 s. Odour was applied using a 20 ml syringe containing a small piece of filter paper loaded with 4 µl of carnation oil. The tip of the syringe was kept about 2 cm from the bee. The odour was presented for 5 s. Sucrose was given to the antenna and extended proboscis 3 s after the onset of odour application, on a toothpick dipped in 1M sucrose solution. If the proboscis was already extended, then sucrose was applied directly to it. In any case, animals were allowed to lick the sucrose for 5 s. After the end of US application bees were left in front of the exhaust tube for 5 s and then put back to the box. The acquisition series consisted either of 1 acquisition trial (1 trial conditioning) or 3 trials (3 trial conditioning) with 2 min intertrial interval (ITI).

During conditioning trials, reaction of the bee was scored as positive when proboscis was extracted to the height of mandibles in a plane parallel to the table during initial 2 s of odour presentation (hence before sucrose application).

During retention tests, bees were presented with carnation oil odour for 5 s. The reaction was considered positive if the insect fully extended its proboscis during odour presentation.

In some experiments, control group was used and these bees received sucrose only and were considered to be the “non-learning” control, opposed to the bees that undergone conditioning procedure. These “sucrose only” bees were treated exactly like conditioned bees with the exception that no odour was presented.

Percentage of bees extending proboscis during odour application was used as a measure of memory performance. Fisher exact or X^2 tests were used to test for significant differences between groups.

2.3.2 Drug injections

Thorax injections:

Drugs in the volume of 1 µl were injected into the thorax hemolymph using a hand operated, siliconised, glass microcapillary (1–5 µl, Selzer). At first, a small hole was poked with a preparation needle in the exoskeleton between mesonotum and scutellum. Then, a microcapillary was used to inject the drug solution. Control animals were treated alike, with the exception that vehicle solution was used as injection.

Brain injections:

Injections to the brain were done using sharp-tip glass microcapillaries. Volume of the injection was 0,2 µl and was applied in 5 pulses during 2 sec using a microinjector Picospritzer II (General Valve Corporation). At first, a small hole was poked in the head exoskeleton in the area between ocelli and then a sharp-tip microcapillary was used to inject the drug solution.

2.3.3 Western blotting

To verify if commercially available antibodies directed against mammalian proteins may be used in honeybee tissues Western blotting technique was used.

Bees in tubes were put for 30 min to 50% ethanol (- 5^0C) to stop all reactions and fix the brain. Later their heads were cut off and mounted on a wax dish. The glands and tracheas covering the brain were removed as well as ocelli, optical lobes and antennal nerves. The central part of the brain was homogenised in 300 µl of ice-cold homogenisation buffer (1 mM EDTA; 1 mM EGTA in PBS) in teflon-glass homogenizer (1 ml) powered electrically (900 rpm) by 15 up-down strokes. Immediately after that, loading buffer was added to the homogenate in the proportion 1:4. Homogenates and prestained markers were heated for 2 min in a boiling water bath.

Then it was checked if antibodies detect in the honeybee and mouse brains proteins with the same molecular weight. Small pieces of mouse forebrains (~50 mg) were taken from liquid nitrogen, defreezed and homogenised in 1 ml of the buffer and treated further like honeybee brains.

To separate the proteins according to the molecular weight and electrical charge electrophoresis was used. The SDS-PAGE gels were poured into a space between two glass plates. 25 µl of bee and mouse homogenate samples as well as molecular weight markers were loaded on the 4,5% stacking gel and 12% resolving gel. The plates were put into a running buffer in a self-made electrophoresis apparatus and 25 mA electric current was

applied. The electrophoresis was stopped after about 30 min, when first proteins reached the lower edge of the gel.

After electrophoresis, gels were removed from the apparatus and were put shortly into the blotting buffer. Next, semi-dry electrotransfer was used to blot the gels on the nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell). Three layers of filter paper wetted in the blotting buffer were placed on the blotting machine (Trans-BlotSD, BioRad). On top of it were placed: wet nitrocellulose membrane, electrophoresis gel and next three layers of wet filter paper. Excess of buffer was removed and electrotransfer was run for 40 min (0,4 mA per membrane).

After electrotransfer, the nitrocellulose membranes were blocked: they were transferred to the blocking buffer (1% BSA; 0,3% TWEEN in PBS) and let for 90 min under mild shaking.

Solutions of primary antibodies (pheEF2, eEF2, phS6K) were prepared in concentration 1:1000 in blocking buffer with small amount of natrium azide. The nitrocellulose membranes were cut into parts, transferred to small foil bags (3-4 ml), filled with solutions of respective antibodies and left overnight at 4^0C under mild shaking.

After binding of the primary antibody, the membranes were washed 3 times for 10 min in PBS and then transferred to the secondary antibody solution (anti-rabbit IgG conjugated with horseradish peroxidase (HRP), 1:10.000 in blocking buffer) for 1 h in room temperature with light shaking.

The localisation of antibody binding was visualized using chemiluminescence reaction. The membranes were washed 3 times per 10 min in PBS to remove unbound antibody. Then they were put for 1 min to 10 ml of Western blot chemiluminescence reagents (ECL Plus, Amersham or Western Lightning, PerkinElmer). Next, they were put between two sheets of foil and placed for varying time (10 s to 5 min) under autoradiography film (Kodak Biomax XAR-5, Kodak) in dark room with red light, until optimal sensitivity/resolution balance was reached. Then, they were washed in developer for 2 min, fixed in fixer for 5 min, washed in water for at least 10 min and finally dried in warm air.

2.3.4 ELISA

ELISA (Enzyme-Linked ImmunoSorbent Assay) was used to measure quantitatively relative amount of proteins (phosphorylated and total amount) after different experimental treatments.

After experimental procedure (e.g. conditioning, glutamate uncaging, feeding etc.) bees were put to -5^0C 50% ethanol for 30 min to 1 h. Then their heads were cut off and placed on a wax dish. The glands and tracheas were removed as well as ocelli, optic lobes and antennal nerves.

The central part of the brain (*inter alia* mushroom bodies, protocerebral lobe, antennal lobes) was homogenised in 1 ml of ice cold homogenisation buffer (1 mM EDTA; 1 mM EGTA; 1 M UREA in PBS) in teflon-glass electrical homogenizer (900 rpm) by 15 up-down strokes. After that the samples were sonicated for 10 s (10% energy output) in a sonicator (HTU Soni 130, G. Heinemann)

In the next step, ELISA plates (Falcon 353915, BD Biosciences or Immulon 1B, Thermo Labsystems) were filled with 50 µl of cold homogenisation buffer. The homogenate samples (50 µl) were added to the first column in a semi-random manner and a series of dilutions was made by removing 50 µl of sample from one column and adding it to the next column. To obtain the blank value, the last column was left without homogenates. Afterwards, 100 µl of homogenisation buffer was added to all wells on the plate. The plates were left for 1 h at 5^0C for coating.

After coating, the samples were removed from the plate and 300 µl of blocking buffer (0,5 – 1% BSA in PBS) was added to each well. The plates were left for 1,5 h in room temperature with gentle shaking.

After blocking, content of the wells was removed and 100 µl of primary antibodies solution (eEF2, pheEF2, phS6K, 1:1000 in blocking buffer) was added. Subsequently the plates were left overnight in a cold room (4^0C) with gentle shaking.

Afterwards, the primary antibody solution was removed and the plates were washed with PBS (3 times for 5-10 min). 100 µl of secondary antibody solution (anti-rabbit IgG–Biotin 1:4000 in blocking buffer) was applied for 1,5 h in room temperature with mild shaking.

The secondary antibodies were removed by washing with PBS (3 times for 5-10 min). Afterwards, 100 µl of alkaline phosphatase (ExtrAvidin – Alkaline Phosphatase, 1:10000 in blocking buffer) was added and plates were left for 45 min in room temperature with gentle shaking.

The alkaline phosphatase was removed by washing with PBS (3 times for 5-10 min) and pNPP colour reaction was used to visualise amount of antigens in wells. 200 µl of 1 mM pNPP in RXN buffer was added to each well and the plates were kept overnight in darkness at 4^0C or at room temperature until the colour reaction was apparent.

Amount of the antigens was quantified by measurement of the optical density of separate wells with ELISA plate reader (SLT 400 ATX or Safire², Tecan) at the wavelength 405 nm versus 620 nm background.

The optical density of series of dilutions resulted in a linear function of concentration. The slope of the function indicated relative concentration of the antigen and was used in further

calculations. Each plate was coated with samples from control and experimental groups in a given experiment. To enable comparison of results from different experiments (and thus different plates), the slopes from experimental and control group were normalized relative to the mean of the plate. Such normalized values from similar experiments were pooled and group means were compared for significant differences.

Values are expressed as:

- means of normalized measurements:
    - eEF2: total amount of eEF2 irrespective of any phosphorylation (eEF2 antibody)
    - pheEF2: amount of eEF2 antigens phosphorylated at Thr56 (pospho-eEF2 antibody)
    - phS6K: amount of S6K antigens phosphorylated at Thr389 (phospho-S6 kinase antibody)
- means of calculated ratios:
    - pheEF2/eEF2: dividing normalised amount of pheEF2 through normalised amount of eEF2, and then taking mean
    - phS6K/eEF2: dividing normalised amount of phS6K through normalised amount of eEF2, and then taking mean

2.3.5 Photolysis of caged glutamate

To determine influence of glutamate on translation machinery in mushroom bodies and protocerebral lobe of the bee brain, glutamate was photolytically released from caged glutamate compounds (CNB and DMNB) with the help of UV light flashes.

Bees with big amount of glands covering brain were excluded from the experiments as this would prevent direct access of the UV light to the brain. Otherwise, residual glands, tracheae and neurolemma covering the brain were left intact.

General procedure was described elsewhere (Locatelli et al., 2005), in particular: thoraces of bees were injected 15 min before photolytic stimulation with 1 µl of mixture of 1 mM CNB [L-glutamic acid, γ-(α-carboxy-2-nitrobenzyl) ester, trifluoroacetic acid salt] and 1 mM DMNB [L-glutamic acid, α-(4,5-dimethoxy-2-nitrobenzyl) ester, hydrochloride] in 5% DMSO/PBS. Controls were injected with vehicle (5% DMSO in PBS) and were treated like experimental group - in particular, they received the same UV light stimulation.

UV illumination was supplied by a UV Flash lamp (T.I.L.L. Photonics) that generated flashes between 340 and 390 nm wavelength, 0,5 ms duration and 10^{15} photons/mm^2 in the back focal

plane of the microscope. The generated light was tunnelled into the photo-adapter port (SZ-CTV, Olympus) of a binocular (SZ40, Olympus). A mask with an aperture in the plane of focus abridged illumination to the mushroom bodies and protocerebral lobe in the specimen plane of the binocular.

Honeybees were killed 15 min after flashing in -5^{0}C 50% ethanol and standard ELISA procedure was used to determine amount of antigens: eEF2, pheEF2 and phS6K.

2.3.6 *In vitro* phosphorylation of eEF2

The bees were caught from winter flight chambers and immediately cooled on ice. Their heads were cut off, the glands and tracheas covering the brain as well as ocelli, optic lobes and antennal nerves were removed. The central part of the brain (*inter alia* mushroom bodies, protocerebral lobe, antennal lobes) was dissected and homogenized in 1 ml of the ice-cold buffer (1 mM EGTA in PBS) in Teflon-glass homogeniser, 15 up-down strokes, 900 rpm. The homogenate of each individual brain was divided into 3 parts, kept on ice and the parts were treated separately from this moment on.

The homogenates were transferred to room temperature for 7 min and the following solutions were added (final concentrations):

- cAMP group: 20 μM bromo-cAMP; 10 mM $MgCl_2$; 50 μM ATP
- Ca^{2+} group: 2,5 mM $CaCl_2$; 10 mM $MgCl_2$; 50 μM ATP
- control group: 10 mM $MgCl_2$; 50 μM ATP

The samples were left for 15 min in room temperature for incubation and the reaction was terminated by addition of 1 M UREA. Subsequently, ELISA was conducted to determine the concentration of eEF2 and pheEF2 antigens.

2.3.7 Immunohistochemistry

The heads of honeybees were cut off, mounted on a wax plate and cooled on ice. The upper part of the head capsule was cut off and trachea and glands were removed with fine tweezers. The whole head capsule with an intact brain was put for 30 min into a 4% paraformaldehyde solution on ice, the brains were dissected and put for 1,5 h into 4% paraformaldehyde at 4^{0}C. Then, the brains were washed 3 times for 15 min in 0,1 M phosphate buffer. Next, they were dehydratated in 60% isopropanol for 1 h in room temperature or left overnight at 6^{0}C with gentle shaking. The organs were put into solutions of increasing isopropanol concentrations (90% and two times 100%) for 1 h each. Next, the brains were put for 1 h into 100% isopropanol at 45°C, and for 1 h into paraffin-isopropanol 1:1 mixture at 70^{0}C. They were

transferred to paraffin and left overnight at 60^0C. Next morning they were put to a fresh paraffin solution for 2 h (60^0C) and then put on ice until stiffening. The blocks of paraffin were cut to fit into the microtome (Autocut 2040, Reichert-Jung) and series of 7 µm slices were cut and placed (with distilled water) onto poly-L-lysin coated glass object holder. Next, they were dried by 40^0C on a heater until drying and incubated at 45^0C in drying chamber overnight. The paraffin on the slices had to be removed to let antibodies penetrate the tissue. At first, the object holders were transferred for 5 min to Roti-Histol and then to the decreasing concentrations of ethanol (96%, 90%, 80%, 70%, 50% 2 min each). At the end they were put for 2 min to the distilled water and then shortly washed in PBS with 0,1% Triton X-100. For blocking, the slices were incubated for 30 min in blocking buffer (PBST + BSA 0,5%) and then they were washed in PBST (3 times x 5 min). Next, they were put into primary antibodies solution (anti-eEF2 1:200) in blocking buffer and left overnight at 6^0C. Next morning, the primary antibodies were washed out with PBST (3 * 5 min) and the slices were incubated for 1,5 h at room temperature with two different antibodies and dyes:

- secondary antibodies (anti-rabbit IgG alkaline phosphatase conjugated) 1:400 in blocking buffer. The slices were washed with PBST (3 * 5 min) and dying solution (NBT) was applied for 0,5 – 2 h and next once again washed with PBST (3 * 5 min). All procedures from blocking on were done in a dark plate. By dyeing warm water was applied additionally to speed up the process. Next, they were shortly washed with distilled water and transferred into a series of increasing ethanol concentrations (50%, 70%, 80%, 96%, 100%) for 2 min each. At the end they were put for 5 min into Roti-Histol and a drop of Roti-Histokitt was applied onto the slice and they were covered with a cover glass.
- secondary antibodies (anti-rabbit IgG Cy3 conjugated) 1:400 in blocking buffer. The slice were once again washed with PBST (3 * 5 min) and next covered in glycerine.

The slices were then inspected using a fluorescence microscope (Leitz DM RB, Leica Microsystems).

2.3.8 Satiation experiments

On the day preceding the experiment, the animals were treated like for ELISA, in particular, head capsule window was cut off and they were fed with 4 drops of sucrose. On the experimental day, the animals were divided into two groups: fed and hungry. The fed animals received as many drops of sucrose as they pleased, while hungry bees did not receive any food. With exception for feeding, both groups were treated alike. Directly after feeding or 30

min later bees were killed in cold ethanol and ELISA was used to determine amount of antigens.

2.4 Data analysis

Data analyses were performed using software: Excel 2000, 2002 and 2003 (Microsoft Corporation) and Statistica 5.5 (StatSoft Inc.).

3 Results

3.1 Commercially available antibodies against eEF2 and S6K can be used in honeybee tissues.

The first part of the project was to determine which of the commercially available antibodies can be used to investigate honeybee proteins taking part in regulation of translation and/or being part of the translation machinery. This was achieved by BLAST-searching for the appropriate amino acid sequences in the Honeybee Brain EST Project library (http://titan.biotec.uiuc.edu/bee/honeybee_project.htm). The next step was Western blotting of honeybee brain homogenates with antibodies raised against mammalian proteins.

3.1.1 eEF2

The eEF2 antibody (Cell Signaling, #2332) recognises N-terminus of the human eEF2 and thus determines total levels of eEF2 protein independently of its phosphorylation status. The phospho-eEF2 (Thr56) (Cell Signaling, #2331) antibody detects sequence around threonine 56 only when the amino acid is phosphorylated. Throughout the thesis, these polyclonal antibodies will be referred to as eEF2 and pheEF2 respectively. Human eEF2 sequence (NP_001952) was blasted against honeybee brain EST database and a sequence showing very high similarity to the N-terminus was detected in Contig499. There were no other sequences in the bee database showing similarity to this fragment of the human eEF2. Region surrounding Thr56 also showed very high similarity in both species. The sequences are aligned and depicted on Figure 3-1. Sequence similarity of the whole contig (1002 nucleotides) to the human eEF2 is 65% identities and 74% positives. This suggests that the detected sequence codes the bee homologue of eEF2 protein. Regions detected by eEF2 and pheEF2 antibodies (N-terminus and Thr56 respectively) show very high sequence similarity, suggesting that both antibodies can be potentially used in the honeybee brain tissue.

Western blotting of mouse and honeybee brain homogenates with eEF2 and pheEF2 antibodies showed that in both species the antibodies specifically detect proteins that migrated to an apparent molecular weight of about 100 kDa (Figure 3-2). This is in agreement with the predicted molecular weight of eEF2 from mammals (95 kDa; P13639, UniProtKB/Swiss-Prot). The pheEF2 antibody has very high specificity only for Thr56 phosphorylated eEF2 in mammalian tissues (Cell Signaling information materials) and sequence similarity at this region between honeybee and human eEF2 is nearly 100% so one can assume that the

antibody specifically detects honeybee eEF2 region around Thr56 only when the amino acid is phosphorylated.

These combined results from database search and Western blotting demonstrate that mammalian-derived eEF2 and pheEF2 antibodies can be used to detect total amount and phosphorylation status of eEF2 in the honeybee.

```
                                                          *
2     VNFTVDQIRAIMDKKANIRNMSVIAHVDHGKSTLTDSLVCKAGIIASARAGETRFTDTRK   61   human sequence
      VNFTVD+IRA+MDKK NIRNMSVIAHVDHGKSTLTDSLV KAGIIA A+AGETRFTDTRK        overlapping sequence
2971  VNFTVDEIRAMMDKKKNIRNMSVIAHVDHGKSTLTDSLVSKAGIIAGAKAGETRFTDTRK   3150 honeybee sequence
```

Figure 3-1 Amino acid sequences of mammalian and honeybee eEF2 protein have very strong similarity in regions detected by eEF2 and pheEF2 antibodies.

