The role of the microRNAs miR-12, miR-124, miR-125, miR-989 and miR-3788 in learning and memory formation processes in the honeybee (*Apis mellifera*)

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It is certain, that memory not only contains philosophy, but all the arts and all the appertain to the use of life.

(*Marcus Tullius Cicero*)

1. Introduction

1.1. Learning, memory, cognition

In the last century, humanity acquired remarkable knowledge in the world of science. Ideas which in their principles go back to antiquity, like the theory that the matter consists of inseparable particles got proven. As we unravel the structure of our world and its nature, we dig deeper into the details and discover even smaller bricks.

Since the ancient world, bright minds wondered about the inimitability of the human consciousness. Philosophers of almost all epochs raised theories about the speciality of the demarcation of the human mind compared to the rest of the animal kind.

The psychologist Herrmann Ebbinghaus contributed the paradigms of the learning and the forgetting curve to the science of learning and memory (Ebbinghaus 1885). Ebbinghaus conducted research on himself, which was not uncommon at the time. In the 20th century the physiologist and Nobel laureate Ivan Pavlov worked on classical conditioning and defined the concept of the "conditioned reflex" (Pavlov 1927). This concept did not only influence neurobiology and physiology but also psychology and changed behavioural approaches in science.

Since the development of methods in cellular biology, we were able to identify the processes of learning and memory formation on the smallest molecular level. Eric Kandel stated, that "One of the most remarkable aspects of an animal's behaviour is the ability to change that behaviour by learning an ability that reaches its highest form in human beings." (Kandel 2000). Our memory defines our personality since it relies on unique experiences and the ability to memorise them. In decades of research on the sea snail *Aplysia californica*, Kandel contributed a groundbreaking piece of knowledge to neurobiology.

With the necessity to understand the molecular processes of memory formation we go back to study smaller brains and simpler minds. Honeybees own the capacity of learning complex behavioural patterns. They can develop an exceptional visual and olfactory memory during foraging. The honeybee brain with about 1 µl volume and 950000 neurons (Witthöft 1967) pictures an ideal model to investigate memory functions.

1.1.1. Implicit and explicit learning

Memory formation and its mechanisms have been studied intensively in the last decades. The acquisition of skills, processes and knowledge which is called learning was categorised into an explicit and an implicit manner (Reber 1967). The implicit learning occurs unconsciously while the explicit learning is a deliberate act and occurs wilfully (Kandel, Schwartz 2014; Squire 1984). The appetitive olfactory conditioning of honeybee foragers provides a perfect paradigm to study the mechanisms of learning and memory formation processes.

1.1.2. Learning and memory formation in the model organism honeybee (*Apis mellifera*)

Honeybee foragers need the ability to learn, to recognise and to memorise food sources not only by visual orientation but also by olfactory perception. The appetitive olfactory conditioning of honeybee foragers provides a perfect paradigm to study the mechanisms of learning and memory formation processes. The most famous example of associative learning might be the classical conditioning after the physiologist Pavlov (Pavlov 1927). The associative olfactory conditioning is also a classical conditioning paradigm. In these conditioning experiments, the honeybees learn to associate an unknown odour stimulus with a known reward stimulus.

In a natural environment, the honeybees forage for nectar which consists of saccharides such as glucose, fructose and sucrose whereby these stimuli act as a reward for the bees. With the appetitive olfactory conditioning paradigm that we use for our experiments, we take advantage of the PER (Proboscis Extension Response) that is triggered by the contact of the bees antennae with sucrose solution (Kuwabara 1957). As sucrose is the preferred food source of the honeybees (Frisch 1934), we use 1 M sucrose solution for our learning experiments. We condition honeybee foragers by associative appetitive olfactory conditioning, pairing of two different stimuli a conditioned stimulus (CS) consisting of clove odour and an unconditioned stimulus (US) consisting of a sugar reward (1 M Sucrose solution). With the sequencing of the honeybee genome (Weinstock et al. 2006), the combination of behavioural and molecular studies opened to new possibilities.

1.1.3. Gustatory sensitivity and non-associative learning

Studying associative behaviour in its complexity does also include the study of non-associative behaviour and the sensitivity to stimuli we use in the experimental setups. The animals were tested for their gustatory sensitivity to sucrose. Depending on the age, the saturation level, the genotype and the task field of the honeybees, the gustatory sensitivity can vary (Page et al. 1998; Pankiw and Page 1999). Sensitisation and habituation belong to the non-associative learning paradigms. Sensitisation means the increase of preparedness to a stimulus. The presentation of a single strong stimulus can lead to a stronger response on a following much weaker stimulus. The honeybee can be sensitised to an odour stimulus through stimulation with concentrated sucrose solution (Erber 1981). Habituation means the accustoming to a longer lasting stimulus, which does not have any positive or negative consequences for the animal and therefore becomes meaningless.

1.2. Molecular mechanisms of learning and memory formation

1.2.1. Short-term and long-term memory

The molecular basics of memory formation processes have been compared in different animal models such as the mouse (*Mus musculus*), the fruit fly (*Drosophila melanogaster*), the sea snail (*Aplysia californica*), the honeybee (*Apis mellifera*) and others (Hernandez and Abel 2009; Menzel 2001). Studies on the patient H.M. revealed, that explicit memory is dependent on certain structures in the cerebral cortex, the medial temporal lobe, which includes the hippocampus (Squire 2009). Memory formation processes are based on different phases, which are induced and maintained through various mechanisms. Understanding the molecular processes regulated during the consolidation phase is one important aspect of explaining the different fundamentals of memory formation. It is a common assumption that long-term memory (LTM) represents the stable modification of neuronal circuit features, including the pattern and strength of synaptic connections. LTM and its activation through strong conditioning can trigger biochemical cascades towards the nucleus, where CREB (cAMP response element binding protein) activates transcription (Müller 2002). Another regulator of transcription in the central nervous system is the Methyl-CpG-binding protein 2 (MeCP2), which has also been observed to play a promoting

role in hippocampal synaptic plasticity (Na et al. 2013). Cellular and molecular studies of learning and memory formation on the sea snail *Aplysia californica* display a detailed network of pathways that are responsible for the storage of memory (Kandel and Schwartz 2014). Short-term sensitisation of the gill-withdrawal reflex induces short-term facilitation (STF) and long-term sensitisation of the gill-withdrawal reflex induces long-term facilitation (LTF). STF lasts for minutes or hours and is triggered in *Aplysia* by a single tail shock or a single pulse of serotonin (5-HT). Resulting covalent modifications of pre-existing proteins like the activation of the adenylyl cyclase, which converts ATP to the second messenger cAMP, which in turn activates the cAMP dependent protein kinase A (PKA) are described as the short-term pathway (Kandel and Schwartz 2014). LTF in *Aplysia* involves a sequence of cellular and molecular mechanisms (see figure 1). The release of neurotransmitter (see figure 1 (1)) and short-term strengthening of synaptic connections as well as the synthesis of new proteins, initiated by PKA (see figure 1 (2)), which recruits the mitogen activated kinase (MAPK) (3) (Kandel and Schwartz 2014; Hawkins et al. 2006). While CREB-1 is an activator of gene expression, CREB-2 inhibits CREB-1 and therewith its activation capability (see figure 1 (4)). For an activation of CREB-1, PKA is able to repress CREB-2. After being transported into the nucleus, PKA can phosphorylate CREB, and through this to activate the transcription (see figure 1 (5)) of cAMP response elements (CRE) in the upstream region of two different cAMP inducible genes (see figure 1 (6)). This gene activation results in the expression of immediate response genes such as the ubiquitin hydrolase, which stabilizes the STF and the transcription factor CCAAT-box-enhanced binding protein (C/EBP) which is important for the formation of LTF. C/EBP and constitutively expressed molecules such as AF (activating factor) induce a second wave of gene expression. The newly synthesised gene products (see figure 1 (7)) can be processed to proteins directly at the active synapses (see figure 1 (8)) and can initiate both, growth of already existing and the formation of new synapses (see figure 1 (9)). In addition, silent synapses can be reactivated (see figure 1 (10)). The repetition of these molecular events leads to persistence of memory (see figure 1 (11)) (Kandel and Schwartz 2014; Hawkins et al. 2006).