The upper row shows human N-terminal eEF2 sequence (np_001952, PubMed), the bottom one the honeybee sequence (translated from Contig499, Honeybee Brain EST Project), and the middle row shows the overlapping sequence between human and honeybee amino acid sequences. The numbers denote nucleotide position. The asterisk and bolded "T" marks threonine at position 56 detected by pheEF2 antibody, and the black line marks sequence presumably detected by eEF2 antibody.

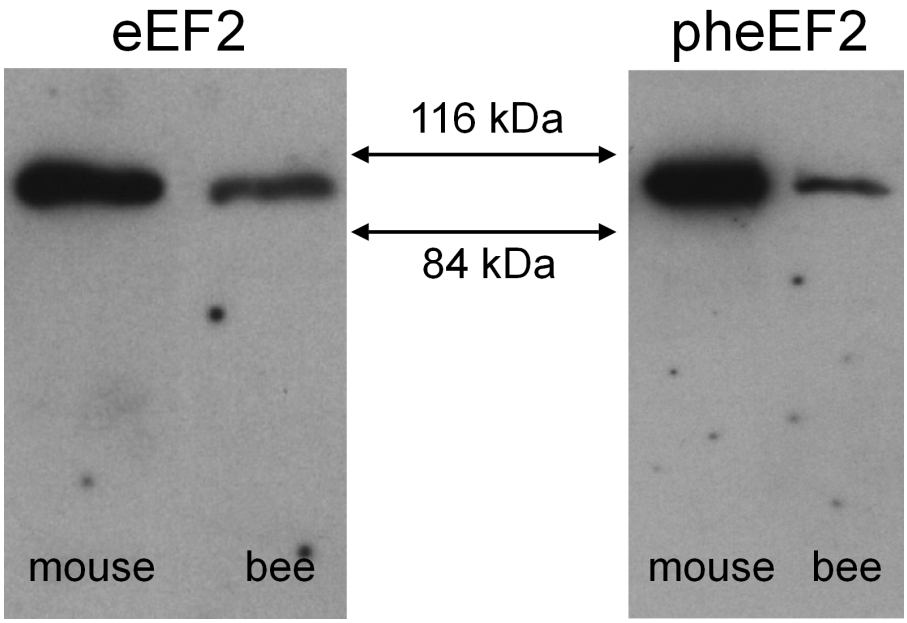


Figure 3-2 eEF2 and pheEF2 antibodies specifically detect in mouse and bee brain tissues a protein with the same molecular weight.

Western blots of mouse and honeybee brain homogenates with eEF2 (left pane) and pheEF2 (right pane) antibodies demonstrate that the antibodies in both species specifically detect a protein with molecular weight ~100 kDa.

3.1.2 S6K

The same procedure as above was used to determine usability of the S6K antibody. The phospho-S6 kinase (Thr389) antibody (Cell Signaling, #9205) detects mammalian (rat) p70 and p85 S6 kinases only when these enzymes are phosphorylated at Thr389 in p70 (or Thr412 in p85). This polyclonal antibody will be referred to as phS6K, throughout the thesis. The rat S6K protein sequence (AAA42103, PubMed) was blasted against honeybee database and aligned with a predicted sequence of honeybee S6K (XP_395876, PubMed). There are 65% identities and 79% positives between these sequences what suggest their homology. In the region detected by phS6K antibody, there is a very high sequence similarity (Figure 3-3) what indicates, that this antibody can be used to detect honeybee proteins.

```
              *
384  VFLGF**T**YVAPSVLE     397  rat sequence
     VF GF**T**YVAPS+LE          overlapping sequence
385  VF*GF**T**YVAPSILE     398  honeybee sequence
```

Figure 3-3 Amino acid sequences of mammalian and honeybee S6K protein have very strong similarity in region detected by the phS6K antibody.

The upper row demonstrates rat S6K sequence (AAA42103, PubMed) surrounding Thr389, the middle row shows the overlapping sequence between rat and honeybee amino acid sequences, and the bottom row presents the honeybee protein sequence (XP_395876, PubMed). Numbers denote amino acid position. The asterisk and bolded "T" marks Thr389 recognised by the phS6K antibody.

Western blot analysis of phS6K antibody (Figure 3-4) detects in both homogenates two proteins of the molecular weight about 70 and 85 kDa, which correspond presumably to the p70 and to the p85 isoform of the S6K. This indicates that the antibody raised against mammalian sequence may be used to detect the honeybee S6K protein when it is phosphorylated at Thr389.

The antibody against total amount of S6K (Cell Signaling #9202) could not be applied as sequence differences between honeybee and rat were too large.

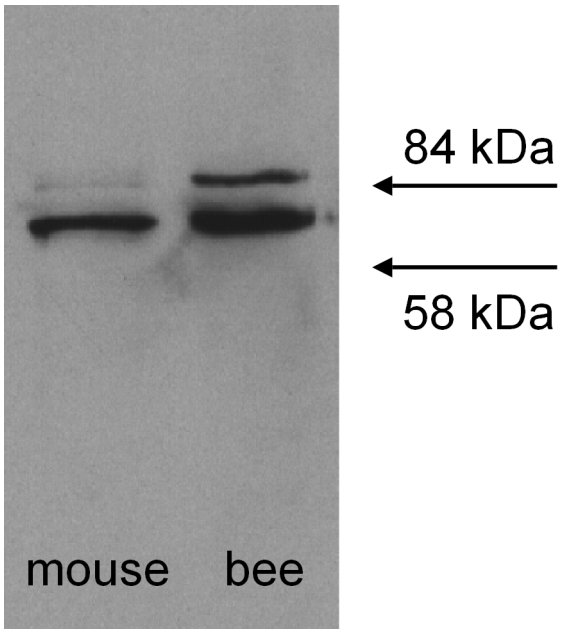


Figure 3-4 phS6K antibody specifically detects in mouse and bee brain tissues proteins with the same molecular weight.

Western blot of mouse (left lane) and honeybee brain homogenates (right lane) with phS6K antibody. The antibody detects the protein only when it is phosphorylated on Thr389. The antibody detects two proteins with molecular weight about 70 and 85 kDa in both species.

3.1.3 Antibodies against other components of the translation control system

Other commercially available antibodies that could possibly be used in honeybee to study translation control system were tested, but either the sequence differences were too big or the Western blots did not give a clear, single bands. The tested candidate antibodies (against total amount and phosphorylation places) were: 4E-BP1, Akt, eEF2K, eIF4E, eIF2A, eIF4G, Mnk, S6, TOR and tuberin.

3.2 eEF2 phosphorylation is modulated by cAMP and Ca^{2+} in vitro

3.2.1 Sequences similar to mammalian eEF2K do not exist in the honeybee

Sequences similar to mammalian eEF2K were not found in insects and the whole α-kinases family seems to be missing in this group of invertebrates (Ryazanov, 2002). Moreover, no eEF2K activity was reported in the moth *Spodoptera* (Oldfield and Proud, 1993). Thus, it was vital to check if sequences homologous to the mammalian kinase exist in the honeybee.

Blasting of human eEF2K sequence (NP_037434, PubMed) against honeybee genome, gave either negative results or only very weak consensus sequences at the C-terminus of eEF2K. This result points out to the conclusion that honeybee, as it seems to be the case in other insects, does not have sequences similar to mammalian eEF2K.

3.2.2 cAMP and Ca^{2+} increase phosphorylation of eEF2 *in vitro*

Honeybee eEF2 has the conserved phosphorylation domain, which is the main control target for eEF2 activity. Therefore, in spite of the suggestions described above, that bees did not possess eEF2K, it seemed very strange, that such an important translation factor lacked its kinase. Besides, phosphorylated eEF2 exists in the bee brain, what can bee seen on Western blots presented on Figure 3-2. Thus, it was crucial to check if eEF2 phosphorylation in the honeybee is regulated in a way known from vertebrates. If this was the case, it would suggest the existence of a functional homologue of the eEF2K.

In mammals cAMP and Ca^{2+} regulate eEF2 phosphorylation through eEF2K (Nygard et al., 1991). cAMP is a natural activator of PKA (Skalhegg and Tasken, 2000) and the latter directly phosphorylates eEF2K and thus inactivates eEF2 (Diggle et al., 2001). To check if eEF2 is regulated in the "eEF2K way", cAMP (20 μM bromo-cAMP) was added to the honeybee brain homogenates and amount of pheEF2 and eEF2 antigens was determined using ELISA.

The result of the experiment (Figure 3-5) shows that cAMP does not influence total amount of the antigen (eEF2), but highly (about 20%) and significantly ($p<0,05$; Wilcoxon signed ranks test) increases both amount of the phosphorylated antigen (pheEF2) and pheEF2/eEF2 ratio.

This finding suggests that in the honeybee brain cAMP induces eEF2 phosphorylation, as it was shown in mammalian tissues containing eEF2K (Proud, 2000).

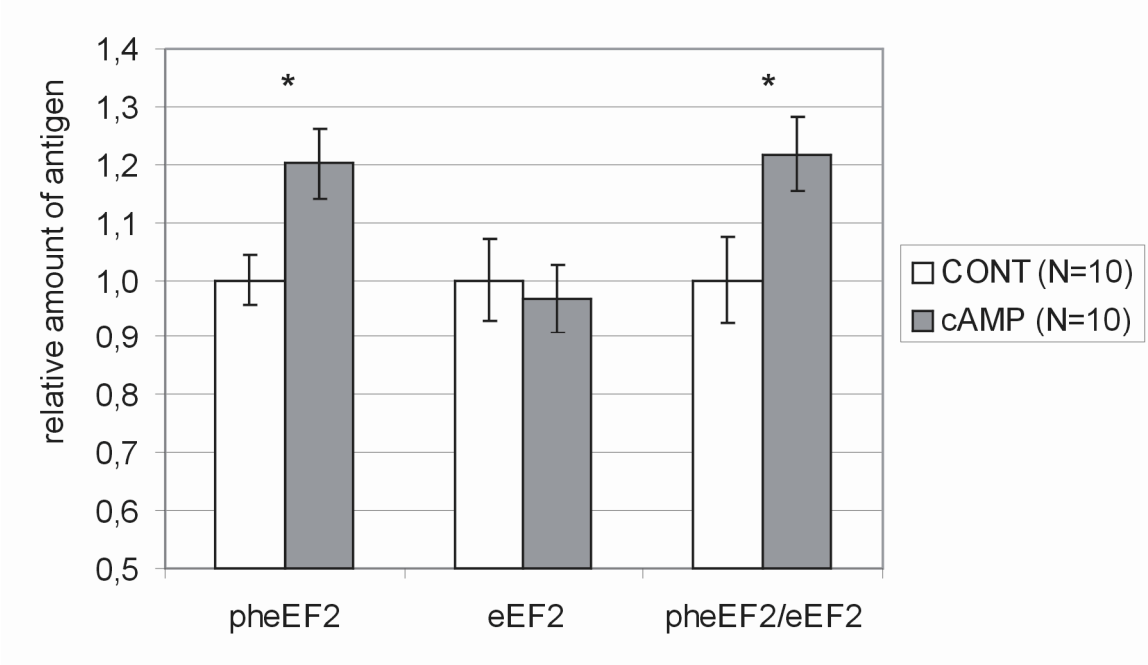


Figure 3-5 cAMP increases phosphorylation of eEF2 leaving total amount of the protein unchanged.
Honeybee brain homogenates were stimulated with 20 µM bromo-cAMP and 15 min later the amount of antigens was determined using ELISA. cAMP increased phosphorylation of eEF2 but left total amount of the protein unchanged. Bars represent the relative amount of Thr56 phosphorylated eEF2 (pheEF2), the total amount of eEF2 (eEF2) and the calculated ratio (pheEF2/eEF2). White bars (CONT) represent the mean (+/- S.E.M) of 10 ELISA measurements in control group and are expressed as 100%. The grey bars (cAMP) represent the mean (+/- S.E.M) of 10 ELISA measurements in cAMP stimulated homogenates and are expressed as % of the control group. * p<0,05 Wilcoxon signed ranks test.

In mammals, the second potent pathway inhibiting eEF2 function depends on Ca^{2+}/calmodulin. Calcium increases eEF2 phosphorylation levels via activation of eEF2K in mammalian, non-neuronal (Proud, 2000) and neuronal (Iizuka et al., 2007) tissues.

To determine if calcium increases eEF2 phosphorylation in the honeybee brain, homogenates from this tissue were stimulated with 2,5 mM $CaCl_2$ and 15 min later the amount of eEF2 antigens was determined. There was a 10% increase in amount of phosphorylated eEF2 (pheEF2) ($p<0,05$ paired t-test), 13% decrease in total amount (eEF2) of the protein ($p<0,05$ paired t-test) and 23% increase in pheEF2/eEF2 ratio ($p<0,05$ paired t-test) (Figure 3-6).

This outcome suggests that calcium increases eEF2 phosphorylation in bee brain and points to the notion that in the bee eEF2 is regulated in a similar way as in mammalian tissues containing eEF2K.

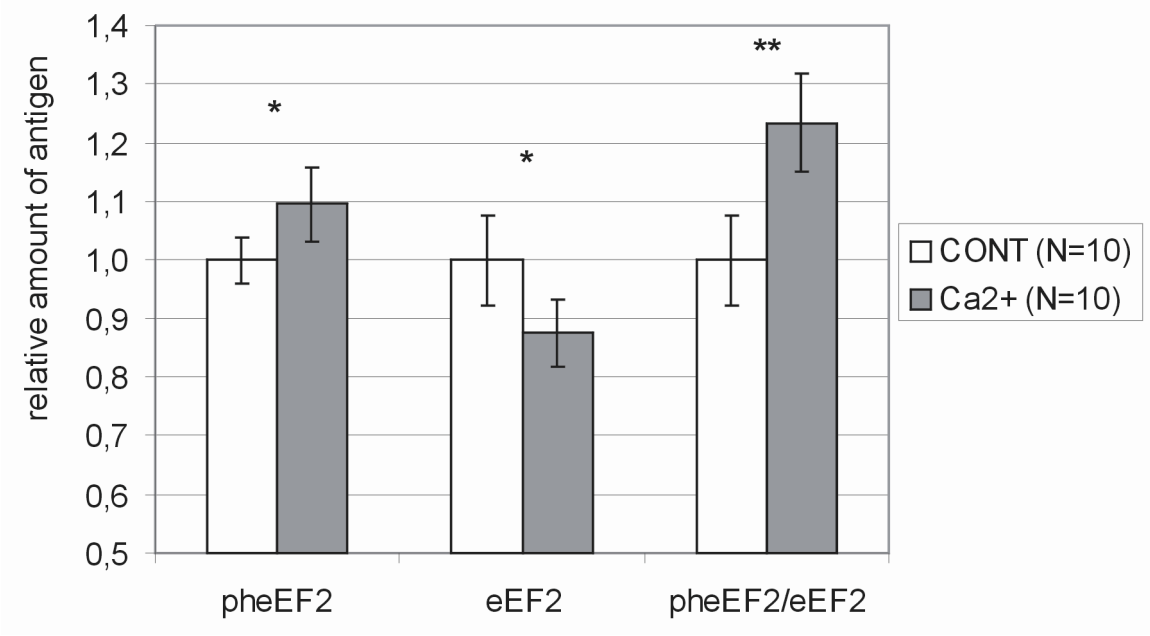


Figure 3-6 Calcium application increases phosphorylation of eEF2 and decreases total amount of the protein.

Honeybee brain homogenates were stimulated with 2,5 mM Ca^{2+} and 15 min later the amount of antigens was determined using ELISA. Calcium application increased phosphorylation and decreased total amount of eEF2.

Bars represent the relative amount of Thr56 phosphorylated eEF2 (pheEF2), the total amount of eEF2 (eEF2) and calculated ratio (pheEF2/eEF2). White bars (CONT) represent the mean (+/- S.E.M) of 10 ELISA measurements of control group and are expressed as 100%. The grey bars (cAMP) represent the mean (+/- S.E.M) of 10 ELISA measurements of cAMP stimulated homogenates and are expressed as % of the control group. * p<0,05; ** p<0,01 paired t-test.

Taken together, the presence of phosphorylated eEF2 as well as increase of eEF2 phosphorylation by cAMP and Ca^{2+} indicate, that the honeybee possesses an enzyme (or enzymes) phosphorylating eEF2. This kinase(s) is regulated in a way characteristic for mammalian eEF2K and thus is a functional homolog of mammalian eEF2K. Non-detection of mammalian eEF2K sequences in bees (as well as in other insects) suggests that the amino acid sequence of insect “eEF2K” is very different from its mammalian counterpart. Although eEF2K has not yet been directly identified in insects the data show that the regulation of eEF2 function seems to be conserved throughout species.

3.3 eEF2 protein in the brain is primarily localised in neuropiles of protocerebral and α-lobes

Since localisation of protein synthesis machinery in the insect brain was not addressed up to day, the next experiment aimed at describing localisation of eEF2 in the honeybee brain.

To address this question immunohistochemistry was used. Bee brains were sliced and incubated with eEF2 antibodies detecting protein independently of phosphorylation status. Two kinds of staining (Cy3 and NBT-BCIP) were applied to visualise eEF2 (Figure 3-7).

The most intensive staining is found in the protocerebral lobes (1-pc) with the lateral protocerebral lobes (1-lpc) and the protein is abundantly detected throughout the structure. Separate bands with strong staining are also present in the α-lobes of the MBs (1-αl). The rest of MBs shows only weak staining with some intermediate labelling in the lip region (3-lip) and basal ring (3-br) of the calyces. Some staining is also located in the peduncles of the MBs (2-mbp). Small amount of the eEF2 is also present in the central complex (4-cc). Parallel experiments without the eEF2 antibody reveal no immunolabelling, confirming that the staining is due to specific binding of the eEF2 antibody to its antigen.

Structures stained with eEF2 antibody consists primarily of neuropil (Bullock and Horridge, 1965), what suggests that in the honeybee brain eEF2 is localised primarily in the axonal and dendritic compartment. This indicates that the outgrowths and not the perikarya are the most intensive places of protein synthesis in the bee brain.

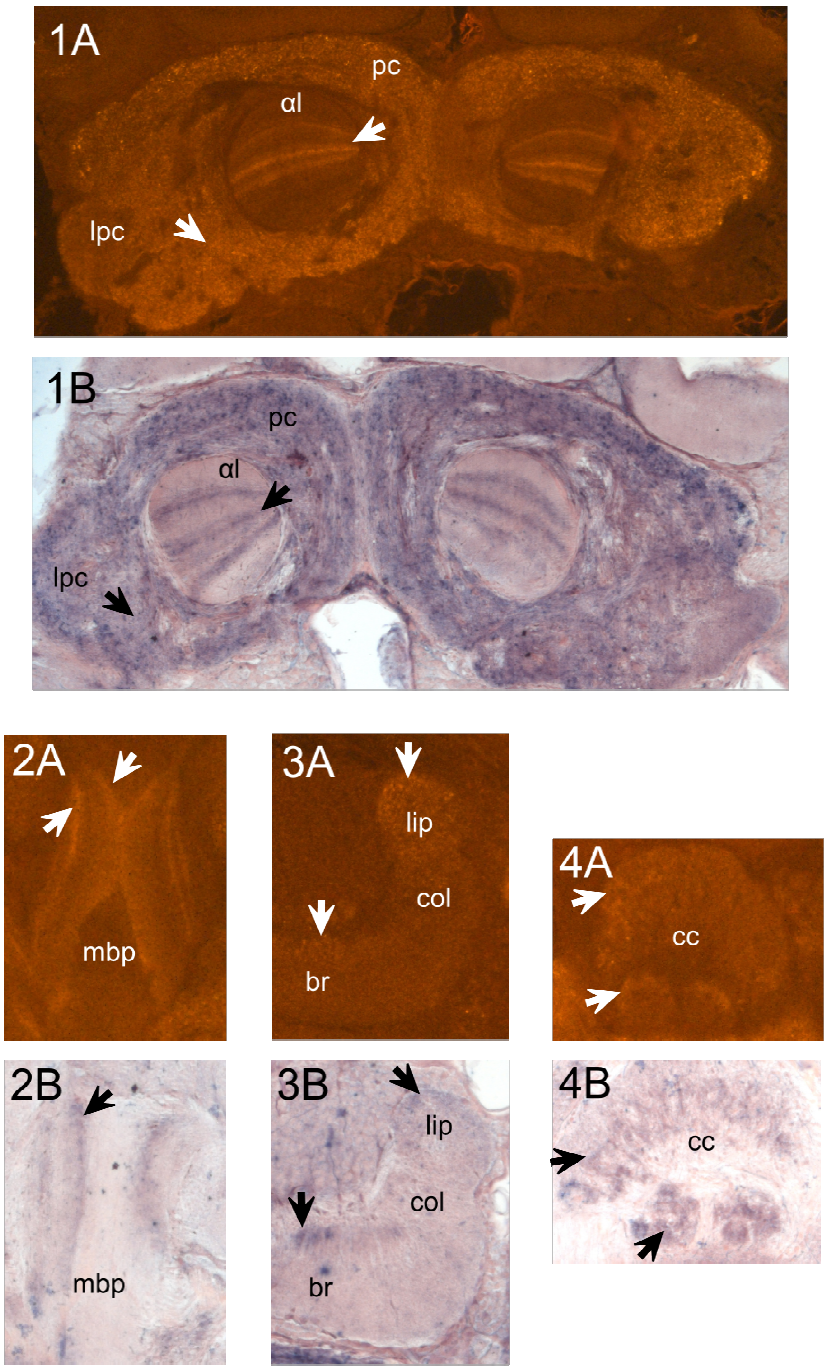


Figure 3-7 eEF2 immunostaining of representative honeybee brain sections reveal most intensive staining in the protocerebral lobes and in the α-lobes of the MBs.

Localisation of eEF2 protein was determined using immunohistochemistry. eEF2 in the honeybee brain is abundant in neuropils of the protocerebral lobe (1 - pc and 1 - lpc) and distinct bands of α-lobe (1 - αl) of the MB. Some eEF2 staining is found in mushroom body peduncles (2 - mbp), basal ring (3 - br) and lip region (3 - lip) of the MB calyces and central complex (4 - cc).

Photos marked with A denote fluorescence staining with Cy3, marked with B – staining with NBT-BCIP.

1 - protocerebral lobe and α-lobes, 2 - mushroom body peduncle, 3 - mushroom body calyx, 4 - central complex;

αl - α-lobe, br - basal ring, cc - central complex, col - collar, lip - lip region, lpc - lateral protocerebral lobe, mbp - mushroom body peduncle, pc - protocerebral lobe.

3.4 Conditioning increases eEF2 activity in the brain as compared to sucrose stimuli

Learning and memory is crucially dependent on new protein synthesis as translational block during or shortly after conditioning deteriorates long-term memory performance (Davis and Squire, 1984). In spite of much work about translation during synaptic plasticity, little is known how memory formation regulates protein synthesis machinery and the translation control system *in vivo*. In addition, most of the memory-related research concentrates on translation initiation, leaving elongation a relatively unexplored subject. Experiments dealing with elongation during plasticity and memory formation are contradictory. For example in *Aplysia* eEF2 phosphorylation level is decreased after induction of synaptic plasticity-like process (Carroll et al., 2004), while in rats learning event induces opposite effect (Belelovsky et al., 2005).

Nothing is known about the regulation of the elongation phase in the honeybee, so I tried to determine, if learning trials, leading to long-term memory formation, influence the elongation phase of protein synthesis. To investigate this and identify time window of potential changes, the amount of eEF2 antigens (phosphorylated and total eEF2) in the brain was measured at different times after learning trials.

The bees were trained according to the PER conditioning protocol. One group of bees was conditioned 3 times with 2 min inter-trial interval (ITI) (COND group). The control group (SUCR group) received 3 times sucrose solution. ELISA was used to determine the amount of antigens in three time windows after training: directly after the end of conditioning (0 min), 10 min or 120 min after conditioning.

When bees were sacrificed directly after learning trials there was no significant difference between conditioned and sucrose group, in terms of eEF2 phosphorylation level, total amount and relative phosphorylation (Figure 3-8, upper graph). 10 min after learning trials, there was no difference in total amount of eEF2 between the conditioned (COND) and control (SUCR) animals, but the relative phosphorylation level (pheEF2/eEF2) was significantly decreased in the conditioned group ($p<0,05$ unpaired t-test) (Figure 3-8, middle graph). 120 min after learning trials the eEF2 phosphorylation level in conditioned (COND) group came back to the control (SUCR) level and the total amount of the protein remained unchanged (Figure 3-8, lower graph).

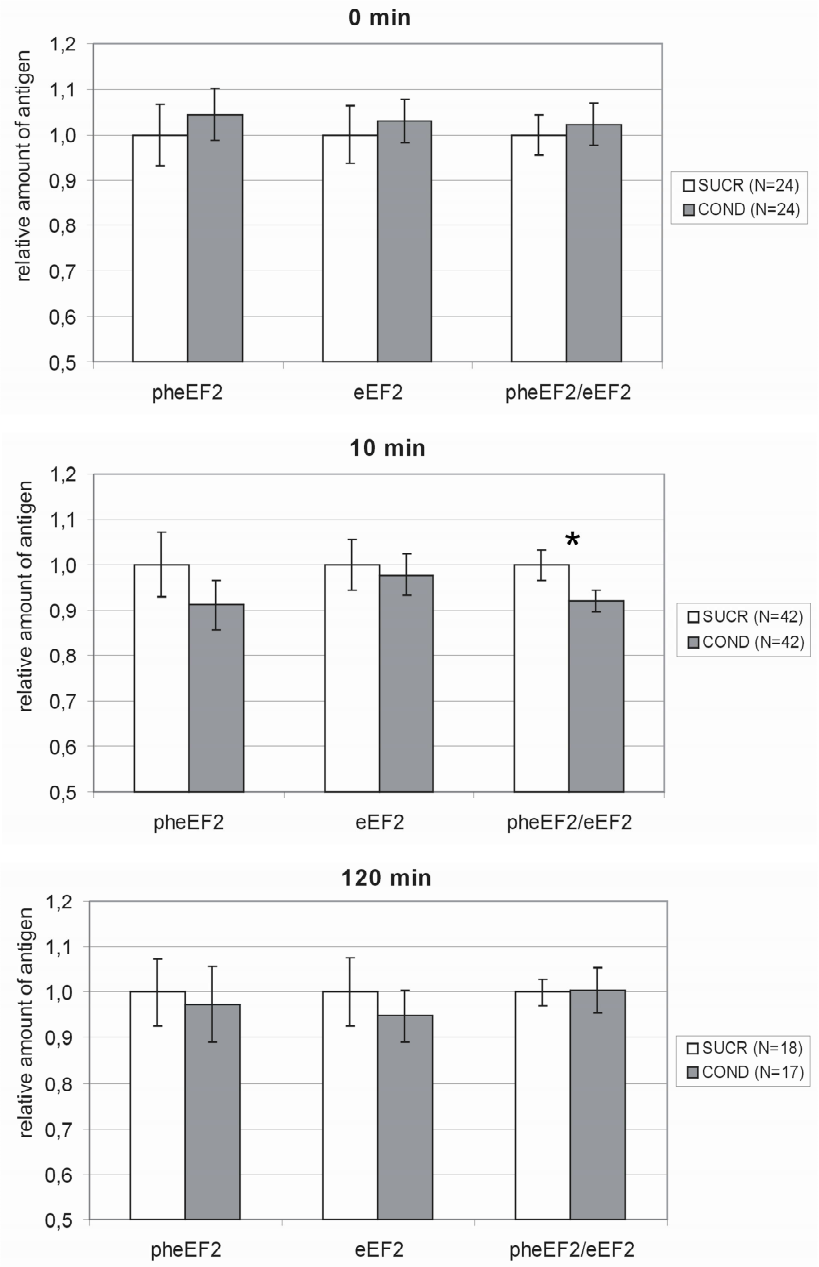


Figure 3-8 Conditioning transiently decreases phosphorylation of eEF2.

Bees were trained according to the 3 trial olfactory conditioning paradigm and amount of eEF2 antigens was determined using ELISA. Directly after 3 trial conditioning, there is no change in either total amount or phosphorylation level of the protein (upper graph – 0 min). 10 minutes later total amount of eEF2 is still unchanged, while phosphorylation of eEF2 (measured as pheEF2/eEF2 ratio) decreases (middle graph – 10 min). The phosphorylation level comes back to the control level by 120 min after conditioning (lower graph – 120 min).

White bars (SUCR) represent mean (+/- S.E.M) of N measurements of control group (bees receiving sucrose only) and are expressed as 100%. The grey bars (COND) represent mean (+/- S.E.M) of N measurements of experimental (conditioned) group and are expressed as percent of the control group. Bars represent relative amount of Thr56 phosphorylated eEF2 (pheEF2), total amount of eEF2 (eEF2) and calculated ratio of pheEF2/eEF2. * p<0,05 unpaired t-test.

To visualise time dependence of these changes, the pheEF2/eEF2 ratios were plotted as function of time (Figure 3-9). As stated above, 3 trial conditioning induces a transient decrease of phosphorylation 10 min and 120 min hours after conditioning this decrement comes back to the baseline.

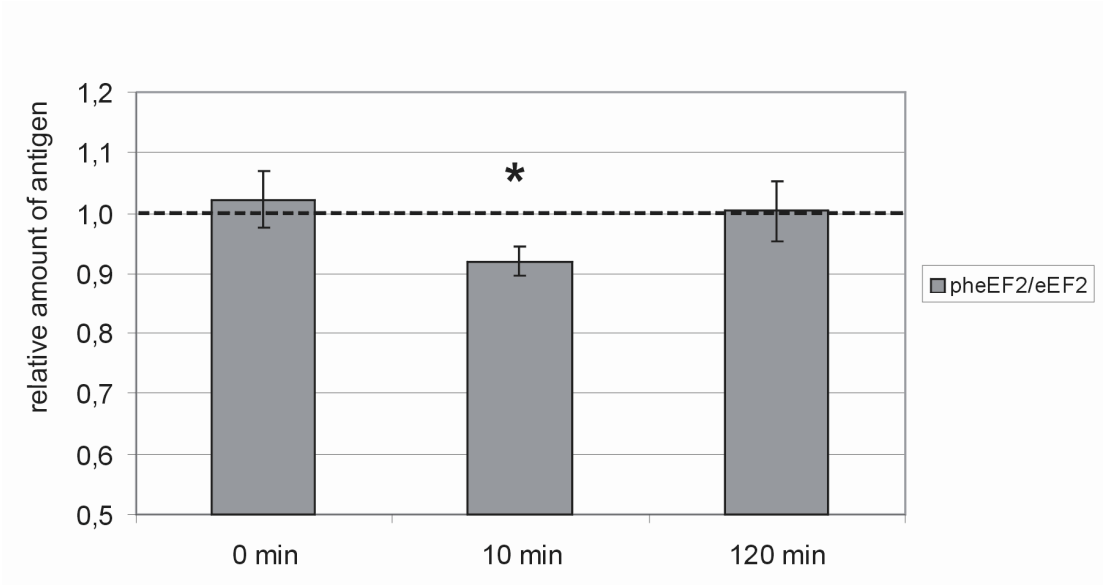


Figure 3-9 Conditioning evokes a transient decrease in phosphorylation of eEF2.

The graph presents the eEF2 phosphorylation status (pheEF2/eEF2 ratio) at specific times after the end of conditioning. Bars represent values from conditioned bees expressed as % of sucrose control. Dashed line denotes the control level. A significant decrease of eEF2 phosphorylation is observed 10 min after 3 trial conditioning, while 120 min later the phosphorylation values come back to the baseline values. * p<0,05 unpaired t-test

As the decrease of phosphorylation at Thr56 is correlated with an increase in activity of the translation factor (Ryazanov et al., 1988), this result suggests that 10 min after multiple conditioning there is an increase in eEF2 function and subsequent acceleration of elongation rate of the proteins synthesis. This process is transient and comes back to the basis level by 120 min after conditioning.

3.5 Identification of the signalling pathways mediating learning induced eEF2 phosphorylation

As conditioning trials transiently decreased eEF2 phosphorylation level in the honeybee brain I tried to determine signalling cascades responsible for the phenomenon. Factors influencing eEF2 in mammalian tissues are depicted on Figure 3-10.

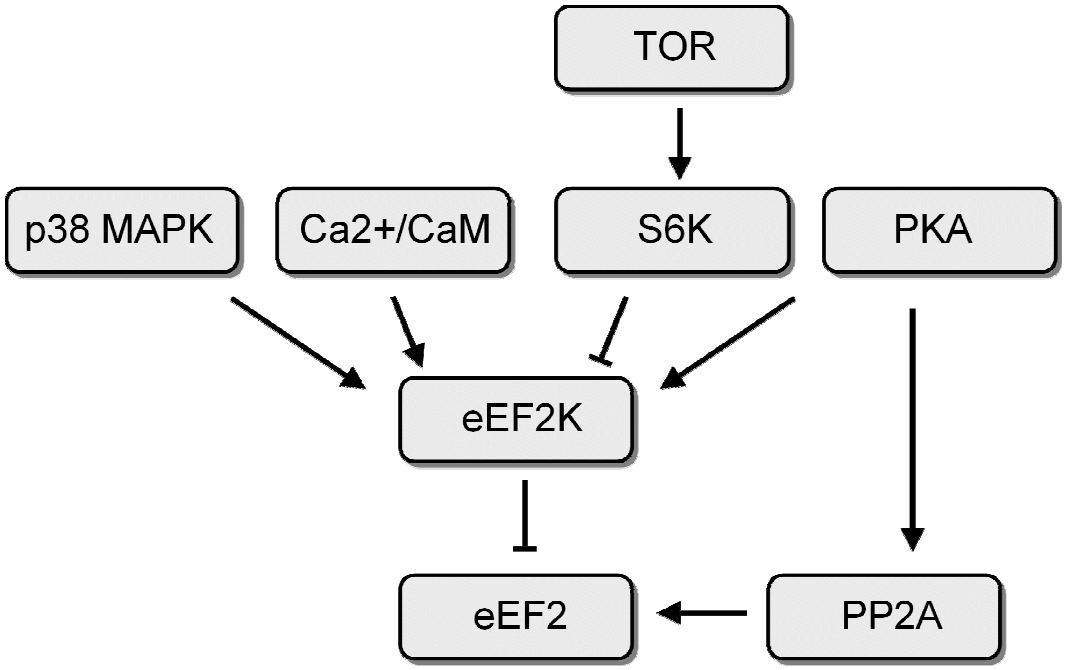


Figure 3-10 Scheme summarizing control of eEF2 activity in mammalian cells.
eEF2 is inactivated by phosphorylation through eEF2K and activated by dephosphorylation catalyzed by PP2A. p38 MAPK and Ca^{2+}/calmodulin activate eEF2K. TOR acting through S6K can activate eEF2; whereas PKA is able to both deactivate eEF2 and probably to activate it through PP2A (but see chapter 1.2.3.2 for details).

3.5.1 S6K does not contribute to eEF2 regulation during memory formation in the honeybee

S6K (S6 kinase) is one of the most important proteins taking part in translational control. In mammals, this enzyme deactivates eEF2K (Proud, 2007) and thus blocks increase in eEF2 phosphorylation. In this set of experiments, I investigated the influence of learning on phosphorylation of S6K at Thr389.

The pre-tests showed that there are no good, commercially available antibodies, detecting S6K independently of phosphorylation status, which could be used in the honeybee. Total

amount of eEF2 is stable after learning trials (see chapter 3.4). For this reason, changes in the levels of phosphorylated S6K induced by learning were evaluated by comparing them to eEF2 levels. Hence, total amount of eEF2 was considered to be an approximation of the protein content of the tissue and was used to calculate phS6K/eEF2 ratio.

Animals were trained according to PER conditioning protocol as described above (see chapter 3.4) and the ELISA technique was used to determine the amount of phS6K.

However, there were no significant differences in phosphorylation level of the S6K (phS6K) between conditioned animals and sucrose control at any investigated time point: directly after the last conditioning trial (0 min), 10 min and 120 min later. Expressing S6K phosphorylation level as phS6K/eEF2 ratio also suggests that learning does not influence S6K phosphorylation (data not shown).

This result hints that S6K does not play a major role in learning-induced eEF2 activation.