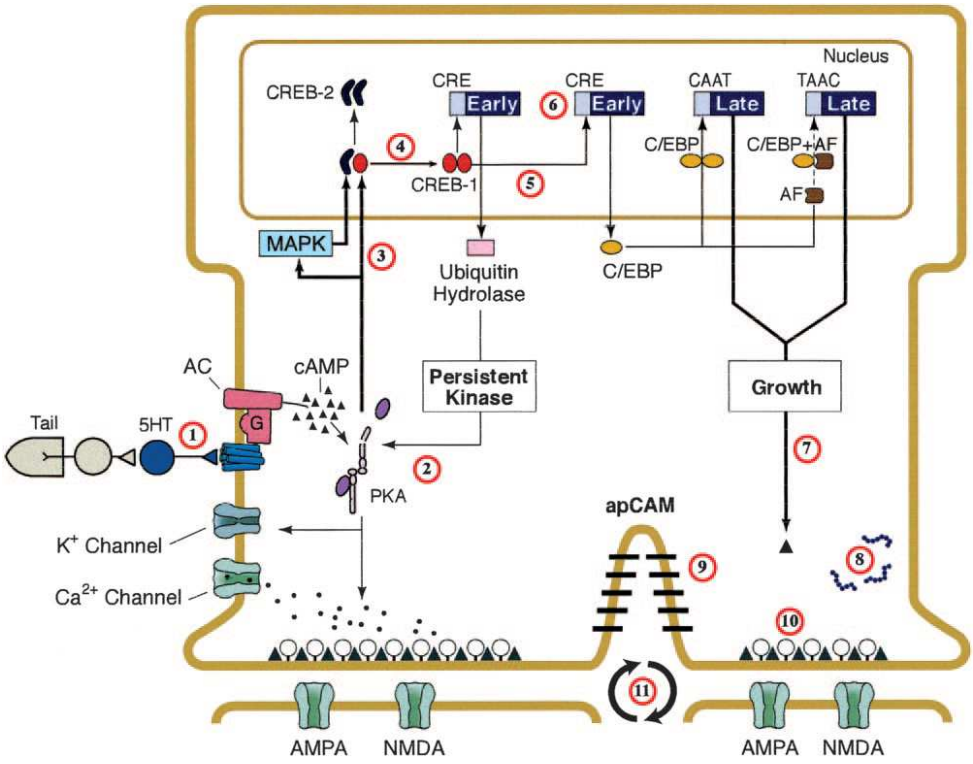

Figure 1 Long-term memory formation mechanisms in the sea snail *Aplysia californica*

The initiation of the mechanism is mediated by neurotransmitter release (1) that activates the adenylyl cyclase and cAMP dependent PKA (2). Therewith, PKA mediates an increase of action potential by enhancing the Ca^{2+} influx, reducing K^+ current and increasing neurotransmitter release. With help of the MAPK, PKA is transported into the nucleus (3), where the transcription factor CREB gets activated (4) and mediates transcription (5). Epigenetic and chromatin changes (6) initiate growth. The newly synthesised gene products (7) can be processed to proteins directly at the active synapses (8) and can initiate both, growth of already existing and the formation of new synapses (9). In addition, silent synapses can be reactivated (10). The repetition of these molecular events leads to persistence of memory (11). (Adapted from Hawkins et al. 2006)

The expression of new genes and therewith synthesis of mRNAs and proteins is essential to form LTM and contributes to changes in neuronal and circuit properties (Ashraf and Kunes 2006). In the honeybee, long-term memory (LTM) is a result of a strong conditioning with repeated presentation of two paired stimuli CS-US and a consolidation phase where transcription happens. Three-trial conditioning that induces LTM leads to a prolonged activation (~3 min) of PKA in the antennal lobes (ALs) of the honeybee (see figure 2). The prolonged activation of PKA is depending on the production of nitric oxide (NO) by the NO synthase (NOS). Through activation of the soluble guanylyl cyclase (sGC), NO can mediate the production of cyclic guanosine monophosphate (cGMP), which acts synergistically on PKA and elongates its activation.

Whereby the photorelease of cGMP or cAMP in the ALs in combination with one trial conditioning is able to induce LTM, it has been demonstrated, that the inhibition of either NOS, cGC, or PKA leads to a specific loss of LTM. The activation of the NO/cGMP and the cAMP/PKA pathways are essential for the induction of LTM (Müller 2013).

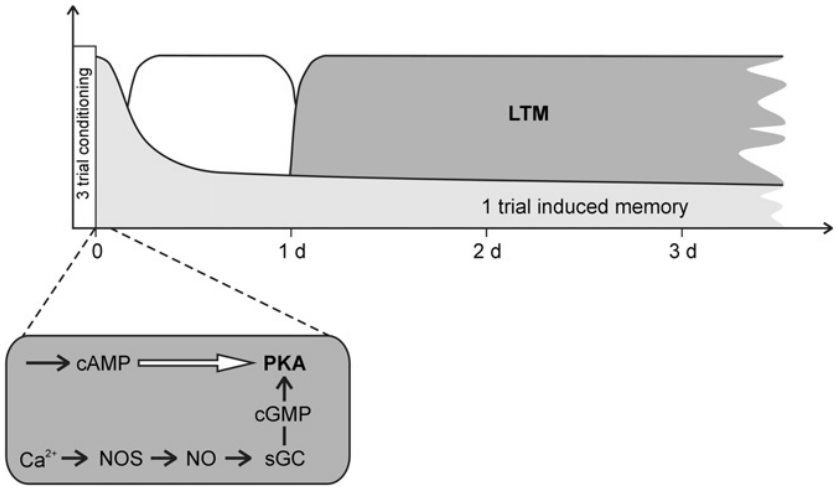

Figure 2 The induction of long-term memory (LTM) in the antennal lobes of the honeybee

The conditioning with one single trial leads to a weak activation of the cAMP/PKA pathway in the Antennal lobes (AL) and induces a form of memory that decays over time. The repeated conditioning with three trials however, induces LTM and activates protein kinase A (PKA) in the ALs for a longer time period (~3 min). After calcium (Ca^{2+}) activation, the NO synthase (NOS) produces nitric oxide (NO) which activates the soluble guanylyl cyclase (sGC) which produces cyclic guanosine monophosphate (cGMP). The cGMP acts synergistically on PKA and extends its activation (Adapted from Müller 2013).

1.3. MicroRNAs

In addition to the well understood functions of the second messenger cascades in memory formation, recent investigations have implicated microRNAs as important players in these molecular processes. MicroRNAs (miRNAs) are small (~22 nt) non-coding RNAs, that are highly conserved throughout species. As a part of the "epigenetic landscape" (McNeill and Van Vactor 2012) they regulate posttranscriptional gene expression through inhibition of translation and destabilisation of their specific targets such as mRNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). MiRNAs play a role in the development of the nervous system

and synaptic plasticity (Nelson et al. 2010) and influence many biological processes like the development of animals and plant, cell proliferation, differentiation, apoptosis (Huntzinger and Izaurralde 2011). One strand is degraded while the other one is loaded into the AGO (Argonaute family protein) and the RISC (RNA induced silencing complex) is formed, which guides the binding of the miRNA to the target mRNA. This leads to the alteration of posttranscriptional gene expression through inhibition of translation and destabilisation of the target mRNA. After incorporation of the guide strand of the mature miRNA into the RNA induced silencing complex (RISC), the complex can silence its mRNA target, either by cleavage mediated through AGO or by resting on the mRNA and blocking the translation process (Bartel 2004). First discovered in 1993 by Lee et al., miRNAs have become more and more interesting due to their fine regulatory functions. Lee et al. (1993) found out, that *lin-4*, a gene that controls the larval development of *C. elegans* is not encoding for a protein but for two small RNA molecules. Also, the smaller RNA molecule binds to several sites of the 3'UTR (*untranslated region*) of *lin-14* and thereby represses the LIN-14 protein synthesis without changing the mRNA amount (Lee et al. 1993). For seven years, the *lin-4* RNA seemed to be the only one of its type, until in the year 2000, Reinhart et al. discovered that the *let-7* gene encoded for a second small regulatory RNA in *C.elegans* (Reinhart et al. 2000). Soon after this discovery, *let-7* gene homologues and many other ~22 nt small RNAs were also found in other species (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). As fine tuners of gene expression, miRNAs have become interesting elements in the decoding of cellular mechanisms. To understand the molecular mechanisms behind learning and memory it is pivotal to uncover those missing pieces and define their roles in the machinery. Previous studies described the importance of miRNAs in synaptic plasticity and in the mechanisms generating memory (Vo et al. 2005; Ashraf and Kunes 2006; Ashraf et al. 2006; Schratt et al. 2006).

1.3.1. MicroRNA biogenesis, mechanism and function

MiRNA genes do mostly appear in clusters of two to seven genes, which are transcribed bi- or polycistronic by the RNA polymerase II (or RNA polymerase III) most of the time and folded into hairpin structures after the transcription (Lee et al. 2002). These hairpins are called primary miRNA (pri-miRNA) and can be more than 1 kb long. The pri-miRNAs are then processed into approximately 70 nt long precursor-miRNAs (pre-miRNAs) by the Drosha RNase III

endonuclease (see figure 3) whereupon the base of the pre-miRNA stem-loop has a 5' phosphate and around 2 nucleotides 3'-overhang (Bartel 2004). The export receptor Exportin-5 and RanGTP transport the pre-miRNAs actively out of the nucleus into the cytoplasm (Lund and Gu 2004; Yi et al. 2003). In the cytoplasm, the DICER RNase III endonuclease recognizes the double stranded pre-miRNA and cleaves both strands of the duplex at about two helical turns away from the base of the stem-loop. The product of this cleavage that lost its loop and 5' phosphate and about 2 nt 3'-overhang consists now of the mature miRNA strand and the miRNA* strand (Lim et al. 2003). This siRNA-like (small interfering RNA) imperfect duplex fragment which is termed as the miRNA: miRNA* duplex gets separated by a helicase into the miRNA and miRNA* strands, the miRNA strand is loaded into the RNA-induced silencing complex (RISC) while the miRNA* strand gets degraded. The RISC complex contains a member of the Argonaute protein family. In humans four different AGO proteins are described (Flores et al. 2014), AGO-1 and AGO-2 were also identified in insects (Lucas and Raikhel 2013). Guiding the RISC to the target mRNA, the mature miRNA can silence its target through cleavage or through translational repression (Bartel 2004). In both cases, the miRNA binds to its target with six to eight bases, the seed sequence, a region between second and the seventh nucleotide of the miRNA. In most cases, the binding happens at the 3' UTR of the mRNA, but it can also appear at the 5'UTR (Orom et al. 2008) and at the ORF (*open reading frame*) (Tay et al. 2008). When the complementarity between the mRNA and the miRNA is sufficient, the RISC can cleave the target mRNA. As one miRNA can bind to several targets but with different intensity, cleavage is not the only mechanism to regulate the amount of mRNAs. The protein GW182, which is recruited to the RISC complex and binds to AGO, plays a role in the degradation of the mRNAs (Behm-Ansmant et al. 2006) by regulating the transport of the whole miRNA-RISC-mRNA complex to so called *P-bodies* (Ipsaro and Joshua-tor 2015). The P-bodies are cytoplasmic domains, in which proteins accumulate for the degradation of mRNAs (Lucas and Raikhel 2013).

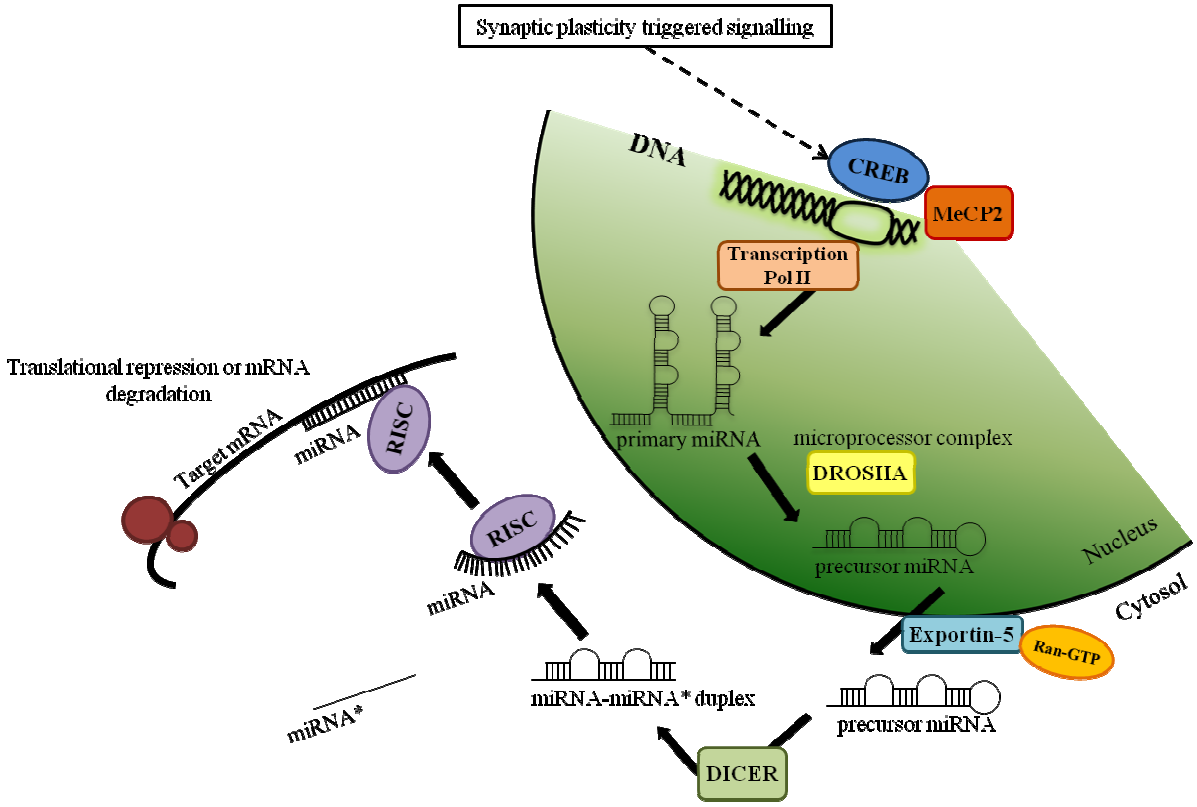

Figure 3 Biogenesis of miRNAs

The biogenesis of miRNAs starts in the nucleus with the transcription of the miRNA gene performed by Polymerase II. The emerging primary miRNA is processed to a precursor miRNA by the microprocessor complex DROSHA and transported out of the nucleus into the cytosol by Exportin-5 and RanGTP. In the cytosol, the precursor is further processed by DICER. The resulting miRNA-miRNA* duplex gets divided by a helicase and the miRNA gets incorporated into the RISC complex while the miRNA* gets degraded. The RISC complex together with the incorporated mature miRNA can either cleave its target mRNA or inhibit translation. Synaptic activation can lead to signalling pathways, which activate CREB and MeCP2, which in turn regulate DNA transcription in the nucleus. Adapted from (Wang et al 2012)

1.3.2. MicroRNAs in learning and memory

Several studies have already described the importance of microRNAs (miRNAs) in neuronal tissues. MiRNAs control both levels and translation of mRNA. They act as fine tuning tools in processes like maturation, connectivity and plasticity of neurons (see figure 4). The fundamentals of neuronal plasticity in the development of learning and memory are strongly influenced by a network of cellular protein cascades. The small non-coding miRNAs influence these neuronal

processes by silencing their target mRNAs, which in turn could lead to the development of memory or impede this process.

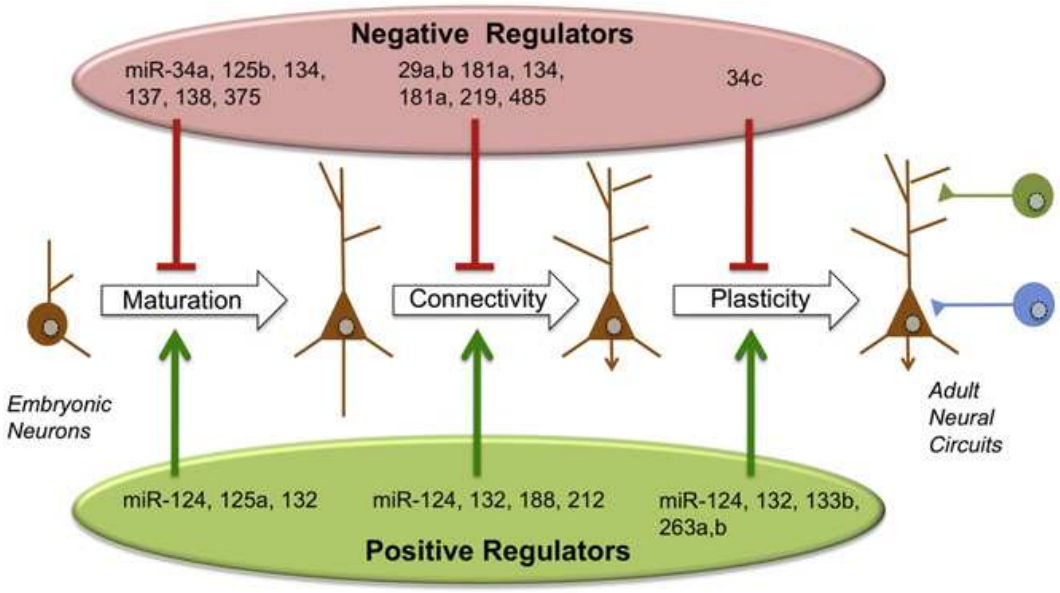

Figure 4 The influence of different miRNAs on neuronal mechanisms

The scheme displays the influence of different miRNAs on maturation, connectivity and plasticity on neuronal cells. The miRNAs can negatively or positively regulate these processes by controlling the levels and the translation of mRNAs (McNeill and Van Vactor 2012).