3.5.2 Rapamycin enhances memory performance

TOR is a protein playing a crucial role in controlling translation at the level of initiation (Proud, 2004) and rapamycin is a potent blocker of this protein (Arsham and Neufeld, 2006;Gingras et al., 2001). Rapamycin not only inhibits translation, but also prevents long-term fear memory formation in mammals (Bekinschtein et al., 2007;Parsons et al., 2006) and LTF formation in *Aplysia* (Casadio et al., 1999). There are however some contradictory results as low concentrations of rapamycin transform E-LTP into L-LTP in mammalian hippocampus slices (Terashima et al., 2000).

It was tested whether the drug interferes with memory performance in the honeybee. The animals were injected with 1 µM (final concentration in the bee) of rapamycin or vehicle solution as control and 30 min later they were conditioned 3 times. Memory performance was measured 3 hours and 1 to 3 days later (Figure 3-11). Rapamycin influenced neither the acquisition phase (conditioning trails), mid-term memory performance (3H testing trial), nor long-term memory performance (1D – 3D testing trials).

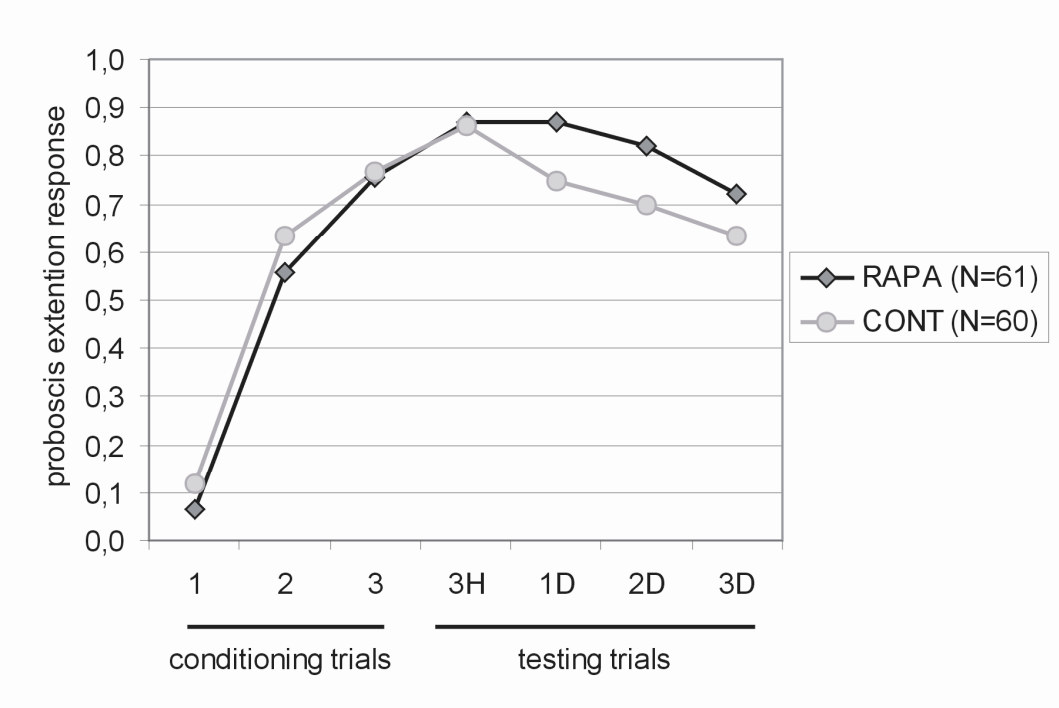


Figure 3-11 Rapamycin influences neither mid- nor long-term memory performance after 3 trial conditioning.

Rapamycin (1 µM) was injected into the thorax of the bees and 30 min later the animals were conditioned using 3 trial olfactory conditioning. During testing trials, insects were presented with odour without sucrose reinforcement, but rapamycin failed to influence memory in any of tested times.

Vertical scale shows proportion of bees showing proboscis extension response during 3 subsequent conditioning trials (1, 2, 3), and testing trials 3 hours (3H), 1, 2 and 3 days (1D, 2D, 3D respectively) after conditioning. RAPA: animals injected with rapamycin, CONT: animals injected with vehicle.

In the aforementioned experiment, there was a surprising, although insignificant, small enhancement of memory in rapamycin-injected animals (1D and 2D $p \approx 0{,}1$ X^2 test). I hypothesised, that rapamycin can enhance memory performance in the honeybee, but the conditioning procedure used (3 trial conditioning) was too strong and caused a ceiling effect, masking the influence of the drug. I decided to check if rapamycin induced memory enhancement after weak conditioning protocol.

Therefore, the insects were treated in the same way as in the previous experiment, with the exception that they were conditioned only once. In this case, memory performance was significantly ($p<0{,}05$ Fisher exact test, two-tailed) enhanced 3 hours, and 3 days after 1 trial conditioning. There was no significant difference between rapamycin and control group 1, 2 and 4 days after conditioning (Figure 3-12).

These experiments lead to surprising conclusion that rapamycin is enhancing memory performance in the honeybee.

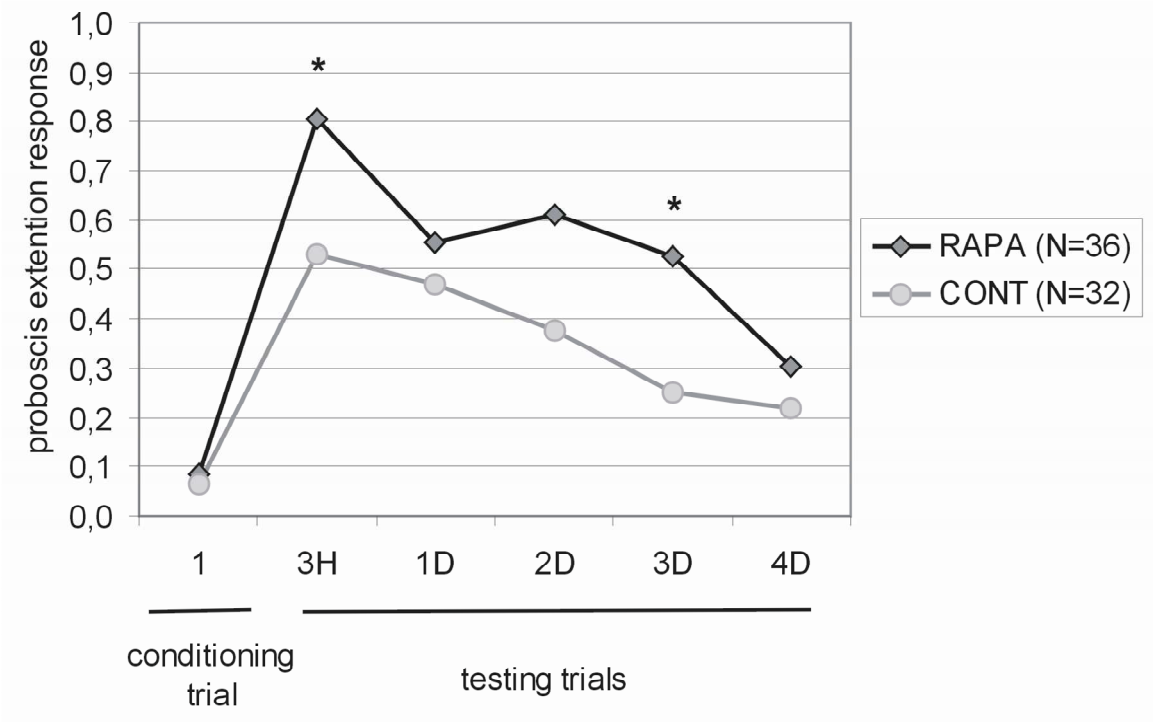


Figure 3-12 Rapamycin increases MTM and a distinct phase of LTM after 1 trial conditioning.

Rapamycin (1 µM) was injected into the thorax of the bee and 30 min later the animal was conditioned using 1 trial olfactory conditioning. During testing trials insects were presented with odour without sucrose reinforcement. 3 hours and 3 days after conditioning there was a significant increase in memory performance in rapamycin injected animals. The enhancement was transient and was not observed on the 4th day.

Vertical scale shows proportion of bees showing proboscis extension response during the conditioning trial and testing trials 3 hours (3H), 1, 2, 3 and 4 days (1D, 2D, 3D, 4D respectively) after conditioning. RAPA: animals injected with rapamycin, CONT: animals injected with vehicle. * p<0,05 Fisher exact test, two-tailed.

3.5.3 Okadaic acid and fostriecin do not influence memory performance

In the bee, eEF2 is dephosphorylated after LTM induction. Enzyme that catalyses dephosphorylation of eEF2 in mammals is PP2A (protein phosphatase 2A) (Sans et al., 2004). Since blocking of PP2A in rodents results in memory impairment (Bennett et al., 2001;Sun et al., 2003), it is possible, that also in honeybees PP2A acts as a positive factor during memory formation.

To test this hypothesis heads of the bees were injected with 20 pmols (0,2 µM in the whole body) of okadaic acid (OA), a potent blocker of PP2A (Boudreau and Hoskin, 2005). This chemical was already successfully used in *Drosophila* (Nowak et al., 2003;Onischenko et al., 2005) so it was probable that it would work specifically in the bee as well. 30 min later, the animals were conditioned to an odour stimulus once or three times. In any case, no memory impairment was found up to 3rd day after training (data not shown).

A similar procedure was used to test other PP2A blocker – fostriecin (Swingle et al., 2007), but this chemical was so far not employed in insects. 30 minutes before 3 trial conditioning 1 or 5 µM of fostriecin was injected into the thoraces of the bees. Also in this case, inhibiting PP2A did not impair memory performance (data not shown).

This leads to the conclusion, that PP2A is not a positive factor in memory formation in honeybees. However, one cannot exclude a possibility that the used chemicals do not specifically inhibit PP2A in the bee.

3.5.4 Okadaic acid does not block learning induced eEF2 dephosphorylation

As presented above (chapter 3.4), conditioning trials transiently decrease the phosphorylation level of eEF2, but the mechanism of this reduction is unknown. Phosphorylation status of the protein in mammals is increased by eEF2K (Ryazanov et al., 1988) and decreased by PP2A (Feschenko et al., 2002). It was hypothesised that blocking of PP2A would inhibit learning-induced changes in eEF2 phosphorylation status.

Thus, 1 µM okadaic acid was injected into thoraces of the bees, and 1 h later the animals were conditioned 3 times. 30 min after learning, changes in eEF2 phosphorylation were determined using ELISA. However, no effect of OA on learning-induced eEF2 changes was observed (data not shown).

Similarly to the experiments described above, it cannot be concluded from present experiment that PP2A mediates learning-induced decrease in eEF2 phosphorylation.

3.5.5 SB 203580 does not interfere with memory formation

One of the kinases activating eEF2K is p38 MAPK (Badger et al., 1998), thus blocking this kinase can activate eEF2 and possibly enhance memory. To address this problem, 100 µM of SB 203580 (p38 MAPK inhibitor (Lee et al., 1999)), was injected into the thorax of the bee. 30 min later the insects received one conditioning trial and memory performance was assessed in following days. Since no difference in the memory performance between drug and vehicle injected bees was found (data not shown), the experiment suggests that p38 MAPK is not implicated in this process.

3.5.6 Rapamycin and LY294002 do not directly influence S6K

S6K is activated, among others, by TOR (Hansen et al., 2005) and PI3-kinase/Akt pathways (Dillon et al., 2007). These pathways are inhibited by rapamycin (Fumagalli and Thomas,

2000) and LY294002 (Djordjevic and Driscoll, 2002), respectively. LY294002 was successfully used in *Drosophila* in earlier reports (Kim et al., 2004).

To check if TOR or PI3-kinase/Akt directly influence S6K *in vivo*, the bees were injected with rapamycin (1 μM) or LY294002 (10 μM) and 30 min later ELISA was used to determine amount of Thr389 phosphorylated S6K in the brain. No clear, direct influence of the drugs on the kinase was found (data not shown).

This suggests that in the bee S6K is not regulated by TOR and PI3-kinase/Akt pathways. The other possibility is that to see an effect, one would have to stimulate S6K at first and only then inhibit TOR and PI3-kinase/Akt pathways. As in other experiments described above one cannot exclude a possibility that LY294002 does not specifically work in the bee.

3.6 Glutamate photorelease increases amount and phosphorylation level of eEF2

Glutamate and its ionotropic (e.g. NMDAR) and metabotropic receptors belong to the most important factors involved in LTP and memory formation. This amino acid plays a positive role in long-term memory formation in many vertebrate and invertebrate species including the honeybee (Locatelli et al., 2005). Moreover, impairment of glutamate re-uptake (Maleszka et al., 2000) or inhibition of metabotropic receptors (Kucharski et al., 2007) in this insect results in amnesia. At the molecular level, LTP induction causes an NMDAR-dependent rapid synthesis of an elongation factor eIF1α in hippocampal slices (Tsokas et al., 2005). NMDAR activation increases phosphorylation of eEF2 in neuronal cultures (Scheetz et al., 2000). On the other hand, there is no work dealing with glutamate influence on translation and phosphorylation of eEF2 *in vivo*.

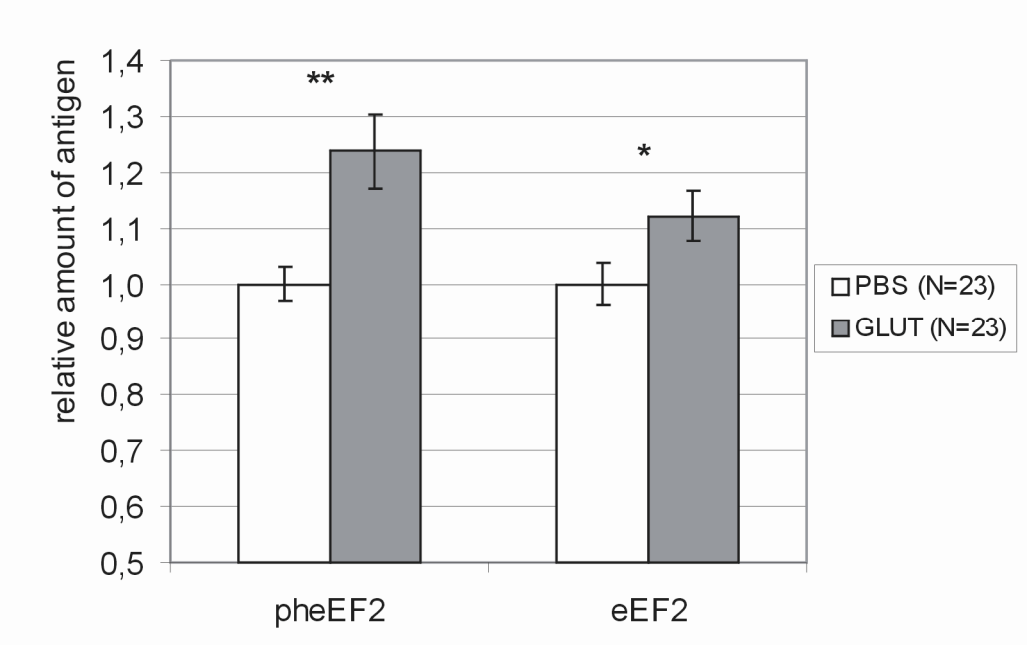


Figure 3-13 Photorelease of glutamate in the honeybee brain elevates the total amount and phosphorylation level of eEF2.

Glutamate was photoreleased in the mushroom bodies and protocerebral lobes of bee brains and 15 min later the total and phosphorylated amount of eEF2 antigens was determined. Glutamate induced a significant increase of phosphorylation level of the protein (pheEF2) as well as of total amount of eEF2 (eEF2).

White bars (PBS) represent (+/- S.E.M.) mean of N measurements of control group (bees receiving vehicle injection) and are expressed as 100%. The grey bars (GLUT) represent mean (+/- S.E.M.) of N measurements of experimental group (bees injected with photoreleasable glutamate). Experimental groups are expressed as % of the control group. pheEF2 – relative amount of phosphorylated eEF2; eEF2 – total amount of eEF2. ** p<0,01; * p<0,05 unpaired t-test.

To tackle this problem glutamate was photoreleased in bee brains (mushroom bodies and protocerebral lobe) and the amount of total and Thr56-phosphorylated eEF2 was determined in stimulated areas 15 min later by ELISA. There was a strong increase in amount of phosphorylated antigen reaching 24% ($p<0,01$ unpaired t-test) over the control level and total amount was increased by 13% ($p<0,05$ unpaired t-test) (Figure 3-13). This result suggests that glutamate can induce quick elevation of the total amount of eEF2 in the honeybee brain, but on the other hand, it phosphorylates eEF2 and thus decreases its function. One has to remember though, that boost in phosphorylation and total amount of the eEF2 may occur in different brain areas or in separate neuronal compartments (e.g. cell bodies vs. dendrites).

S6K is one of the proteins suggested to be the main control point of eEF2 synthesis and function (Fumagalli and Thomas, 2000). To check if glutamate-rendered raise in eEF2 phosphorylation and amount is correlated with an increase in S6K phosphorylation, samples collected in previous experiment were also tested with an antibody against phosphorylated S6K. After glutamate release there was no significant change in phosphorylation level of the protein. This suggests that S6K is not involved in regulating glutamate-induced increase in eEF2 amount and phosphorylation level.

3.7 Feeding destabilises the balance between phosphorylated and nonphosphorylated eEF2

Feeding is a factor deteriorating LTM performance in honeybees (Friedrich et al., 2004). Among metabolic processes greatly influenced by feeding status are the initiation and the elongation phases of the protein synthesis cycle, but these processes are poorly investigated in brain tissues (Proud, 2002). Since conditioning trials transiently activate eEF2 in the honeybee (see chapter 3.4) it was tempting to speculate, that feeding inhibits eEF2 function in the bee brain. Such an interaction could be a possible mechanism explaining the inhibitory influence of food intake on memory performance. To test this hypothesis, I checked if feeding influenced total amount and phosphorylation level of eEF2.

Experimental design consisted of two groups: hungry bees that did not receive food on the experimental day and fed bees that were given sucrose solution *ad libitum*. Insects were killed either directly or 30 min after feeding and ELISA was used to determine the amount of eEF2 antigens. There were no differences between fed and hungry animals (in terms of total or phosphorylated amount of eEF2) directly after feeding nor 30 min later (Figure 3-14). Bees have eaten on average 8,7 drops of sucrose solution, but this number was highly variable. There was no correlation (regression) between the number of ingested sucrose drops and levels of eEF2 and pheEF2 antigens ($r<0,1$ Pearson correlation coefficient).