A number of studies deliver evidence of the importance of the miRNA machinery in synaptic plasticity and learning and memory formation tasks. Enhanced cognition in aversively and appetitively motivated tasks was observed in *Dicer1* mutant mice lacking miRNAs in mature neurons in the adult brain (Konopka et al. 2010). MiRNAs also play a critical role in learning and memory formation processes via regulating important proteins such as CREB and Mef2 (myocyte enhancing factor 2) (Wang et al. 2012). The overexpression of different miRNAs in transgenic mice resulted in impaired memory, impaired synaptic plasticity and deficits in recognition (Scott et al. 2012; Gao et al. 2010; Hansen et al. 2010). The first dendritic miRNA identified, miR-134 does regulate dendritic spine size negatively (Bicker et al. 2014). Controlled via the histone deacetylase sirtuin 1 (SIRT1), miR-134 has been described to be involved in hippocampus-dependent memory by targeting CREB (Gao et al. 2010) and in spine shrinkage via targeting the LIM domain kinase 1 (LimK1) (Siegel et al. 2011).

1.3.3. The miR-124

The miR-124 is a well-studied, neuron-specific and plenty expressed miRNA (Conaco et al. 2006) which is highly conserved from worm to human (Li et al. 2010). Weaver et al. (2007) provided computational and transcriptional evidence (q-RT-PCR) of the existence of miR-124 in the honeybee and it has already been described in the literature to be found in the honeybee (Behura and Whitfield 2010; Greenberg et al. 2012; Qin et al. 2014) as well as in other insect species like *Drosophila melanogaster* (Aravin et al. 2003; Sempere et al. 2003; Ruby et al. 2007; Stark et al. 2007). It is a likely candidate to uncover missing pieces of the molecular mechanisms behind learning and memory formation. MiR-124 was previously described in relation to synaptic plasticity, learning and memory (Cao et al. 2007; Cheng et al. 2009; Rajasethupathy et al. 2009) and is known to promote neuronal differentiation (Makeyev et al. 2007) and identity (Conaco et al. 2006). MiR-124-target interaction was previously described for GluA2 (AMPA-type glutamate receptor) in the hippocampus of mice by Ho et al. 2014. They showed that miR-124 regulates GluA2 in the cell-bodies before the GluA2 protein is transported to synapses and dendrites. They were also able to locate the miR-124 in cell-bodies and dendrites and the GluA2 mRNA in the somata (Ho et al. 2014). Furthermore miR-124 was also shown to constrain synaptic plasticity in absence of serotonin through regulation of CREB (cAMP response element-binding protein) in *Aplysia californica* by binding to its 3'UTR (Rajasethupathy et al. 2009). MiR-124 was shown to be upregulated in young nursing bees in comparison to old nursing bees and young or old forager bees (Behura and Whitfield 2010). Qin et al. (2014) described that the miR-124 was upregulated after maze based visual pattern learning. Cristino et al. (2014) found an upregulation of miR-124 after learning in the honeybee.

1.3.4. The miR-125

The miR-125 plays an important role in vertebrate neuronal differentiation and in synaptic plasticity and –function (Le et al. 2009; Edbauer et al. 2010; Boissart et al. 2012) and it is highly conserved from worm to human (Li et al. 2010; Kiezun et al. 2012). The miR-125 has already been described in the honeybee (Behura and Whitfield 2010; Greenberg et al. 2012; Qin et al. 2014) as well as in other insect species like *Drosophila melanogaster* (Aravin et al. 2003; Sempere et al. 2003; Ruby et al. 2007; Stark et al. 2007). In mammals, there is a differentiation

between the miR-125a and miR-125b. Those two miRNAs regulate synaptic plasticity in different ways (see figure (4)). The miR-125a was shown to be a positive regulator of synaptic plasticity by controlling PSD-95 and thus regulating the density and branching of spinous processes in neurons (Muddashetty et al. 2011). The miR-125b is described as a negative regulator of maturation in neurons (McNeill and Van Vactor 2012), as a negative regulator of p53 in zebra fish and humans (Le et al. 2009) and as a promoter of neuronal differentiation in human cells (Le et al. 2009). It has been shown in mice, that overexpression of miR-125b resulted in longer and thinner processes of hippocampal neurons and that the miR-125b targets the Eph receptor A4 (EphA4) (Edbauer et al. 2010). A loss of EphA4 leads to filopodia-like protrusions in neuronal cells of the hippocampus (Edbauer et al. 2010). According to Sempere et al. (2003), miR-125 is known as a putative homologue of lin-4 miRNA in *Drosophila melanogaster*. MiR-125 is clustered with miR-100 and let-7 within an 800 bp region on chromosome 2 L in *Drosophila melanogaster* and the upregulation of miR-125 miR-100 and let-7 and downregulation of miR-34 requires the hormone ecdysone (Ecd) and the activity of the Ecd inducible gene *Broad-Complex* (Aravin et al. 2003; Sempere et al. 2003). MiR-125 expression was upregulated in young nursing bees in comparison to old nursing bees and young or old forager bees (Behura and Whitfield 2010). Qin et al. (2014) described that the miR-125 was upregulated after maze based visual pattern learning. An upregulation of miR-125 in inactive ovaries of Apis mellifera virgin queens (compared to mated queens) and inactive ovaries of worker bees (compared to activated worker ovaries) has been described by (Macedo et al. 2016).

1.3.5. The miR-132, miR-138 and the miR-329 in neuronal tissues

The miR-132 plays a role in neuronal plasticity and synapse formation (Bicker et al. 2014) and is also known to be conserved through species (Kiezun et al. 2012). It has been shown, that miR-132 expression is regulated by the Brain-derived neurotrophic factor (BDNF) through the transcription factor CREB (Vo et al. 2005). Furthermore, via neuronal activation, the miR-132 regulates neuronal morphogenesis in developing neurons by repressing the translation of p250GHP a member of the Rho family GTPase-activating protein (Wayman et al. 2008). MiR-132 transgenic mice, which overexpress miR-132 in forebrain neurons, showed deficits in hippocampal-dependent novel object recognition memory and exhibited an impaired expression of the methyl CpG binding protein 2 (MeCP2), a protein implicated in Rett Syndrome and other

disorders of mental retardation (Hansen et al. 2010). The specific overexpression of miR-132 in the perirhinal cortex of the rat resulted in impaired short-term recognition memory associated with reduced long-term depression and long-term potentiation (Scott et al. 2012). Hansen et al. (2013) found enhanced cognitive capacity while sensitively over-expressing miR-132 in the hippocampi of doxycycline regulated miR-132 transgenic mice.

The miR-138 is another conserved, intensively studied neuronal miRNA (Kiezun et al. 2012). The miR-138 is highly enriched in the brain, localized within dendrites and it negatively regulates the size of dendritic spines in rat hippocampal neurons (Siegel et al. 2013). High levels of miR-138 in the mouse hippocampus are correlated with better short-term recognition memory performance (Tatro et al. 2013). The miR-138 controls acyl protein thioesterase1 (APT1) translation (Siegel et al. 2013) and through this affects short-term object recognition memory (Tatro et al. 2013). SIRT1 has been identified as a target of miR-138, and both of them have been described to regulate mammalian axon regeneration *in vivo* (Liu et al. 2013). NMDA (*N*-methyl-*D*-aspartate) dependent chemical- long-term potentiation (LTP) is described to induce a reduction and –long-term depression (LTD) and an increase of miR-138 expression levels in cultured hippocampal neurons (van Spronsen et al. 2013).

The miR-329 which was demonstrated to play several roles in neurons, is conserved through more than 25 species (Kiezun et al. 2012). The transcription factor Mef2 mediates transcription of the miRNA 379-410 cluster which is co-regulated by neuronal activity in hippocampal neurons of rats (Fiore et al. 2009; Khudayberdiev et al. 2009). The miR-329 as well as the miR-134 is a part of this genetic cluster. Fiore et al. (2009) inhibited the miR-329 and found that it is necessary for dendritic outgrowth triggered by KCl in hippocampal neurons. In association with Alzheimer's disease, miR-329 was upregulated on H_2O_2-induced hippocampal neurons and different strains of senescence accelerated mice (Zhang et al. 2014). The potential tumor suppressing miR-329 was shown to decrease cell viability, proliferation, migration, and invasion of neuroblastoma cells in vitro through inhibition of its target lysine-specific demethylase 1 (KDM1A) (Yang et al. 2014).

1.3.6. The miR-12

The miR-12, has already been described in the honeybee (Behura and Whitfield 2010; Greenberg et al. 2012; Qin et al. 2014) as well as in other insect species like *Drosophila melanogaster* (Lagos-Quintana et al. 2001; Aravin et al. 2003; Sempere et al. 2003; Ruby et al. 2007; Stark et al. 2007; McCann et al. 2011; Nishihara et al. 2013) and *Aedes aegypti* (Osei-Amo et al. 2012). It has also identified in the Marek's disease virus (Xu et al. 2008). MiR-12, was upregulated in young nursing bees in comparison to old nursing bees and young or old forager bees (Behura and Whitfield 2010). An upregulation of miR-12 in inactive ovaries of *Apis mellifera* virgin queens (compared to mated queens) has been described by Macedo et al. (2016). In addition to the aforementioned studies that proved the existence of the miR-12 and its localisation in different tissues, there is also evidence for the functional roles of miR-12. Qin et al. (2014) described that the miR-12 was upregulated after maze based visual pattern learning in honeybees.

1.4. Manipulation of microRNA function

The silencing of miRNAs *in vivo* is an important step in uncovering miRNA function. Many different approaches have been made to acquire this goal.

Strong effects were shown by knock-out of miRNA genes (miRNA KO) (Park et al. 2010) (see figure 5), knock-outs of the miRNA processing proteins with for example the Cre-loxP inducible knock-out system (Konopka et al. 2010), or the systemic generation of miRNA deletion mutants in *Drosophila melanogaster* (Weng and Cohen 2012). However, the knock-out of one miRNA alone does not necessarily contribute to the functional understanding of the respecting miRNA in synaptic plasticity, because a deletion or inducible knock-out is not reversible but permanent. Additionally, those knock-outs can be lethal, like the knock-out of the let-7 miRNA in *C. Elegans* (Reinhart et al. 2000).

Overexpression through transient transfection of for example a synthetic miRNA precursor, or by stable introduction of a lentiviral miRNA expression construct can cause false positive results (Thomson et al. 2011). The specific overexpression of miR-132 in the perirhinal cortex of the rat resulted in impaired short-term recognition memory associated with reduced long-term depression and long-term potentiation (Scott et al. 2012). The lentiviral transduction of miR-132

into the perirhinal cortex of rats had been performed three weeks before the actual behavioural experiments (Scott et al. 2012). Contrarily, Hansen et al. (2013) found enhanced cognitive capacity while sensitively over-expressing miR-132 in the hippocampi of doxycycline regulated miR-132 transgenic mice. These *in vivo* manipulations were also not transient but permanent and irreversible after induction.

The performance of miRNA overexpression in a cellular system, which is not natural for the respecting miRNA may be suboptimal for the effectiveness of that respecting miRNA because of the differences of miRNA-mRNA target expression patterns in different cell types (Thomson et al. 2011). The overexpression of the neuronal miR-124 in ovarian cancer (HeLa) cells (Lim et al. 2005) represents a misperformance for this example. In an *in vitro* approach with HeLa cells, transfection with miR-1 and miR-124 resulted in the downregulation of 100 and more mRNAs (Lim et al. 2005). Methods using those above-mentioned overexpression models to study miRNA function can be performed *in vitro* and also in genetically manipulable organisms but not in the honeybee. The use of those methods alone can be misleading because of their irreversibility and the permanent manipulation of miRNA function (McNeill and Van Vactor 2012). Those techniques can be combined with other methods described below to clarify miRNA roles (Makeyev et al. 2007).

Expression of miRNA mimics (see figure 5) can be used to artificially raise the levels of miRNA but show controversial effects in *in vitro* experiments (Osei-Amo et al. 2012). The use of miRNA mimics and miRNA-Inhibitors in combination showed expected effects in cell culture of *Aplysia* neurons (Rajasethupathy et al. 2009).

MiRNA sponges (see figure 5) were designed for the transient transfection into cultured cells and act like competitive miRNA-Inhibitors by binding the seed sequences of many different miRNAs or of miRNA seed families and thus inhibit their functions (Ebert et al. 2007).

The “tough decoy” constructs carry a miRNA seed complement in between a degradation resistant overall RNA structure (see figure 5) and provide a method for the *in vitro* as well as the *in vivo* inhibition of miRNAs, but they have not yet been tested in the CNS (Haraguchi et al. 2009; McNeill and Van Vactor 2012).

When the function of a specific miRNA has already been defined, target protectors (TPs) (see figure 5) can be used. Target protectors, consisting of an oligonucleotide, prevent the miRNAs from binding to their specific endogenous target and was designed by Staton and Giraldez (2011) for the *in vivo* use with reporter vectors in zebrafish embryos.

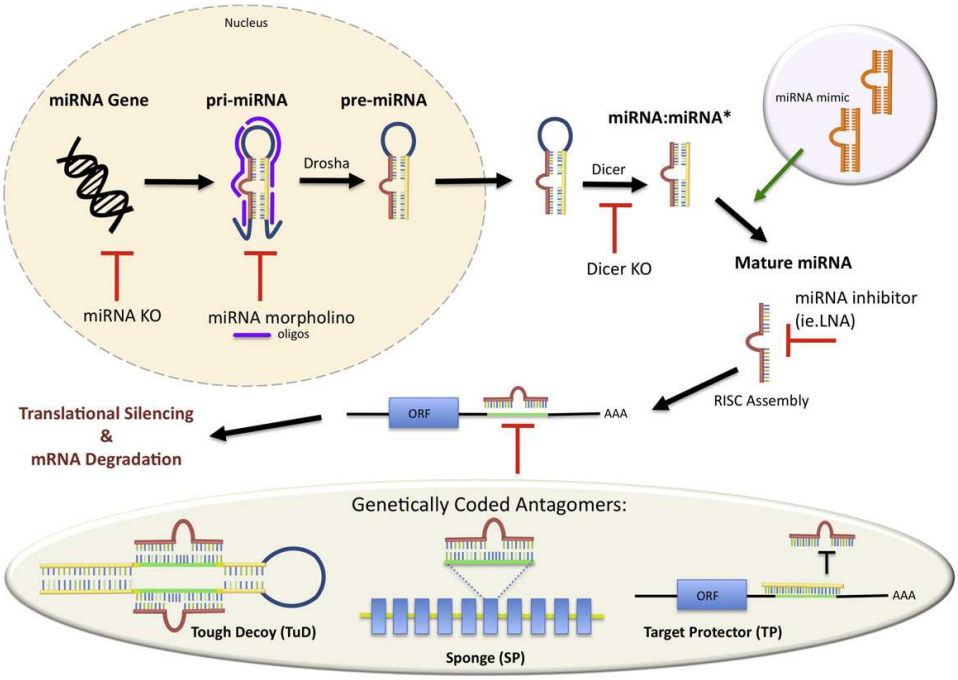

Figure 5 Multiple methods for miRNA manipulation

From the knock-out of miRNA genes (miRNA KO) on the DNA level, the blocking of the primary miRNA with LNA morpholinos and the disruption of the Drosha /Pasha microprocessor complex in the nucleus, to the knock-out of DICER and therewith the processing to mature miRNAs, the inhibition of mature miRNAs with miRNA-Inhibitors in the cytosol, there are many different techniques to manipulate miRNA functions. The use of genetically encoded tough decoys (Tuds), miRNA sponges (SPs) and target protectors (TPs) were developed to reduce or compete with mature miRNA levels or miRNA-mRNA target complexes. Adapted from (McNeill and Van Vactor 2012)

As methods like creating genetic knock-outs and the *in vivo* use of reporter assays are difficult to transfer to *in vivo* experiments with the model organism of the honeybee, a controlled and transient manipulation of miRNA function would be absolutely essential to study the specific effects of single miRNAs on acquisition and consolidation phases. Of special interest in the context of this work, is the model of "Anti-miRNA-oligonucleotides" (AMOs). The small molecules are designed to match with the sequence of the miRNA of interest. They bind to their specific target miRNAs and can thus impede their functions. Due to the instability of RNA molecules, which is caused by rapid degradation mediated through RNAses, there have been several concepts in designing modifications to stabilize the AMOs (Esau 2008). The "basic" Anti-miRNA oligonucleotide is designed with a 2'–O-methyl (2'-OMe) chemical modification

and was used in cultured cells (Hutvágner et al. 2004; Meister et al. 2004; Cheng et al. 2005) and *Drosophila* embryos (Leaman et al. 2005). Improved AMOs with a 2'-OMe mixed phosphorotioate backbone called "Antagomirs" (Krützfeldt et al. 2005), or "Antisense oligonucleotides" (ASOs) (Esau et al. 2006) were tested in mice. The following designs were "locked nucleic acids" (LNAs) which have an additional bridge between the 2'-O and 4'-C and show increased stability (Koshkin et al. 1998) LNA's were tested in cultured cells and in mice (Orom, et al. 2006; Davis et al. 2006) and "morpholinos" were designed for the use in zebra fish embryos (Kloosterman et al. 2007).