These results led to the conclusion that feeding does not affect eEF2. However, a careful evaluation of the regression between pheEF2 (dependent variable) and the total amount of the eEF2 (independent variable) in individual bees (Figure 3-15) revealed differences between hungry and fed animals.

When measured directly after feeding, Pearson correlation coefficient of pheEF2-eEF2 regression is significantly higher ($p<0,05$ one-tailed Fisher r-to-z transformation) in hungry ($r=0,890$), than in fed animals ($r=0,500$) (Figure 3-15, two upper graphs). This is also true for the results 30 min after feeding: correlation coefficient is higher in hungry ($r=0,864$) than in fed ($r=0,705$) animals and the difference between two groups remains significant ($p<0,05$ one-tailed Fisher r-to-z transformation) (all of the above correlations are statistically significant). However, it seems that 30 min after feeding the difference between fed and hungry group is smaller than directly after feeding.

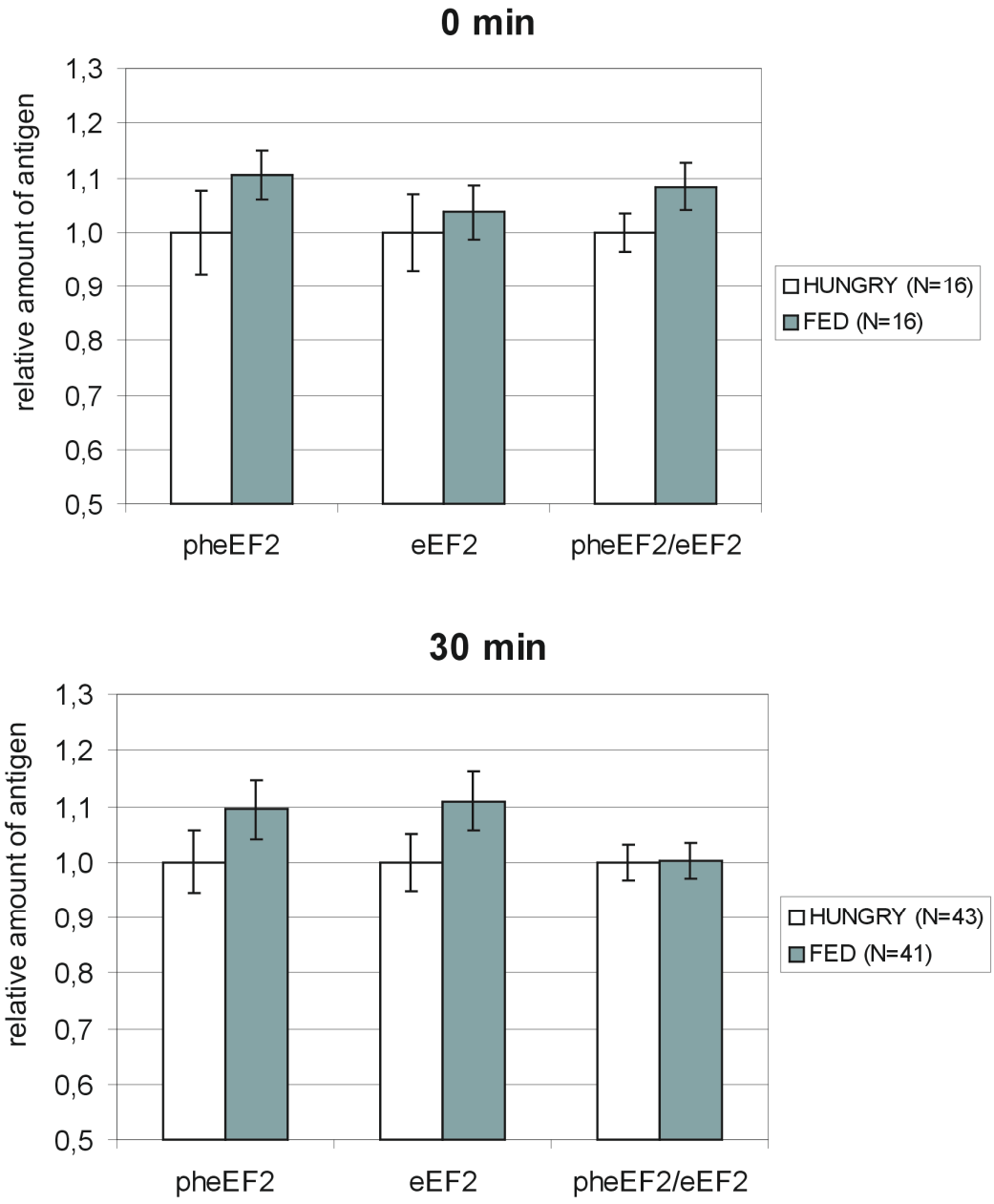


Figure 3-14 Feeding influences neither amount of total nor amount of phosphorylated eEF2 antigens.

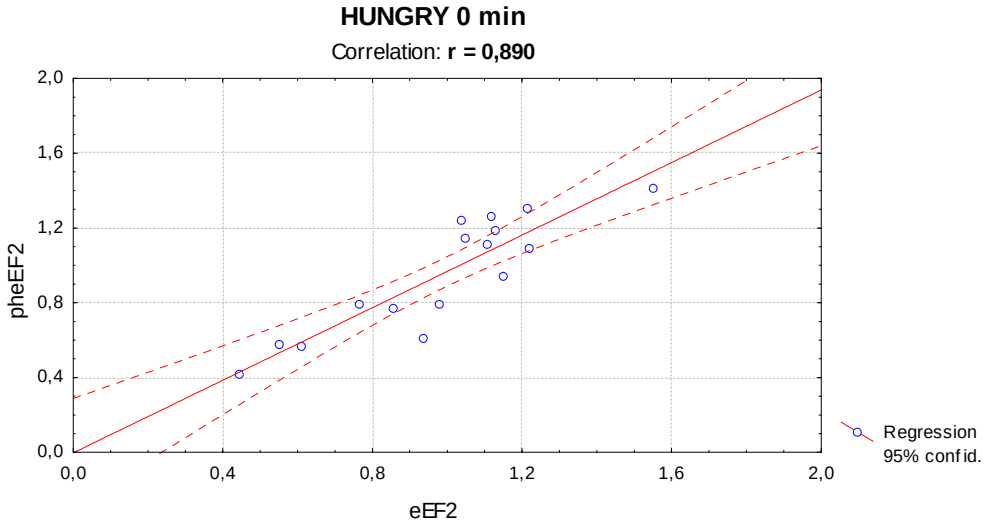
Animals in the control group (HUNGRY) did not receive any food, while animals in the experimental (FED) group were fed until satiation. The bees were killed directly after feeding (0 min – upper graph) or 30 min later (lower graph). There is no significant difference neither in phosphorylation (pheEF2) or total amount of eEF2 (eEF2) between fed and hungry animals, measured directly (0 min, upper graph) and 30 min (30 min, lower graph) after feeding.

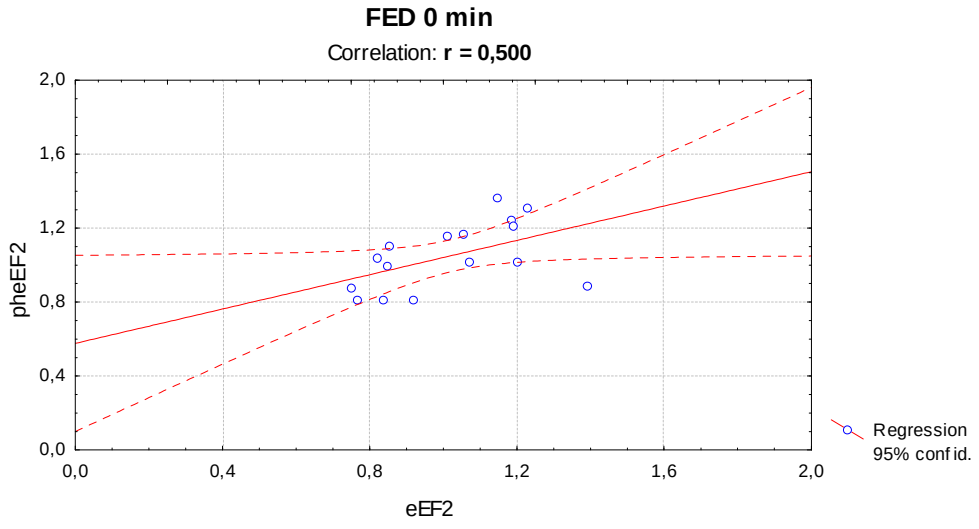
White bars (HUNGRY) represent mean (+/- S.E.M.) of N measurements of control group and are expressed as 100%. The grey bars (FED) represent mean (+/- S.E.M.) of N measurements of experimental group and are expressed as % of the control. pheEF2 represents amount of Thr56 phosphorylated eEF2; eEF2 - total amount of eEF2 and pheEF2/eEF2 stands for calculated ratio of pheEF2 and eEF2.

If the correlation (regression) between eEF2 and pheEF2 antigens is high, then bees that have e.g. ten eEF2 proteins will have, say, seven proteins (out of the ten) phosphorylated. If the bee has six eEF2 proteins, four of them will be phosphorylated and so on. The higher the amount of eEF2, the higher the amount of phosphorylated eEF2. Knowing the total amount of the protein (eEF2) we can predict the amount of phosphorylated eEF2 proteins (pheEF2).

If the correlation between eEF2 and phEF2 antigens is low, then bees that have e.g. ten eEF2 protein may have, let's say, ten phosphorylated eEF2s just like they may have four phosphorylated eEF2s. If the bee has six eEF2 proteins, maybe six of them will be phosphorylated or maybe only two of them will be phosphorylated. In this case, knowing total amount of the protein (eEF2) we cannot predict amount of phosphorylated eEF2 proteins (pheEF2).

Characteristically, the mean level of phosphorylation and total amount in both cases is not different.





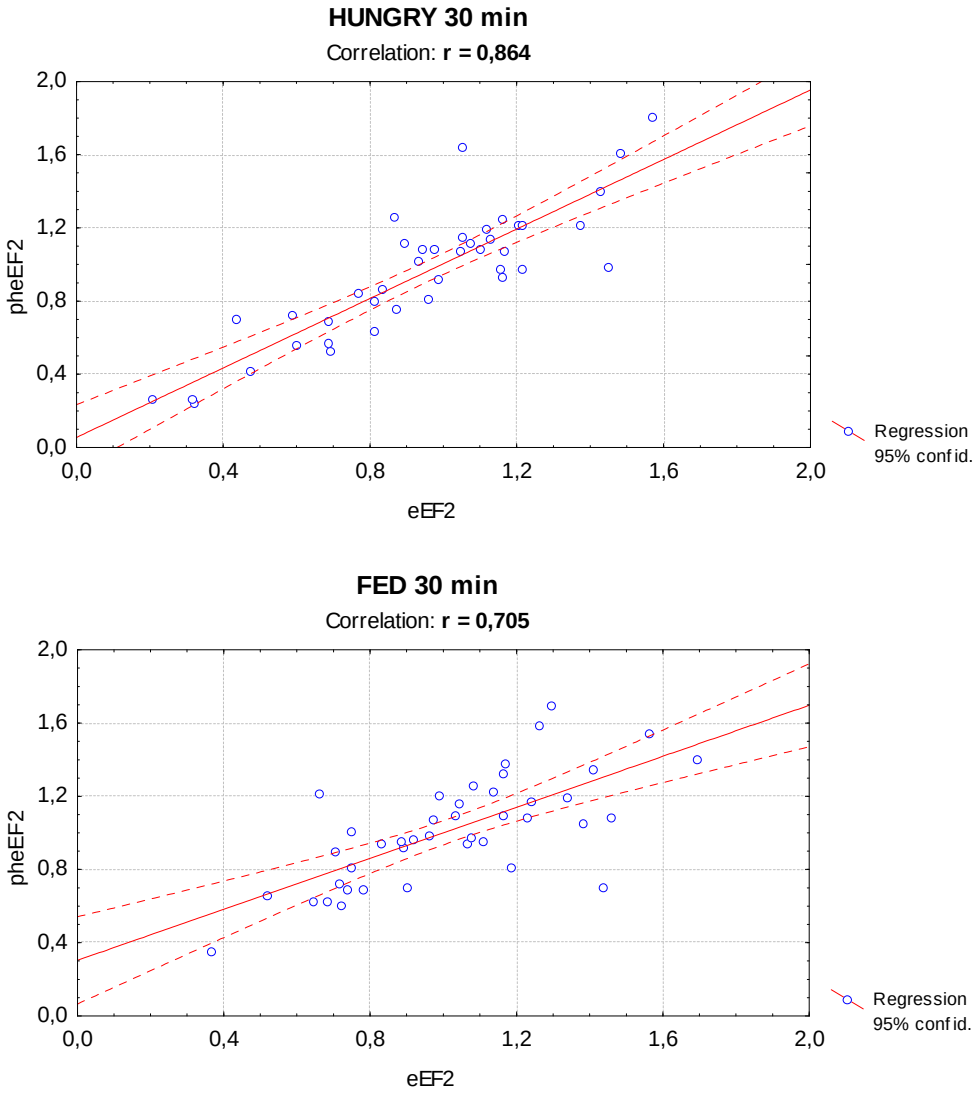


Figure 3-15 Feeding decreases correlation between the amount of phosphorylated eEF2 antigens and total eEF2 antigens.

Graphs show regression between total amount of eEF2 antigens (eEF2) and the amount of phosphorylated eEF2 antigens (pheEF2) measured in animals fed *ad libitum* (FED) directly after feeding (0 min) or 30 min later or in hungry animals (HUNG) killed at the same times. Each point denotes single animal.

Directly after feeding, the Pearson correlation coefficient is much higher in hungry(r=0,890) than in fed (r=0,500) bees (panes on the previous page). 30 min later the correlation coefficient in hungry group stays on virtually the same level (r=0,864), while correlation in the fed group increases (r=0,705) (panes on this page). Differences between fed and hungry groups are statistically significant at both time points (p<0,05 one-tailed Fisher r-to-z transformation).

3.8 Blocking the ubiquitin-proteasome complex enhances memory performance

The control of gene expression is not reduced to the production of proteins (translation and precedent transcription) but it comprises the protein degradation as well. The final stage of gene expression is thus proteolysis mediated by e.g. ubiquitin-proteasome complex (UPC). This protein degradation machinery was reported to be involved in synaptic plasticity processes and act either as a positive (Karpova et al., 2006) or negative (Zhao et al., 2003) factor. Nothing is known about the function of UPC during memory formation in the bee, so I checked the influence of MG132, a potent proteasome blocker (Lee and Goldberg, 1998), on memory performance in this insect. In addition to mammals, MG132 has been successfully used to block proteasome in many invertebrates: *Aplysia* (Kurosu et al., 2007), crab *Chasmagnathus* (Merlo and Romano, 2007) and *Drosophila* (Lundgren et al., 2005), so one can premise that this drug works similarly in the honeybee.

First hypothesis assumed that MG132 would deteriorate LTM, so the bees were injected with 100 µM of the drug and 30 min later they were trained using strong (3 trial) conditioning paradigm. This treatment however, did not affect memory performance (data not shown).

The next hypothesis presumed that proteasome functions as an inhibitory constraint on memory and blocking it would enhance memory performance. In this experiment, the weak (1 trial) conditioning protocol was used and the bees were injected with the drug 30 min before single conditioning trial. MG132 did not influence general sensitivity to the olfactory stimuli, as there is no difference between control and drug-injected bees during conditioning trial. In contrast, the drug clearly enhanced memory performance at 3 hours ($p<0,05$ Fisher exact test, two-tailed) after conditioning and this increase returned to the baseline at day 1 (Figure 3-16 upper graph).

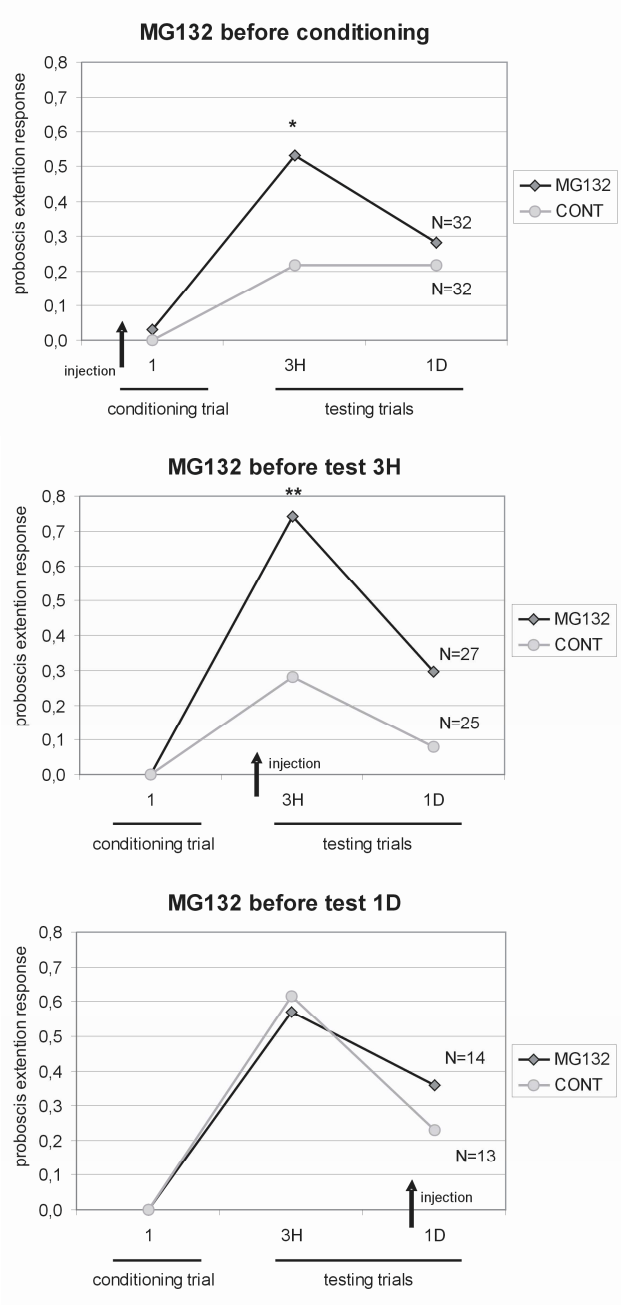


Figure 3-16 Blocking of ubiquitin-proteasome complex in the honeybee enhances mid-term memory performance.