Lennox and Behlke (2010) compared the potencies of 15 AMO designs, inter alia the designs described above, *in vitro* in HeLa cells with luciferase reporter assays. Lennox et al. (2013) introduced an improved AMO that showed high potency and low toxicity in cell culture with a 2' -OMe backbone and the new compound N,N-diethyl-4-(4-nitronaphtalen-1-ylazo)-phenylamine ("ZEN") which is stable for at least 24 h.

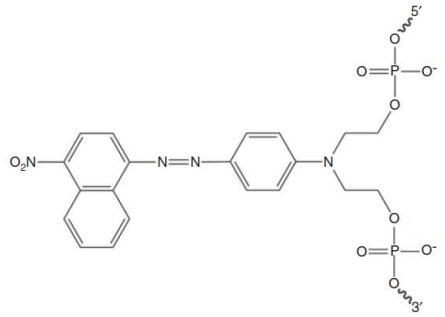

Figure 6 Structure of the ZEN modifier

N,N-diethyl-4-(4-nitronaphtalen-1-ylazo)-phenylamine is connected via phosphate linkages to the ribose backbone of the oligonucleotide (modified after Lennox et al. 2013).

Another basic concept for the *in vivo* inhibition of parts of the miRNA machinery was provided by Watashi et al. (2010). They screened for chemical compounds that supress small RNA-mediated gene silencing and described two promising chemicals: Poly-L-Lysine (PLL) and Trypaflavine (TPF). The study showed, that PLL inhibits DICER-mediated processing of pre-miRNA/shRNA (small hairpin RNA) to miRNA/siRNA, which means mature miRNA levels get reduced while the amount of pre-miRNAs is increasing. Furthermore they showed, that TPF reduces the association of siRNA/miRNA with AGO2, so that many of the mature miRNAs can not associate with the RISC and inhibit their targets.

2. Aim of this work

Various investigations have shown the relevance of miRNAs in the neuronal tissues by overexpression, cell culture models or knock-out mutants but more *in vivo* loss of function studies are necessary to define the function of specific miRNAs in the brain. Because little is known about miRNAs in learning, I want to identify the role of miRNAs involved in the formation of STM and LTM in the honeybee brain. Due to her need to learn and memorise new food sources and to pass the information to others, the honeybee (*Apis mellifera*) pictures an ideal model organism to study learning and memory mechanisms. Of special interest are the changing levels of miRNAs triggered by weak and strong associative learning.

The first approach of this work will be the analysis and quantification of the levels of ame-miR-12, ame-miR-124, ame-miR-125, ame-miR-989, ame-miR-3756, ame-miR-3769 and ame-miR-3788 in the central honeybee brain after strong and weak conditioning. The miRNAs will be selected for the previously described roles of their homologues in relation to synaptic plasticity, learning and memory. To show, that learning and memory processes have an influence on miRNA levels, I want to measure the amounts of those miRNAs by q-RT-PCR (quantitative-Real-Time-Polymerase Chain Reaction) and to compare their levels between naive and conditioned honeybees.

A second approach will be, to use a transient *in vivo* inhibition of selected miRNAs by Anti-miRNA-oligonucleotides (AMOs) and to analyse the function of those individual miRNAs on the behaviour of the honeybees. I want to investigate the differences in memory formation due to transient and dynamic manipulation of miRNA function during the acquisition or the consolidation phase of weak (single-trial CS-US) and strong (three-trials CS-US) appetitive olfactory conditioning. Comparing miRNA inhibited and control animals in non-associative conditioning tasks and gustatory sensitivity allows specifying the contribution of distinct miRNAs in associative learning processes.

In addition to the inhibition of specific miRNAs, I want to test unspecific inhibitors of the miRNA machinery and compare the differences between the specific and the unspecific inhibition of miRNAs (PLL/TPF). Furthermore, the effects of AMOs and unspecific inhibitors on the levels of selected miRNAs will be examined by quantification with q-RT-PCR to identify interactions between single miRNAs. The changes of miRNA amounts after conditioning and

AMO/inhibitor treatment will hereby also be addressed not only to identify miRNA interactions but also to verify the functionality of the inhibitors.

Identifying changes of individual miRNAs by learning combined with the transient manipulation of individual miRNAs by AMOs during distinct phases of learning will reveal a better understanding of the role of distinct miRNAs in memory formation and consolidation.

3. Materials and methods

3.1. Materials

Table 1 Apparatus

Binocular Leica10x/23	Leica (Wetzlar, Germany)
Cold Light Lamp KL 1500 LCD	Schott (Mainz, Germany)
Eppendorf Centrifuge 5417R	Eppendorf (Hamburg, Germany)
Eppendorf Centrifuge 5804R	Eppendorf (Hamburg, Germany)
Eppendorf Mastercycler personal Eppendorf	Eppendorf (Hamburg, Germany)
Heating block	Labnet (Dülmen, Germany)
Heating chamber	Memmert (Schwabach, Germany)
Homogenisation Shaker modified Power Drill	Self-made
Homogenisation Shaker modified Vortex Device VX100	Labnet (Dülmen, Germany)
pH meter (inoLab pH 730)	WTW (Weilheim, Germany)
Reader infinite F200 Pro	Tecan (Männedorf, Switzerland)
Real-Time cycler BioRad CFX Cycler	Bio-Rad (Munich, Germany)
Real-Time cycler BioRad My iQ5 Cycler	Bio-Rad (Munich, Germany)
Soldering iron	Self-made
Table Centrifuge Spectrafuge 24 D	Labnet (Dülmen, Germany)
Vortex Mixer Device VX-100	Labnet (Dülmen, Germany)
Weight Balances CP3202S and CP225D	Sartorius (Göttingen, Germany)

Table 2 Miscellaneous materials

96-Well PCR High Profile Plates	Biozym (Oldendorf, Germany)
Adhesive film for 96 well qPCR plates	Biozym (Oldendorf, Germany)
Cannules 0,40x 20 mm	Braun (Melsungen, Germany)
Catching Tubes Plexi Glass	Greiner (Kremsmünster, Austria)
Dental Wax Blocks	Gebdi Dental Products (Engen, Germany)

Falcon Tubes 15 ml	Greiner (Kremsmünster, Austria)
Falcon Tubes 50 ml	Greiner (Kremsmünster, Austria)
Filter Tips	Peqlab (Erlangen, Germany)
Forceps	Dumont (Switzerland)
Glass Capillaries	Brand (Wertheim, Germany)
Honeybee Harnessing Tubes	Self-made
Honeybee Standing Racks	Self-made
Light Protection Reaction tubes (1,5 ml)	Greiner (Kremsmünster, Austria)
Metal Pestles for Glass Capillaries	Self-made
Multiply PCR stripes with lids (0,2ml)	Sarstedt (Nürnbrecht, Germany)
Paper towels	Supermarket (Saarbrücken, Germany)
Plastic tubs with lids	Supermarket (Saarbrücken, Germany)
Plexi Glass Pyramids	Self-made
Q-Tips	Supermarket (Saarbrücken, Germany)
Razor Blade breakers	Nopa Instruments (Tuttlingen, Germany)
Razor Blades	Faulhaber (Schöneich, Germany)
Reaction tubes (0,2ml, 1,5ml, 2ml)	Sarstedt (Nürnbrecht, Germany)
Reaction tubes (0,5ml, 5ml) safelock	Eppendorf (Hamburg, Germany)
Single channel pipettes	Abimed (Langenfeld, Germany)
Spatula	Roth (Karlsruhe, Germany)
Surgical Disposable Scalpels	Braun (Melsungen, Germany)
Syringe 20 ml Omnifix	Braun (Melsungen, Germany)
Syringe 5 ml Omnifix	Braun (Melsungen, Germany)
Tooth Picks	Supermarket (Saarbrücken, Germany)

Table 3 Chemicals

Chloroform	Sigma Aldrich (Munich, Germany)
Clove oil	Pharmacy (Saarbrücken, Germany)
DEPC (Diethylpyrocarbonat)	Roth (Karlsruhe, Germany)
EDTA	Roth (Karlsruhe, Germany)
Ethanol absolute	Sigma Aldrich (Munich, Germany)

KCl	UdS Central chemical Supply (Saarbrücken, Germany)
KH_2PO_4	Grüssing (Filsum, Germany)
miRNA-Inhibitors	Integrated DNA Technologies (Coralville, USA)
$Na_{2\,h}PO_4$	VWR International (Dublin, Ireland)
NaCl	VWR International (Dublin, Ireland)
Oligo nucleotides	Sigma-Aldrich (Munich, Germany)
Poly-L-Lysine	Sigma Aldrich (Munich, Germany)
Real-Time qPCR Mastermix: Kapa Probe Fast Universal, Kapa SYBR Fast Universal	Peqlab (Erlangen, Germany)
RNAse Exitus Plus	AppliChem (Darmstadt, Germany)
Saccharose	Supermarket (Saarbrücken, Germany)
Trizma-base	Sigma Aldrich (Munich, Germany)
TRIzol Reagent Solution	Ambion Life Technologies (Darmstadt, Germany)
Trypaflavine	Sigma Aldrich (Munich, Germany)
Ultra Pure Water RNAse/DNAse free	Fisher Scientific (Schwerte, Germany)
Universal ProbeLibrary Probe #21	Roche (Mannheim, Germany)

Table 4 Solutions and buffers

1 M Tris-HCl (pH 8,0) for 200 ml	3,64 g Trizma-base
	Dilute in 160 ml H_2O_{bidest}
	Set pH with HCl to 8,0 and fill up
	With H_2O_{bidest} to 200 ml
1 x Phosphate buffered Saline	2,7 mM KCl
	137 mM NaCl
	10,1 mM $Na_{2\,h}PO_4$
	1,8 mM KH_2PO_4
H_2O-DEPC 0,1%	1 l H_2O_{bidest}
	1 ml DEPC

	Shake at 37°C over night, autoclave
TE- Buffer	10mM Tris-HCl (pH 8,0)
	1 mM EDTA

Table 5 Kits

KAPA SYBR FAST qPCR Kit Master Mix (2x) Universal	Peqlab (Boston, USA)
Revert Aid RT CDNA Synthesis Kit	Thermo Fisher Scientific (Waltham, USA)

Table 6 Solutions for injection

MicroRNA-Inhibitors	
miR-12-Inhibitor 0.5 µM miRNA-Inhibitor (Anti-ame-miR-12 (Ref.Nr.: 66388547): 5nmol solved in 50µl TE-Buffer diluted to 0,5 µM in PBS sterile filtrated	5'-mA/ZEN/mCmCmAmGmUmAmCmCmUm GmAmUmGmUmAmAmUmAmCmUmC/3ZEN/-3'
miR-124-Inhibitor 0.5 µM miRNA-Inhibitor (Anti-ame-miR-124 (Ref.Nr.:66477088)): 5nmol solved in 50µl TE-Buffer diluted to 0,5 µM in PBS sterile filtrated	5'-mC/ZEN/mUmUmGmGmCmAmUmUmCm mAmCmCmGmCmGmUmGmCmCmUmU/3ZEN/-3'
miR-125-Inhibitor 0.5 µM miRNA-Inhibitor (Anti-ame-miR-125 (Ref.Nr.:68529874)) 5nmol solved in 50µl TE-Buffer diluted to 0,5 µM in PBS sterile filtrated	5'-mU/ZEN/mCmAmCmAmAmGmUmUmAm GmGmGmUmCmUmCmAmGmGmG/3ZEN/-3'
NC1 Negative Control (human) 0.5 µM miRNA-Inhibitor (Ref.Nr.:66388548): 5nmol solved in 50µl TE-Buffer diluted to 0,5 µM in PBS sterile filtrated	5'mG/ZEN/mCmGmUmAmUmUmAmUmAm GmCmCmGmAmUmUmAmAmCmG/3ZEN/-3'

Other Solutions for Injection	
Trypaflavine (Acriflavine hydrochloride) Sigma Aldrich (Munich, Germany)	5 mM (diluted in 1xPBS sterile filtrated)
Poly-L-Lysine Sigma Aldrich (Munich, Germany)	1 mM (diluted in 1xPBS sterile filtrated)

3.2. Software and databases

- Bio-Rad CFX Manager ™ 3.1
- Bio-Rad iQ5
- BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)
- Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)
- Galaxy Server (https://usegalaxy.org/)
- Mendeley Desktop Version 1.15.2
- Microsoft Office 2007
- miRBase (http://www.mirbase.org/)
- MxPRo QPCR software for Mx3000P (v 4.1.0.0)
- Oligo Calc (http://www.basic.northwestern.edu/biotools/oligocalc.html)
- RefSeq (http://www.ncbi.nlm.nih.gov/refseq/)
- RNA hybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/)
- UniProtKB (http://www.uniprot.org/)
- Vassarstats (http://vassarstats.net/)

3.3. Methods

3.3.1. Animals

The honeybees (*Apis mellifera*) were kept throughout the whole year in the apiary of the Saarland University in Saarbrücken, Germany. In summertime the foragers were caught in the botanical garden in front of the hive with a UV light-permeable plexi glass pyramid (Felsenberg 2011) and transferred into plastic vials. In wintertime, the honeybees were kept indoors in the winter bee house, the animals were allowed to fly out of their hives and collect pollen and 1 M sucrose solution freely. The hives were surrounded by a thin gossamer fabric in which we caught them immediately into plastic vials. The temperature in the winter bee house was kept constantly at 23°C - 25°C with a lower temperature during the night time and 50% humidity. In both cases, the plastic vials were transported immediately to the lab, where the honeybees were immobilised on ice and transferred into plastic harnessing tubes. Fixed with textile adhesive film between the caput and the thorax and with another film that covered the abdomen (see figure 7), the bees in their harnessing tubes were kept together in bee racks. The bees were fed with 1 M sucrose solution and kept in plastic tubes, moistened with water on the ground and covered with a dark lid.

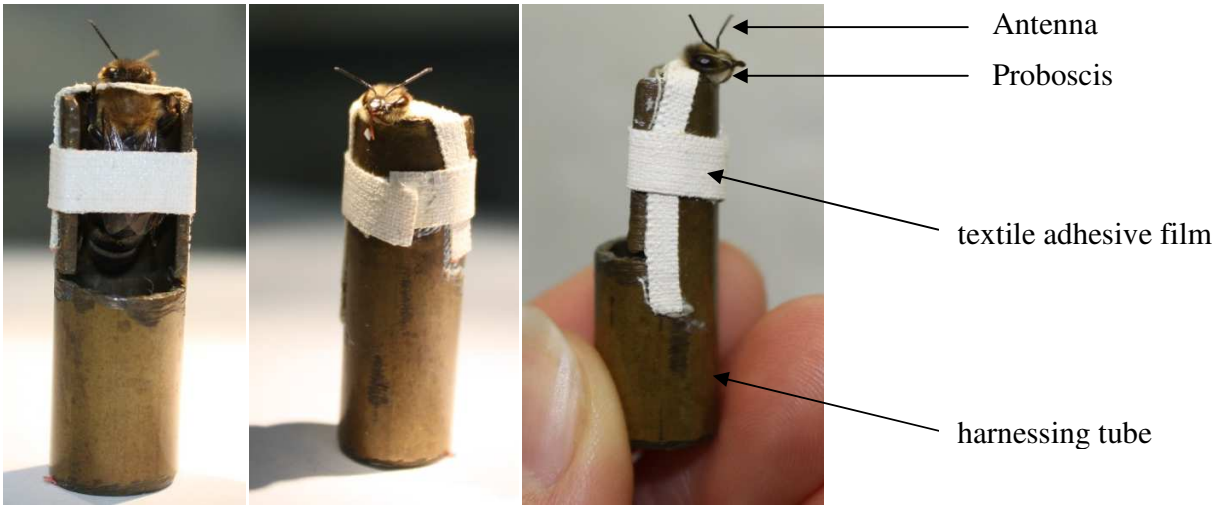

Figure 7 Honeybee in harnessing tube

The honeybee is fixed in a harnessing tube, the antennae and the proboscis can move freely (Source image: Michely).

3.3.2. Associative olfactory conditioning

The conditioning experiments were carried out one day after collecting the honeybees. The animals were starved over night for at least 16 h. Acquisition trials consisted of an odor stimulus (CS conditioned stimulus) paired with a sucrose reward (US unconditioned stimulus). The presentation of the CS with a 20 ml syringe containing clove oil for 5 s was paired after 3 s with an US. The antennae of the honeybee were touched with a 1 M Sucrose moistened toothpick, after proboscis extension the bees were allowed to lick the sucrose for 3 s. One CS-US pairing (single-trial) is described as weak training (Müller 2002) whereas 3 CS-US pairings with a 2 min interval between each trial is described as strong training. 2 h, 1 d and 2 d memory recalls were performed with the same animals. The memory recall consisted of presentation of the CS alone. Animals that did not respond to the US during the conditioning and animals that showed the proboscis extension response to the odour alone were excluded from the experiments.