Honeybees were injected with 100 µM MG132 (ubiquitin-proteasome complex blocker) at different times before or after 1 trial conditioning and memory performance was tested at 3 h or 1 day after conditioning. Injection 30 min before single trial conditioning does not influence odour sensitivity, but transiently enhances memory performance 3 hours later (MG132 before conditioning - upper graph). Blocking of proteasome 30 min before test at 3 hours also transiently increases memory performance (MG132 before test 3H – middle graph). Blocking before test on day 1 has no effect (MG132 before test 1D – lower graph).

Vertical scale shows proportion of bees showing proboscis extension response during the conditioning trial and testing trials 3 hours (3H) and 1 day (1D) later. MG132: animals injected with MG132, CONT: animals injected with vehicle. * p<0,05; ** p<0,01 Fisher exact test, two-tailed

To test if MG132 enhances memory scores when applied at later times, the drug was injected 30 min before the 3 hours test. In this situation, blocking of the proteasome greatly increased memory performance at 3H test ($p<0,01$ Fisher exact test, two-tailed) and this effect decayed to the control level at day 1 (Figure 3-16 middle graph). It seemed that the chemical boosted memory retrieval, so the next question was whether it increased only unconsolidated memory or already consolidated one as well. To address this problem, animals were trained with 1 trial conditioning and the drug was injected on the next day 30 min before day 1 test. In this case blocking of proteasome did not have a significant influence on memory performance (Figure 3-16 lower graph).

One can conclude that in the honeybee, blocking of UPC enhances mid-term memory scores.

4 Discussion

4.1 Learning induces transient activation of eEF2

During this work it was shown, that 3 trial conditioning in the honey bee transiently decreases phosphorylation ratio of the eEF2, while total amount of eEF2 remains unaffected. When the total amount of the protein remains stable, then phosphorylation ratio (pheEF2/eEF2) is a reliable, normalised measure of changes in phosphorylation level of a given protein. As decrease in eEF2 phosphorylation is directly correlated with an increase of eEF2 activity and elongation rate (Ryazanov et al., 1988), it may be concluded that associative conditioning results in a transient increment of the elongation rate during protein synthesis cycle.

The only paper addressing the involvement of eEF2 in synaptic plasticity-like processes in invertebrate species comes from the Sossin lab, and investigates the influence of serotonin (5-HT) on *Aplysia* synaptosomes (Carroll et al., 2004). Serotonin induces long-term facilitation of the sensory-to-motor synapses in the mollusc - a process believed to underlie long-term sensitisation of gill and siphon withdrawal response (Casadio et al., 1999). Experiments in *Aplysia* showed that 10 min of the 5-HT stimulation resulted in an eEF2 dephosphorylation in the synaptosomes, while there was no change in the steady-state levels of the protein (Carroll et al., 2004). These results are in good agreement with outcomes presented in this thesis. I showed that learning in the honeybee transiently activated the eEF2 without changing total amount of the protein. The same effect was observed in *Aplysia* neurites using procedure evoking cellular analogue of learning. This suggests that common molecular events underlie memory formation processes in honeybee and synaptic plasticity in *Aplysia*.

The only publication dealing with changes in eEF2 activity related to memory formation processes was done on rats using taste learning paradigm (Belelovsky et al., 2005). In this paper authors showed that after incidental taste learning, there was an increase in phosphorylation level of the eEF2 (and hence decrease in elongation rate) in the taste (insular) cortex. This change was restricted to the synaptoneurosomal fraction, whereas there was no alteration in the perikaryon. Moreover, total amount of the protein remained unchanged.

In another research dealing with mammals, BDNF-induced LTP was succeeded by a boost in phosphorylation level of eEF2 (Kanhema et al., 2006). The authors reported that 15 min after BDNF application to the dentate gyrus of anesthetised rats there was a transient increase in eEF2 phosphorylation, while the total amount of the protein was constant. This effect presumably occurred outside of synapses and dendrites, as BDNF treatment of

synaptodendrosomes did not influence eEF2 state. The authors suggested that during synaptic plasticity, the initiation of translation was upregulated while elongation remained steady at synaptic and dendritic sites. In other compartments (mainly perikaryon), initiation would be upregulated and elongation downregulated at the same time.

Comparison of mammalian *in vivo* results (Belelovsky et al., 2005) with honeybee finding presented in this thesis, shows that the direction of the changes is opposite: after 3 trial olfactory learning in the honeybee, eEF2 phosphorylation is decreased and hence elongation rate is enhanced, while in mammals learning decreases elongation rate. This contradiction could be explained by the differences in learning procedure: incidental taste learning vs. appetitive olfactory conditioning. There is also a possibility that in some regions of the honeybee brain, eEF2 is activated upon learning while in other structures its function is diminished or unchanged. In this case, the observed decrease in honeybee whole-brain eEF2 phosphorylation would be a mixture of these opposing effects. However, a more plausible explanation is that elongation phase during memory formation is regulated differentially in mammals and invertebrates. This idea is corroborated by the *in vitro* experiments. After mammalian LTP there is a decrease (Kanhema et al., 2006), while after synaptic plasticity-like treatment in *Aplysia* there is an increase in eEF2 function (Carroll et al., 2004). This difference is strikingly similar to dissimilarity between *in vivo* results, what suggests, that during synaptic plasticity or memory formation, the elongation rate of translation is augmented in invertebrates and decelerated in mammals.

Apart from differences there are also similarities between experiments in rats (Belelovsky et al., 2005;Kanhema et al., 2006) and in the honeybee presented in this thesis: eEF2 changes its activity in the range of dozens of minutes after the learning trial and this alteration disappears within few hours. The relative strength of the change is similar in both *in vivo* cases. In the rat, the change is about 20% in brain region specifically involved in learning. In the honeybee the change is about 10% in the whole brain, what probably corresponds to higher values in structures explicitly involved in learning. Just like in the bee, in the mammalian experiments changes are restricted to phosphorylation, and the total amount of eFE2 remains stable.

In this thesis I have shown that conditioning trials induce dephosphorylation of eEF2 in the honeybee brain. This effect can be explained either by activation of PP2A-mediated dephosphorylation, or by inhibition of eEF2K-mediated phosphorylation. However, application of PP2A blockers did not prevent learning-induced eEF2 dephosphorylation, nor it deteriorated learning. This suggests that PP2A is not involved in memory processes in the

bee, what is in contradiction to results showing that inhibition of PP2A leads to memory impairment (Bennett et al., 2001;Sun et al., 2003). The other possibility explaining learning-induced eEF2 dephosphorylation is inhibition of eEF2K. I was unable to tackle experimentally this option, as so far eEF2K was reported to be non-existent in insects. Only this thesis brings first suggestions that eEF2K activity is present in the honeybee, but this activity is most probably is mediated by a kinase with different sequence. Because of this, eEF2K inhibitors known from mammalian models are unusable in the bee (see chapter 4.3 for discussion about presence of eEF2K in honeybee).

Comparison of the results presented in this manuscript with findings obtained in *Aplysia* plasticity (Carroll et al., 2004), mammalian learning (Belelovsky et al., 2005) and hippocampal BDNF-dependent LTP (Kanhema et al., 2006) suggests an opposite way of regulating the elongation rate in mammals (decrease of elongation rate) and invertebrates (increase in elongation rate) during memory formation. Function of such difference remains obscure.

4.2 eEF2 is abundant in distinct neuropils in honeybee brain

Using immunohistochemistry it was shown, that the eEF2 protein in the honeybee brain is localized primarily and in protocerebral lobes and in distinct bands of α-lobes of the MBs. Such location suggests that these structures are loci of intensive protein synthesis. These parts of the honeybee brain are composed predominantly of neuropil (processes and synapses) (Bullock and Horridge, 1965), although some glial cell bodies are also present in the protocerebral lobe (Hahnlein and Bicker, 1996). Histological patterns of eEF2 localisation resemble to some extent the localisation of NR1 subunit of NMDA receptor - much in α-lobe and the protocerebral lobe, and little in the rest of MBs (Zannat et al., 2006).

eEF2 was shown many times to be positioned in dendrites and synapses in neuronal tissues of other species: eEF2 was found in close vicinity of postsynaptic sites in tadpole brain (Scheetz et al., 1997), as well as in dendritic lipid rafts of postsynaptic compartment in rat forebrains (Asaki et al., 2003). In the bee brain eEF2 is placed in structures composed primarily of neuropil what suggests that indeed outgrowths are the dominant location of eEF2 in the bee brain.

As protein synthesis is indispensable for LTM formation and learning transiently modulates eEF2 in bee brain it is tempting to speculate that learning-induced change in protein synthesis is localised in dendrites of protocerebral lobe or α-lobes. If this speculation was true, these structures would be a place where persistent, translation-dependent forms of memory were stored. This proposition is substantiated by reports showing that protocerebral structures are indeed involved in learning-related plasticity (Okada et al., 2007) and in LTM formation (Menzel and Manz, 2005) in the honeybee and in *Drosophila* (Gerber et al., 2004;Keene et al., 2006;Liu et al., 2006;Wu et al., 2007).

However, one has to remember that engrams were also suggested to be localised in mushroom bodies (Gerber et al., 2004;Menzel et al., 1974) and antennal lobes (Gerber et al., 2004;Menzel et al., 1974;Thum et al., 2007) in the insect brain. The other contradiction to the abovementioned hypothesis comes from the only publication directly addressing localisation of learning-induced protein translation in insects: in *Drosophila* the ALs are structures involved in memory-related protein (CaMKII) synthesis (Ashraf et al., 2006).

4.3 Evidence for a functional homologue of eEF2K in the honeybee

The eEF2 kinase (eEF2K) described in mammals has unusual properties, as its catalytic domain does not have any similarity to the sequences of the superfamily of orthodox kinases. eEF2K and related enzymes form a separate group of kinases called α-kinases (Proud, 2007;Ryazanov, 2002).

eEF2K was so far not detected in insect tissues using biochemical techniques and genomic databases approaches. I presented results showing that eEF2 can be phosphorylated in the insect tissues what suggests that eEF2K-like activity exists also in the honeybee brain. Moreover, this phenomenon is evoked by Ca^{2+} and cAMP and thus phosphorylation of eEF2 in the bee seems to be regulated in the same way as in mammalian systems.

4.3.1 Arguments for the non-existence of eEF2K in insects

First line of arguments suggesting that insects lack eEF2K (and even the whole family of α-kinases) is derived from genomic database searches. Honeybee and *Drosophila* genome databases screens give consistently no hits (or rarely very weak C-terminal consensus sequences, at best). "There are no α-kinases in *Drosophila*" - as the discoverer of this kinase writes (Ryazanov, 2002). This is somewhat surprising, as other invertebrates such as *Caenorhabditis elegans* possess the kinase. *Aplysia* also seems to have the enzyme as blasting its database (SlimeBase, http://dlc-genomics.rsmas.miami.edu/~tfiedler/) gives some hits, although with medium sequence similarity only. However, one has to keep in mind that the database search algorithms are not perfect and may not detect existing similarities between mammalian and insect eEF2K sequences, what would result in a false negative outcome.

Second line of argumentation against the existence of eEF2K in insects comes from experimental research and is based on a publication by Oldfield and Proud (1993). The authors reported that in *Noctuidae* moth *Spodoptera*, they did not manage to induce phosphorylation of the insect eEF2, although in a similar experimental system the insect eEF2 could be phosphorylated by mammalian eEF2K (Oldfield and Proud, 1993). This would point to a conclusion that this moth lacks eEF2K activity.

However, it has to be stressed that, it is methodologically very hard to proof the "non-existence" of something, as the investigated phenomenon may simply be to small to be detected by a given method or the method may be inappropriate. This seems to be the case in this publication. Oldfield and Proud (1993) used insect cell extracts to which they added ATP and eEF2 protein in the presence or absence of Ca^{2+} ions. They reported technical problems in

Western blot detection of phosphorylated eEF2, so they separated phosphoproteins and blotted only them, but they failed to detect phosphorylated eEF2 either. However, as they noticed themselves, the kinase might have been present in the system, but was simply too diluted to be detected using their method. To directly verify the existence of the kinase, they tried to purify insect eEF2K, but this purification was conducted in a system designated for mammalian eEF2K (Redpath and Proud, 1993). So they would detect insect eEF2K only if its properties (sequence) were similar to that of mammalian eEF2K, and this, according to genomic databases searches, is not true. Hence, their conclusion that the moth possesses no eEF2K can be reinterpreted that *Spodoptera* has no eEF2K that is similar to its mammalian equivalent. In my opinion, one cannot derive from these experiments the conclusion that there is no eEF2K in any form in the moth.

4.3.2 Arguments for the existence of eEF2K in insects

On the other hand, insects may possess a functional homologue of eEF2K, which does not share sequence similarity to its mammalian equivalent. There are examples from other systems, that the same function can be mediated by, in terms of amino acid sequence, totally unrelated kinases that share the same regulatory properties. This may indeed be the eEF2K case, as crystallographic investigation of one of the α-kinases suggested, that in spite of totally different sequence, 3D-structre interactions may be similar to the orthodox protein kinases (Proud, 2007;Yamaguchi et al., 2001).

This hypothesis is in a good agreement with results presented in this thesis. I showed that in honeybee brain homogenates, eEF2 may be phosphorylated upon stimulation, which requires the existence of an appropriate kinase. Moreover, the experiments presented above suggest that eEF2 in this insect is regulated by cAMP and Ca^{2+}, in a way similar to mammals. Since genomic databases algorithms are not able to identify significant equivalents of mammalian eEF2K in insects, one could speculate that the putative honeybee eEF2K does not share sequence similarities to α-kinases. This suggests the existence of an orthodox kinase capable of performing functions typical for α-kinases.

To the best of my knowledge, this is the first suggestion, that eEF2K activity is present in insect tissues. One needs further experiments, which are out of scope of this thesis, to support the idea that a putative eEF2K exists in insects. On the one hand, one could apply a modelling approach to elucidate which insect protein has structural properties similar to mammalian

eEF2K, and on the other hand, one could use biochemical methods to purify and sequence the kinase(s) that phosphorylate insect eEF2.

4.3.3 cAMP increases phosphorylation of eEF2 *in vitro*

Apart from showing the existence of eEF2K activity in the honeybee I tried to establish the signalling pathways by which eEF2 is regulated in the honeybees and found that cAMP raises eEF2 phosphorylation level in the brain homogenates.

In mammals, phosphorylation of eEF2 is increased by the specific kinase - eEF2K (Ryazanov et al., 1988) and decreased by the phosphatase - PP2A (Browne and Proud, 2002) (Figure 4-1). Thus, increase in phosphorylation may be mediated either by direct phosphorylation (increase in eEF2K activity while PP2A activity remains unchanged) or by inhibition of dephosphorylation (decrease of PP2A activity while eEF2K activity remains unchanged).

cAMP induces eEF2 phosphorylation (deactivation) in non-neural vertebrate cells and this process is mediated by eEF2K (Gutzkow et al., 2003;Hovland et al., 1999;McLeod et al., 2001). PKA was also shown to directly phosphorylate eEF2K in rat leukaemia cells (Hovland et al., 1999).

On the other hand the cAMP/PKA system activates PP2A (Ahn et al., 2007) and PP2A decreases eEF2 phosphorylation (Everett et al., 2001). Interestingly, cAMP was shown to dephosphorylate eEF2 in a non-PKA way (Feschenko et al., 2002). Anyway, there is no data showing that cAMP or PKA can block PP2A and thus, it is improbable, that inhibition of PP2A contributes to the cAMP-induced increase of eEF2 phosphorylation.

Observed cAMP-induced phosphorylation of eEF2 in the bee brain may be plausibly explained by PKA-dependent activation of the putative eEF2K. The other explanation is that PKA directly phosphorylates eEF2, although this explanation seems less probable as PKA was not reported to directly influence eEF2 in mammalian tissues.

Results presented in this thesis are the first suggestion that, in neural tissues eEF2 is phosphorylated as a result of cAMP application. Moreover, it seems that, just like in mammals, in the honeybee eEF2 phosphorylation is regulated by the cAMP/PKA system.

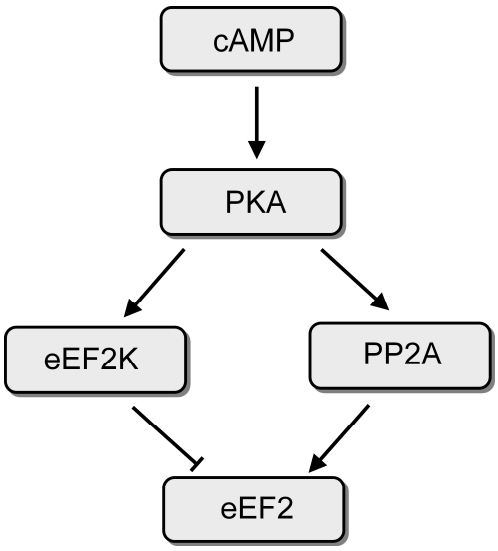


Figure 4-1 cAMP modulates eEF2 activity in mammalian tissues.

cAMP is able to deactivate eEF2 (increase eEF2 phosphorylation) through PKA and eEF2K. On the other hand cAMP or PKA can activate eEF2 (decrease eEF2 phosphorylation) through PP2A (but the latter possibility is controversial - see text for details).

4.3.4 Ca^{2+} increases phosphorylation of eEF2 *in vitro*

In experiments presented in this thesis, I have shown that application of calcium to the honeybee brain homogenate induces phosphorylation of eEF2 but at the same time decreases total amount of this protein.

Similar effects were reported in mammalian non-neural tissues. Calcium application evokes eEF2 phosphorylation, subsequent diminishment of elongation rate and this process is dependent on eEF2K (calcium/calmodulin dependent kinase III) (Hincke and Nairn, 1992;Nairn and Palfrey, 1987;Ryazanov, 1987). Strikingly similar phenomenon was published in a paper studying axon growth cones in vertebrate cell culture (Iizuka et al., 2007). The authors showed that high Ca^{2+} levels induced fast and transient phosphorylation of eEF2 and concurrent decrease of the total amount of the protein. These alterations were paralleled by an increase in eEF2K activity. Unfortunately, the paper does not address mechanisms of calcium-evoked decrease in total eEF2 amount.

A possible explanation of calcium-induced decrease in eEF2 amount involves the activation of calcium-activated proteases - calpains. Interestingly, these proteases play an important role in synaptic plasticity (Wu and Lynch, 2006). Verification if the observed decrease in the amount of eEF2 is mediated by these proteases would require the application of a calpain inhibitor (e.g. calpastatin (Carragher, 2006)) along with calcium.

Results presented in this thesis suggest that eEF2 in honeybee brain is regulated not only by cAMP but also by calcium. This corroborates the hypothesis that eEF2 in the insect is controlled in the same way as in mammals.

4.4 Possible influence of cAMP and Ca^{2+} on eEF2 during learning

4.4.1 Possible interactions between cAMP and eEF2 during learning

In this thesis, I have shown that cAMP inhibits eEF2 activity in honeybee brain homogenates, but on the other hand, eEF2 is activated upon learning. Earlier experiments showed that cAMP was positively involved in LTM formation in ALs in honeybees (Muller, 2000), so there is a clear contradiction between the data.

There are a few probable explanations of this discrepancy. First of all, the learning-induced changes in cAMP/PKA system are restricted to the honeybee ALs (Muller, 2000), while the immunohistochemical data presented in this thesis show, that eEF2 is hardly detectable in these brain structures. It may well be, that cAMP/PKA and protein translation systems are both activated during memory formation, but due to the different spatial localization, they do not interact with each other. This is a general problem, as we very often do not know if pathways involved in learning and plasticity exist and interact within the same neurons or neuronal networks. Only the single neuron labelling techniques (e.g. in *Drosophila* (Keene and Waddell, 2007)) can help to tackle this problem.

Second explanation of this discrepancy points to an idea that regulation pathways investigated in *in vitro* preparations (e.g. brain homogenates) do not necessarily directly correspond to the regulation *in vivo*. Homogenisation of a tissue causes dramatic changes in cellular homeostasis and the spatial organisation of regulatory pathways is destroyed by such procedure. In mammals, PKA can directly phosphorylate PP2A (Ahn et al., 2007), and PP2A induces dephosphorylation of eEF2 (Sans et al., 2004) what results in augmented elongation. On the other hand, PKA activates eEF2K and increases phosphorylation of eEF2 (Gutzkow et al., 2003) (see Figure 4-1). It is possible that only pathways leading to cAMP-induced increase in eEF2 phosphorylation (cAMP/PKA-“eEF2K”-eEF2) survive homogenisation in a functional state while pathways decreasing eEF2 phosphorylation (cAMP/PKA-PP2A-eEF2) are disrupted by this procedure (but see chapter 1.2.3.2 for remarks whether the latter pathway exists).

Assuming that during learning, PKA and protein synthesis are activated in the same neurons, PKA stimulation could be able to exert two opposing effects on translation: intensify or decrease protein synthesis by bidirectionally modulating eEF2. Such bidirectional changes in eEF2 activity in mammalian neurons were presented and eEF2 was reported to function as a

postsynaptic decoder of neural activity. eEF2 was activated by action potentials, but on the other hand inhibited by miniature neurotransmission (Sutton et al., 2007).

Direct influence of cAMP on brain eEF2 was not addressed *in vivo*. It would be valuable to use cAMP uncaging in living honeybee brains to address the effects of the cAMP pathway on eEF2 regulation *in vivo*.

4.4.2 Possible interactions between Ca^{2+} and eEF2 during memory formation

In experiments presented in this thesis, I have shown that calcium application to the honeybee brain homogenates increased eEF2 phosphorylation (deactivated the factor). Calcium is an all-important element in molecular memory formation processes. Why does it inhibit eEF2 and as consequence decelerate protein translation? There are a few possible explanations to this conundrum. First, as written in chapter 4.4.1, we cannot directly compare changes of eEF2 phosphorylation in bee brain homogenates with such changes in neurons *in vivo*. Secondly, distinct changes in synaptic activity change eEF2 activity in opposing directions. As synaptic activity modulates calcium concentrations, there is a possibility that calcium takes part in bidirectional regulation of eEF2 phosphorylation *in vivo* (Sutton et al., 2007). Therefore, calcium-induced phosphorylation of eEF2 observed in honeybee brain homogenates would represent only one, probably most significant, of the two possible directions of change that exist *in vivo*.

4.5 Glutamate induced eEF2 synthesis as a possible mechanism of LTM formation

In experiments presented above, I have shown that glutamate uncaging in the *Apis mellifera* brain on the one hand rapidly increased total amount of the eEF2, but on the other hand increased phosphorylation level of eEF2 and hence reduced its activity.

Glutamate is an important factor in long-term memory and LTP formation in a wide variety of experimental animals ranging from molluscs to rodents (Kandel, 2001). This neurotransmitter was shown to be positively involved in long-term memory formation also in the honeybee (Kucharski et al., 2007;Locatelli et al., 2005;Maleszka et al., 2000;Si et al., 2004) and *Drosophila* (Wu et al., 2007). Moreover, glutamate NMDA (Zannat et al., 2006) and metabotropic (Kucharski et al., 2007) receptors were identified and localized in the bee brain. Specifically, uncaging the amino acid in the honeybee mushroom bodies shortly after single conditioning trial leads to an enhancement of LTM (Locatelli et al., 2005).

Molecular effects of glutamate receptors stimulation are extensive, and protein translation is one of the processes regulated by this amino acid. Glutamate was shown to boost local, dendritic synthesis of eEF1α (elongation factor 1α). Five minutes after L-LTP induction by high-frequency stimulation of the CA1 area of the rat hippocampus, there is a strong increase in dendritic eEF1α synthesis. This increment is glutamate-specific, since it can be blocked by NMDA receptor inhibitors (Tsokas et al., 2005). A recent paper from the same group reported a rapid synthesis of eEF2 (as well as other proteins translated from a group of mRNAs (5'TOP mRNAs) coding part of translation machinery) in dendrites after similar stimulation (Tsokas et al., 2007). These *in vitro* experiments are in very good accordance with the *in vivo* results presented in this thesis, corroborating the idea that glutamate induces eEF2 synthesis in the bee brain. However, experiments using protein synthesis blocker are necessary to substantiate the idea of glutamate induced eEF2 synthesis in the bee brain. It would be also valuable to test if other components of the translation machinery (translation factors, ribosomal proteins) are synthesised in the bee brain in a response to glutamate treatment. A still unresolved question is the exact localisation of eEF2 synthesis – comparison of immunohistochemical stainings of bee brains with and without glutamate uncaging could answer this question. Interestingly, histological patterns of eEF2 localisation resemble to some extent the localisation of NR1 subunit of NMDA receptor (much in the α-lobe and the protocerebral lobe, and little in the MBs) (Zannat et al., 2006).

Whether glutamate-induced eEF2 translation in bee brain occurs in neuronal dendrites or cell bodies is an open question. Dendritic protein synthesis requires similar localisation of its mRNA and indeed, such localization was reported: eEF2 mRNA is present in postsynaptic densities (PSD) in rat forebrains (Suzuki et al., 2007). Nothing is known about this aspect in the bee yet.

What is the role of such eEF2 synthesis in the brain? Its mRNA belongs to the group (5'TOP mRNAs) that encodes part of the translation machinery (Meyuhas, 2000;Meyuhas and Hornstein, 2000) and can be quickly shifted from translationally quiescent (mRNP) into translationally active state (polysoms) upon stimulation (Meyuhas and Hornstein, 2000). It enables cells to produce the protein synthesis apparatus quickly. Such a mechanism of fast increase in translation capacity was proposed to be yet another mechanism of synaptic plasticity (Tsokas et al., 2007). Dendrites and synapses that increase the number of translation machineries (ribosomes, translational factors etc.) are capable of a quick, local and on demand synthesis of PRPs (plasticity related proteins). This would constitute the basis for permanent changes in strength and number of synapses.

In the bee brain glutamate enhances memory performance (Locatelli et al., 2005) as well elevates the amount of eEF2 (my own experiments). Taken together, these results make a correlational argument for the hypothesis that increasing amount of translational factors in the brain is a possible mechanism of memory formation and/or maintenance in the honeybee. A casual evidence is missing and a critical experiment testing this hypothesis would be the measurement of LTM performance after inhibition of dendritic translation of eEF2 (and other 5'TOP) mRNAs, e.g. by RNAi. To test the role of NMDA receptors in glutamate induced eEF2 synthesis, this experiment should be done in presence of glutamate (iono- and metabotropic) receptors inhibitors. To corroborate the hypothesis that glutamate induces in the bee brain synthesis of eEF2, and not releases the protein from a kind of store, glutamate uncaging should be done in presence of protein synthesis inhibitors.

4.5.1 Glutamate increases eEF2 phosphorylation

Results presented in this thesis show, that apart from increasing total amount of eEF2, glutamate induces its phosphorylation. This is in good accordance with literature data as glutamate was demonstrated to increase eEF2 phosphorylation and diminish general translation in neuronal (Gauchy et al., 2002;Marin et al., 1997;Scheetz et al., 2000) and non-neural (Cossenza et al., 2006) tissues in a transient way (Gonzalez-Mejia et al., 2006).

Glutamate-dependent slowdown of translation elongation seems contradictory to the well-known dependence of memory formation on new protein synthesis and concomitant involvement of glutamate receptors in this process. Such process however, could play a very important role in regulating translation in neurons. In mammalian neurons, NMDAR stimulation transiently inhibits elongation by phosphorylating eEF2, what subsequently decreases general translation rate, but paradoxically increases synthesis of CaMKII (Scheetz et al., 2000) – a protein ubiquitously involved in memory formation. Glutamate may function as a switch inhibiting synthesis of non-PRP (non-plasticity related proteins) and increasing translation of PRPs. To address this hypothesis it would be necessary to check CaMKII levels after glutamate uncaging in the bee brain.

This proposition is not necessarily in contradiction to learning-induced decrease in eEF2 phosphorylation described in this thesis. *In vivo*, glutamate-induced phosphorylation of the eEF2 may be only short-lived, resulting in a switch to the synthesis of PRPs, followed by an increase in elongation rate. This speculative model makes an exciting experimental proposition.

4.5.2 Glutamate does not modulate S6K

In experiments presented in this thesis, I was not able to prove that glutamate induces change in phosphorylation level of the S6K. This is contradictory to other findings showing that glutamate did render phosphorylation of S6K in neurons (Lenz and Avruch, 2005) and glial cells (Gonzalez-Mejia et al., 2006).

There are results suggesting that translation of elongation factors and other 5'TOP mRNAs depends on phosphorylation of S6 ribosomal protein (rpS6) and antecedent activation of its kinase (S6 kinase, S6K) (Fumagalli and Thomas, 2000;Jefferies et al., 1997). However, this has recently been questioned, and it was suggested that rpS6 does not control translation of 5'TOP mRNAs (Ruvinsky and Meyuhas, 2006). In my experiments, glutamate enhances eEF2 translation without influencing S6K, what suggests that S6K is not involved in regulation of synthesis of eEF2. This in agreement with the second possibility (Ruvinsky and Meyuhas, 2006), suggesting that S6K does not control translation of eEF2. To clear the problem of S6K involvement in eEF2 translation one could block activity of this kinase during glutamate uncaging and measure the synthesis of eEF2.

4.6 Feeding destabilises the balance between phosphorylated and dephosphorylated eEF2

Relation between feeding status, translation and memory formation seems to be a conundrum. On the one hand, nutrients stimulate protein synthesis and the latter process is necessary for long-term memory formation (Proud, 2002;Proud, 2007). On the other hand, satiated animals do not build normal LTM what results in poor LTM performance in the range of days (Friedrich et al., 2004).

There is not much research investigating the influence of feeding on memory formation and performance, although common assumption is that animals are to be moderately hungry during training. One of the few works that explicitly addresses this problem was done in the honeybee: LTM was impaired when bees were well fed compared to more hungry animals (Friedrich et al., 2004).

Feeding influences not only learning, but also energy status and thus translation. Therefore, I tried to establish the common biochemical factor that is influenced by both feeding and learning. In experiments presented in this thesis, I have shown that feeding destabilises the balance between phosphorylated and dephosphorylated eEF2 in honeybee brains. This effect is immediate and profound directly after feeding and can be observed in a less intense form 30 min later. This transient destabilisation of eEF2 correlates with inhibitory effects of feeding on memory formation (Friedrich et al., 2004). As eEF2 is modulated by both learning and feeding one could hypothesise, that inhibitory effects of feeding on memory performance are mediated by the destabilisation of eEF2 phosphorylation in the bee brain.

There is also a possibility that changes in pheEF2/eEF2 correlation in the bee do not reflect changes in energy/feeding status, but are induced by mere sucrose perception and reflect sensitisation rather than feeding processes.

4.7 Rapamycin enhances memory performance

In experiments presented in this thesis, I have shown that rapamycin does not influence memory performance after 3 trial conditioning, but it significantly enhances memory performance evoked by 1 trial conditioning.

4.7.1 Rapamycin does not impair memory performance after 3 trial conditioning

Injection of rapamycin to the thoraces of bees, before the strong training procedure (3 trial conditioning) that leads to LTM formation did not impair memory performance. This is in disagreement with some published results, as rapamycin prevents long-term fear memory formation in mammals (Bekinschtein et al., 2007;Parsons et al., 2006) and deteriorates LTF in *Aplysia* (Casadio et al., 1999). One can argue that rapamycin does not deteriorate memory, as it is not specific in the honeybee. While the specificity of rapamycin in the bee has not been directly demonstrated, this drug was successfully used in other insects, such as *Drosophila* (Hennig et al., 2006), molluscs such as *Aplysia* (Carroll et al., 2004;Carroll et al., 2006) and even yeast (Zinzalla et al., 2007). Therefore, it seems justified to assume that rapamycin specifically works in the honeybee.

On the biochemical level rapamycin is an inhibitor of TOR (target of rapamycin) protein which is one of the main protein translation controllers (Arsham and Neufeld, 2006). TOR activates translation through intensifying the elongation phase (Sataranatarajan et al., 2007) and stimulating the initiation phase in a cap-dependent way (Proud, 2007).

TOR activates elongation phase of translation through S6K (Proud, 2007), eEF2K and finally activation of eEF2 (Browne and Proud, 2002) (see Figure 4-2). If rapamycin effectively blocked this pathway, then it would erase learning-induced activation of eEF2 and this could inhibit memory performance. However, this was not the case, and TOR/S6K pathway seems not to be involved in memory formation in the bee. This notion is corroborated by my results showing that conditioning trials did not modulate S6K.

Rapamycin blocks not only elongation phase of protein synthesis but also the cap-dependent initiation of translation (Proud, 2007). This process seems not to be involved in memory formation in the bee either, as rapamycin does not cause a memory impairment.

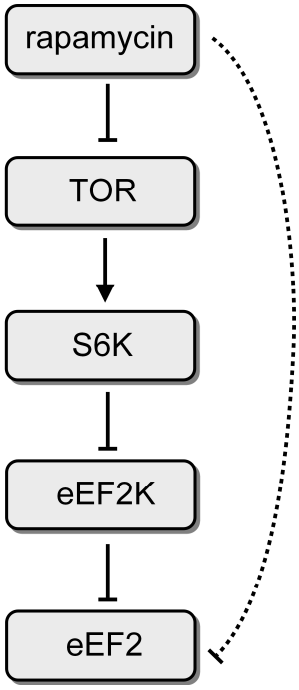


Figure 4-2 In mammals rapamycin inhibits TOR/S6K pathway what possibly results in deterioration of eEF2 activity.

Rapamycin is a potent blocker of TOR. TOR activates elongation phase of translation by releasing eEF2K inhibition over eEF2. Therefore, application of rapamycin may result in deterioration of eEF2 activity.

4.7.2 Rapamycin enhances memory after 1 trial conditioning

Rapamycin did not deteriorate LTM induced by 3 trial conditioning in the bee. Unexpectedly, one could observe a small, although statistically insignificant, memory enhancement. This was quite surprising, but there are some results suggesting that low concentrations of rapamycin may enhance synaptic plasticity. For example this drug transformed E-LTP into L-LTP in mammalian hippocampus slices (Terashima et al., 2000). Hence, I decided to check if rapamycin improved memory in the bee. It turned out that rapamycin applied before the weak training procedure (1 trial conditioning) did increase this cognitive process in the insect and boosted MTM and a distinct phase of LTM. This seems surprising, as rapamycin is generally thought to inhibit translation and particularly its initiation phase. However, the observation may be parsimoniously explained by taking into consideration that rapamycin blocks only cap-dependent form of initiation, at the same time augmenting cap-independent (IRES-dependent) initiation of translation.

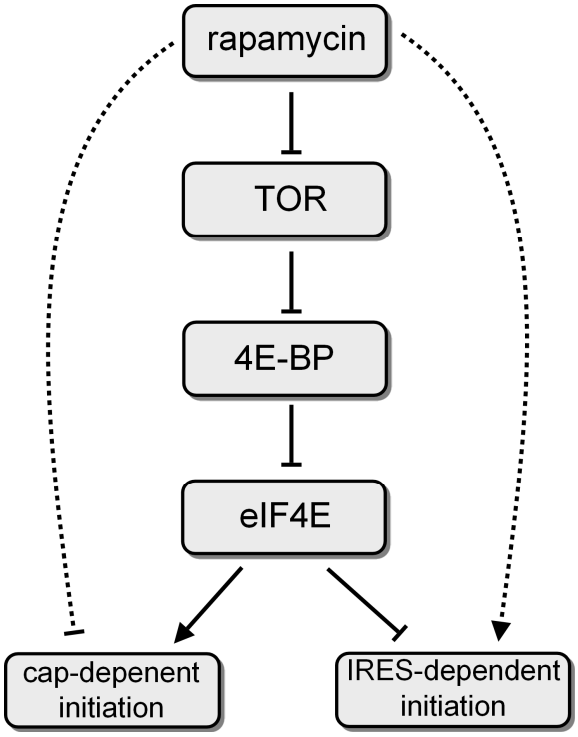


Figure 4-3 Rapamycin inhibits cap-dependent, but augments cap-independent (IRES-dependent) initiation of translation.

Rapamycin blocks TOR what results in sequestration of eIF4E. This leads to inhibition of cap-dependent and augmentation of cap-independent (IRES-mediated) initiation of protein synthesis.

Cap-dependent initiation of translation is by far the most typical way of initiating translation, but there is a group of mRNAs that may be initiated in a cap-independent (IRES-dependent) way as well (Lopez-Lastra et al., 2005). Rapamycin by inhibition of TOR (target of rapamycin protein), blocks eIF4E (eukaryotic initiation factor 4E) (Raught et al., 2000). eIF4E is an initiation factor responsible for mRNA cap binding (Proud, 2007) and was reported to function as a switch from cap-dependent to cap-independent (IRES-dependent) initiation of translation in *Aplysia* neurons (Dyer et al., 2003) and in picornavirus infected mammalian cells (Svitkin et al., 2005). As a result, rapamycin blocks cap-dependent initiation while at the same time it facilitates cap-independent (IRES-mediated) initiation (Figure 4-3).

Rapamycin was not described to directly induce translation of IRES-containing mRNAs, but it was reported to spare translation of such mRNAs during general inhibition of protein synthesis in mammals (Chang et al., 2007) and *Drosophila* (Vazquez-Pianzola et al., 2007). This chemical was also shown to augment IRES-mediated initiation in mice (Frost et al., 2007).

Many of the dendritic mRNAs involved in memory and synaptic plasticity contain IRES sequences and have possibility to be initiated in the cap-independent (IRES-dependent) manner. To this group belong CaMKII, MAP2, Arc, dendrin and neurogranin (Pinkstaff et al., 2001). CaMKII, neurogranin and Arc have been reported to play a positive and crucial role in synaptic plasticity and memory formation: CaMKII is synthesised in *Drosophila* ALs upon learning (Ashraf et al., 2006); neurogranin enhances LTP and learning in mice (Huang et al., 2004) and Arc is essentially involved in LTP formation (Tzingounis and Nicoll, 2006). Interestingly, in terms of localisation, cap-independent initiation of these mRNAs is more efficient in dendrites than in the cell body.

It is very tempting to formulate a hypothesis that rapamycin-induced memory enhancement in the honeybee is mediated by an increase in translation from IRES-containing mRNAs, such as CaMKII. A similar hypothesis was actually formulated a few years ago: “A switch to IRES usage in sensory neurons may be important for an increase in synaptic strength that depends on proteins that are translated at synapses during a general decrease in translation rate” (Dyer et al., 2003).

To test this hypothesis, it would be necessary to eliminate IRES structures (from e.g. CaMKII) and check if this procedure leads to memory/synaptic plasticity deterioration. Determining of CaMKII levels after rapamycin application would also help to verify this hypothesis. It is also possible that pharmacological activation of TOR (at least in a certain range) leads to memory deterioration. Proposed model assumes that only small concentrations of rapamycin boost memory, while big concentrations of the substance may deteriorate this process. To check if this assumption holds, it is necessary to investigate influence of a rage of rapamycin concentrations on memory performance.

Rapamycin-induced mid- and long-term memory enhancement is most plausibly explicable by an augmentation of IRES-dependent initiation of translation. This proposition remains an exciting hypothesis and has to be addressed experimentally.

4.8 Inhibition of proteasome enhances MTM performance

Results presented in this thesis showed that MG132, blocker of ubiquitin-proteasome complex (UPC) (Lee and Goldberg, 1998), applied shortly before or within a time window of 3 h after 1 trial conditioning, enhanced MTM, but left LTM intact. Application of the drug on the next day after learning did not influence memory.

MG132 has been successfully used in many invertebrate species: *Aplysia* (Kurosu et al., 2007), crab *Chasmagnathus* (Merlo and Romano, 2007) and *Drosophila* (Lundgren et al., 2005), so one can premise that this substance specifically works in the honeybee.

The idea that proteasome and protein degradation play an important role in memory and plasticity processes gained momentum only recently (Hegde, 2004;Patrick, 2006), however the results are contradictory. On the one hand blocking UPC during training disrupts LTM in crab *Chasmagnathus* (Merlo and Romano, 2007), deteriorates L-LTP in rat hippocampal slices (Fonseca et al., 2006) and LTF in *Aplysia* (Hegde et al., 1993). On the other hand chronic proteasome inhibitors enhance LTF, strengthen the synapses and even induce outgrowth of neurites in *Aplysia* (Zhao et al., 2003). Even more interestingly, UPC blockers rapidly elevate presynaptic (Speese et al., 2003) and postsynaptic (Haas et al., 2007) transmission efficacy in *Drosophila* neuromuscular junctions. Results resembling the effects presented in this thesis, were published in a recent paper. Blocking of proteasome led to reversal of the anisomycin-induced memory impairment after retrieval of consolidated fear memory in the rat. Moreover, injection of proteasome blockers before extinction trials, impairs fear memory extinction what results in higher memory scores (Lee et al., 2008). Particularly this last result is in a very good accordance with the effect reported in this thesis.

The published results suggest that the role of the ubiquitin-proteasome complex is not straightforward and this structure may function either as a positive or negative factor in memory formation/retrieval and synaptic plasticity. *In vivo* results obtained during this work in the honeybee are in accordance with the idea that proteasome functions as a negative constraint and blocking its function leads to enhancement of memory scores. They are in agreement with suggestion that UPC destabilises retrieved memory (Lee et al., 2008).

4.9 Proposition for a mechanism explaining memory enhancement after cycloheximide treatment in the honeybee.

Experiments presented in this thesis suggest that, like in other experimental animals, long-term memory formation crucially depends on translation of new proteins.

Initial experiments in the honeybee seemed to contradict this idea, as application of a potent protein synthesis inhibitor (cycloheximide) did not deteriorate memory performance in a series of experiments (Menzel et al., 1993;Wittstock et al., 1993;Wittstock and Menzel, 1994). Moreover, cycloheximide raised memory performance 6 hours after 2 trial olfactory conditioning (Wittstock et al., 1993). When bees were trained 3 times, cycloheximide enhanced LTM performance on the next day after conditioning (Menzel et al., 1993). These surprising results suggested that honeybee is an exception and memory processes in this species do not depend on new protein synthesis. It has to be noted, that in these experiments, protein synthesis reduction observed in the bee brain was never complete and reached 95% (Wittstock et al., 1993). Therefore, there was a possibility that this residual protein synthesis was responsible for rescuing memory performance in conditions of dramatically reduced protein synthesis.

Cycloheximide is a substance that inhibits protein synthesis, but there is an important publication reporting that this chemical rescued and even stimulated synthesis of CaMKII during general protein synthesis inhibition. In cell cultures of mammalian neurons, mild concentration of this drug dramatically reduced general protein synthesis, but at the same time, increased translation of CaMKII by 40%. Lower doses of cycloheximide induced even bigger synthesis of CaMKII (Scheetz et al., 2000). Concentrations of this PSI used in the experiments with honeybees *in vivo* (Menzel et al., 1993;Wittstock et al., 1993;Wittstock and Menzel, 1994) were only about 8 times higher than those used in mammalian cell cultures *in vitro* (Scheetz et al., 2000). Most probably, the effective concentration in the honeybee brain was even lower, approaching conditions similar to the neuronal culture. If this was the case, then cycloheximide could have blocked general protein synthesis and coincidently boosted synthesis of CaMKII in the bee brain. As CaMKII is a crucial component of memory formation processes (Wang et al., 2006) this mechanism could explain the surprising cycloheximide-induced memory enhancement in the bee.

mRNA of CaMKII contains IRES sequences (Pinkstaff et al., 2001) and active IRES formation is enhanced after cycloheximide treatment (Fernandez et al., 2005). Hence, cap-

independent initiation of translation could explain increase in CaMKII synthesis. To test this hypothesis, one would have to investigate the influence of low doses of cycloheximide on CaMKII synthesis in the brain, and subsequently conduct the same experiment eliminating IRES structures.

5 Summary

The aim of this work was to describe the involvement of protein translation processes in the formation of long-term memory during classical, olfactory conditioning in the honeybee (*Apis mellifera*). Special attention was given to the elongation phase of translation and its main regulatory factor: eEF2 (eukaryotic elongation factor 2).

Since the eEF2 kinase (eEF2K) responsible for phosphorylating and thus regulating of eEF2 was not yet detected (and even suggested not to exist) in insects, I designed a set of *in vitro* experiments to test the existence of a kinase capable of phosphorylating eEF2. cAMP and Ca^{2+} increase eEF2 phosphorylation in mammals, so they were added to the honeybee brain homogenates and raised eEF2 phosphorylation level. This experiment provides a strong argument for the existence of a functional homologue of the eEF2K in the honeybee and is the first indication that such a kinase exists in insects.

To check if memory processes modulate the elongation phase of translation in the honeybee, the animals were trained using classical, olfactory conditioning paradigm. Learning trials transiently decreased eEF2 phosphorylation (increased its activity) 10 min after learning and this augmentation came back to the baseline levels 2 h later. Total amount of eEF2 remained constant. This suggests that conditioning trials transiently, in the range of a dozen of minutes, boost the elongation phase of translation.

Trying to elaborate signalling pathways involved in learning-induced eEF2 activation TOR protein (a known regulator of translation system) was inhibited with rapamycin. After strong conditioning protocol (3 trial conditioning) memory performance was not deteriorated. Surprisingly, when a weak protocol (1 trial conditioning) was applied in the presence of rapamycin, memory was enhanced in the range of hours and days. This memory increment may be explained by the rapamycin-induced activation of the cap-independent initiation of translation.

In order to establish pathways concomitantly regulating eEF2 and memory processes, glutamate was uncaged in the honeybee brain. This action increased total amount as well as phosphorylation level of eEF2 within 15 min after stimulation. Increase in total amount of eEF2, a central component in the translation process, should increment translational capacity, what according to a new hypothesis may represent a novel mechanism contributing to synaptic plasticity and LTM formation.

To examine the involvement of proteasome, the protein degradation machinery, in memory formation and maintenance processes, this structure was blocked by MG132. This chemical injected either before 1 trial conditioning or before the retrieval test at 3 hours increased memory performance at 3 hours, but was ineffective when injected before the retrieval test at day 1. This experiment suggests that in the honeybee, proteasome acts as a negative constraint on memory.

In order to determine the localisation of eEF2 protein in the honeybee brain, tissue slices were incubated with anti-eEF2 antibodies. The most intensive staining was found in the protocerebral lobes and in separate bands of the α-lobes of the mushroom bodies. These structures consist mainly of neuropil, suggesting that dendritic rather then perikaryal translation dominates in the honeybee brain.

Experiments presented in this thesis provide first insights into protein translation processes in the honeybee brain in the context of LTM formation. They suggest an increase in the elongation rate of protein synthesis cycle after learning and point to an important role of rapamycin and glutamate-dependent pathways in these processes. Existence of a functional homologue of eEF2K in the bee is postulated as well as the involvement of proteasome in memory.

6 Zusammenfassung

Ziel dieser Arbeit war es, die Beteiligung der Proteinsynthese bei der Bildung des Langzeitgedächtnisses nach klassischer, olfaktorischer Konditionierung in der Honigbiene (*Apis mellifera*) zu beschreiben. Besondere Aufmerksamkeit galt der Elongationsphase der Translation und ihrem wichtigsten regulierenden Faktor: eEF2 (eukaryotic elongation factor 2).

Da die Kinase (eEF2K), die für die Phosphorylierung und folglich für die Regulation von eEF2 verantwortlich ist, in Insekten noch nicht bekannt ist (es wird sogar vermutet, das sie überhaupt nicht existiert), habe ich eine Reihe von in vitro Experimenten durchgeführt, um die Existenz einer solchen Kinase, die eEF2 phosphorylieren kann, zu prüfen. Wie bei Säugetieren steigert cAMP und Ca^{2+} die Phosphorylierung von eEF2 in der Honigbiene. Dies ist der erste eindeutige Befund dass ein Funktionshomolog der eEF2K auch in der Honigbiene (Insekten) existiert.

Um zu überprüfen ob die Elongationphase der Translation in der Honigbiene während der Bildung eines Gedächtnisses moduliert wird, wurden die Tiere nach einem klassischen, olfaktorischen Paradigma konditioniert. Zehn Minuten nach dem Lernen verringerte sich die Phosphorylierung von eEF2 vorübergehend (Aktivitätserhöhung). Dieser Anstieg kam 2 h später zum Ausgangsniveaus zurück. Die Gesamtmenge von eEF2 blieb konstant. Dies lässt vermuten, dass die Elongation im Zeitraum von einigen Minuten nach dem Lernen verstärkt wird.

Um herauszufinden, welche Signaltransduktionwege bei der lerninduzierten Aktivierung von eEF2 eine Rolle spielen, wurde TOR (Target of rapamycin), ein bekannter Regulator des Translationsystems mit Rapamycin blockiert. Während sich die Gedächtnisleistung nach einem 3-fachen Konditionierungsprotokoll, nicht verändert, führt Rapamycin nach 1-facher Konditionierung (schwaches Trainingsprotokoll) im Zeitraum von Stunden bis Tagen zu einer verbesserten Gedächtnisleistung. Diese Gedächtnisverbesserung liegt wahrscheinlich an einer Rapamycin-verursachten Aktivierung der cap-unabhängigen Initiation der Translation.

Da bekannt ist, dass Glutamat-abhängige Signaltransduktionwege an der Verbesserung der Gedächtnisleistung beteiligt sind, wurde der Einfluß von Glutamatfreisetzung auf eEF2 im Honigbienengehirn untersucht. Es zeigte sich, dass sowohl die Gesamtmenge als auch das Phosphorylierungsniveau von eEF2 innerhalb von 15 Minuten nach der Stimulation erhöht wurden. Eine Erhöhung der Gesamtmenge von eEF2 würde nach neuen Vorstellungen die

Translationskapazität erhöhen. Mit der Glutamat-induzierten Mengenänderung von eEF2 ergeben sich somit erstmals Hinweise auf einen neuen Mechanismus der Tranlationsregulation der zur synaptischen Plastizität und zur Langzeitgedächtnisbildung (LTM) beitragen könnte.

Um die Beteiligung der Proteasome, der Proteindegradationsmaschinerie, bei der Gedächtnisbildung und -erhaltung zu untersuchen, wurden die Proteasome mit MG132 blockiert. Blockierung der Proteasome vor der 1-fachen Konditionierung oder vor dem Gedächtnistest nach 3 Stunden, erhöhte die Gedächtnisleistung nach 3 Stunden. MG132 hatte jedoch keinen Effekt, wenn es vor einem Test an Tag 1 (24 h später) injiziert wurde. Dieses Experiment lässt vermuten, dass Proteasome eine hemmende Wirkung auf das Gedächtnis im Stundenbereich haben.

Immunhistologische Untersuchungen mit anti-eEF2 Antikörpern zeigen, dass die intensivste Färbung in den protozerebralen Loben und in einigen Bändern der α-Loben der Pilzkörper zu finden ist. Diese Strukturen bestehen hauptsächlich aus Neuropil, was darauf hindeutet, dass dendritische Translation gegenüber perikaryaler Translation in der Honigbiene dominiert.

Zusammengefasst gewähren diese Untersuchungen erste Einblicke in die Regulationsprozesse der Proteinbiosynthese im Honigbienengehirn während der Bildung eines Langzeitgedächtnisses. Sie weisen auf eine Zunahme der Elongationsrate des Proteinsynthesezyklusses hin und legen eine wichtige Rolle der Rapamycin-abhängigen Signaltransduktionswege und des Glutamates bei diesen Prozessen nahe. Darüber hinaus wird erstmals die Existenz eines Funktionshomologes von eEF2K in Insekten beschrieben und Hinweise für die Beteiligung der Proteasome bei der Gedächtnisbildung präsentiert.

7 Abbreviations

4E-BP - eIF4E binding protein

5’TOP - 5’-terminal oligopyrimidine (mRNA)

5-HT - 5-hydroxytryptamine (serotonin)

aa-tRNA - amino acid-tRNA complex

ACT - antennal-cerebral tract

AGT - antenno-glomerularis tract

AMPK - AMP-activated protein kinase

ARM - anaesthesia resistant memory

BDNF - brain-derived neurotrophic factor

BLAST - Basic Local Alignment Search Tool

BSA - bovine serum albumin

CaMKII - calcium/calmodulin-dependent protein kinase II

cAMP - cyclic adenosine monophosphate

CPE - cytoplasmic polyadenylation element

CPEB - cytoplasmic polyadenylation element binding protein

CR - conditioned response

CREB - cyclic AMP response element binding protein

CS - conditioned stimulus

dsRNA - double-stranded RNA

DTE - dendritic targeting element

eEF - eukaryotic elongation factor

eEF2 - eukaryotic translation elongation factor 2

eEF2K - eEF2 kinase

eIF - eukaryotic initiation factor

eIF4E - eukaryotic initiation factor 4E

ELISA - enzyme-linked immunosorbent assay

E-LTD - early phase of long-term depression

eLTM - early long-term memory

E-LTP - early phase of long-term potentiation

EST - expressed sequence tag

HRP - horseradish peroxidase

IgG - immunoglobulin G

IRES - internal ribosome entry site

ITI - intertrial interval

KC - Kenyon cell

LH - lateral horn

L-LTD - late phase of long-term depression

lLTM - late long-term memory

L-LTP - late phase of long-term potentiation

LPL - lateral protocerebral lobe

LTD - long-term depression

LTF - long-term facilitation

LTM - long-term memory

LTP - long-term potentiation

LTS - long-term sensitisation

MAP2 - microtubule-associated protein 2

MAPK - mitogen-activated protein kinase

MB - mushroom body

miRNA - microRNA

mRNP - messenger ribonucleoprotein

MTM - mid-term memory

NMDAR - NMDA receptor

NO - nitric oxide

NOS - nitric oxide synthetase

OA - okadaic acid

PABP - poly(A) binding protein

PBS - phosphate buffered saline

PER - proboscis extension reflex

pheEF2 - eEF2 phosphorylated at Thr56

phS6K - p70 S6 kinase phosphorylated at Thr389

PKA - cAMP-dependent protein kinase A

PKC - protein kinase C

PN - projection neuron

PP1 - protein phosphatase 1

PP2A - protein phosphatase 2A

PP2B - protein phosphatase 2B

PRP - plasticity-related protein

PSD - postsynaptic density

PSI - protein synthesis inhibitor

RISC - RNA-induced silencing complex

RNAi - RNA interference

RNP - ribonucleotide protein

rpS6 - ribosomal protein S6

S6 - see rpS6

S6K - S6 kinase

SITA - synaptic integration in translational activation

STF - short-term facilitation

STM - short-term memory

TOR - target of rapamycin

UPC - ubiquitin-proteasome complex

UR - unconditioned response

US - unconditioned stimulus

VUM - ventral unpaired median (neuron)

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