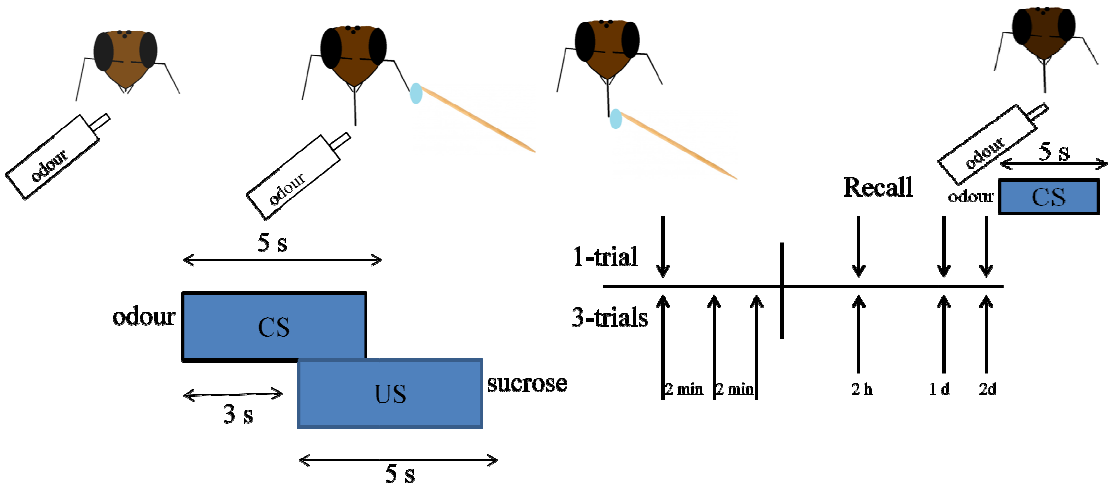

Figure 8 Conditioning: Odour and sucrose paired presentation

The honeybees were conditioned with a paired presentation of a CS (clove oil odour) and a US (1 M sucrose solution). The CS was presented for 5 s, after 3 s of CS presentation; the US was presented for 5 s as well, so the paired presentation of the two stimuli lasted for a total time of 2 s. The single-trial conditioning consists of one CS-US pairing, whereas the three-trial conditioning consists of three CS-US pairings with an inter trial interval of 2 min. The memory recall was tested by presentation of only the CS and was performed 2 h, 1 d and 2 d after the conditioning (Source image: Michely).

3.3.3. Gustatory sensitivity

The animals were tested for their gustatory sensitivity to sucrose. Depending on the age, the saturation level, the genotype and the division of labour of the honeybees, the gustatory sensitivity can vary (Page et al. 1998; Pankiw and Page 1999). First, a pre-test was conducted in order to classify the honeybees to their corresponding gustatory sensitivity and to distribute them into homogenous groups. The responsiveness to the sucrose stimulus was tested by touching the antennae with toothpicks moistened with sucrose solutions of increasing concentration (0 M, 0,03 M, 0,1 M, 0,3 M and 1 M). The honeybees were sorted according to their scores into different groups. When too many of the honeybees were responding to small concentrations, the bees were all fed with 1 M sucrose solution and the pre-test was repeated after one hour. To test for altered gustatory sensitivity due to treatment with different miRNA-Inhibitors, PLL and TPF, the bees were injected at different points in time before the responsiveness tests (the different points in time and experiments are described directly in the Results section). The responsiveness to the sucrose stimulus was tested by touching the antennae with toothpicks moistened with sucrose solutions of increasing concentration (0 M, 0,03 M, 0,1 M, 0,3 M and 1 M). The test was carried out at different points in time after the treatment. For statistical evaluation of data, the gustatory response score (sum of reactions of every single animal (0-5 reactions possible)) was calculated and tested by a Mann Whitney test (http://vassarstats.net/). A p-value $\leq$ 0,05 was hereby considered as significant.

3.3.4. Non-associative learning

To study the influence of the treatment with the different chemical compounds the animals were tested with two different non-associative learning paradigms: the habituation and the sensitisation. For the 1 d treatment with miRNA-Inhibitors, the animals were caught in the afternoon, fed till satiation and then injected with the appropriate solutions. For the 2 h and the 4 h test; the animals were caught the day before, fed till satiation and treated in the morning. The tests were then carried out exactly 2 h or 4 h after the treatment. The habituation and the sensitisation are both non-associative learning paradigms in which the animals learn to value and distinguish a stimulus' context and relevance.

3.3.5. Habituation

The animals were habituated by touching one antenna repeatedly (inter-stimulus-interval 1 s) of the animals with a toothpick moistened in 1 M sucrose solution. The animals were considered habituated, when the animals stopped the reaction with the PER for five times in a row. After reaching the habituation criterion, bees were dishabituated by touching the other antenna with the same toothpick. The dishabituation test is necessary to distinguish animals that showed fatigue or sensory adaptation from those, that were actually habituated. The number of stimuli until habituation was noted for evaluation. Bees that showed more than 50 PERs (cut off), that did not react at all or did not show dishabituation, were excluded from the evaluation of data.

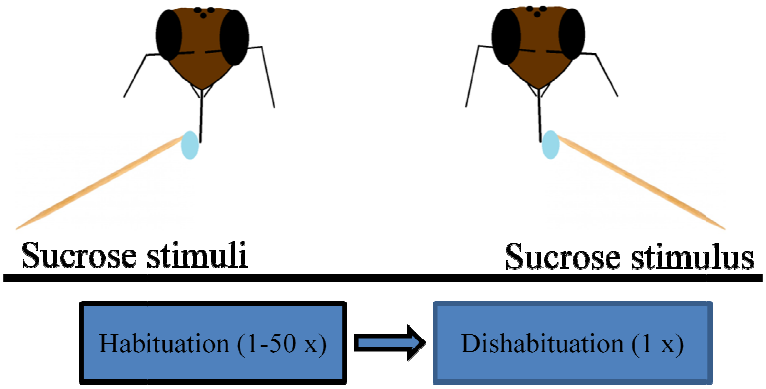

Figure 9 Habituation scheme

The honeybees were habituated, by repeatedly touching one antenna with an interval of 1 s with a toothpick moistened with 1 M sucrose. After reaching the habituation criterion and a response to the dishabituating stimulus, bees were considered habituated (Source image: Michely).

3.3.6. Sensitisation

Before sensitisation, the bees were stimulated with clove odour to test for spontaneous response to the odour and the PER was noted. After 2 min the bees were touched with a toothpick moistened in 1 M sucrose solution. 20 s after the sucrose stimulus, the clove odour was presented to the animals again and the PER was noted. The bees that responded to the first presentation of the odour or did not react to the sucrose stimulus were excluded from evaluation.

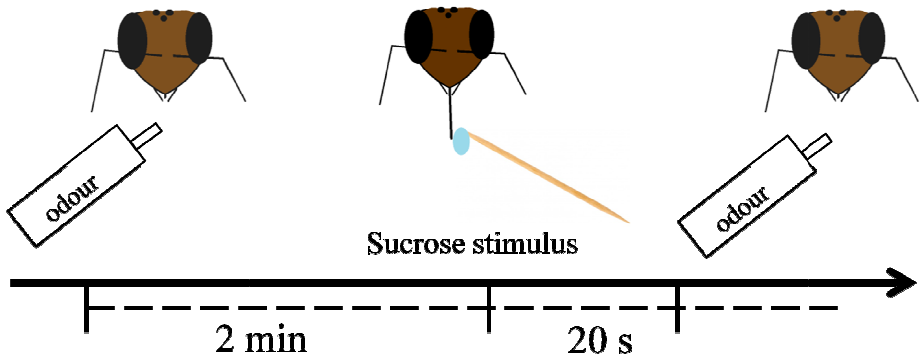

Figure 10 Sensitisation scheme

The odour (clove oil) was presented to the honeybees. After 2 min, an antenna was stimulated with a toothpick moistened with 1 M sucrose solution followed by the presentation of an odour 20s later. The PER was noted with each presentation (Source image: Michely).

3.3.7. Drug application

Drug application was performed at different points in time before or after conditioning experiments as indicated in the results section. Substances were applied by injection of 1 µl volume each with a calibrated glass capillary into the hemolymph of the thorax of the honeybee. Prior to injection, the thorax of the honeybee was pricked with a cannule to enable access to the hemolymph. 0.5 µM miRNA-Inhibitors Anti-ame-miR-12, Anti-ame-miR-124, Anti-ame-miR-125: 5 nmol solved in 50 µl TE-Buffer) by Integrated DNA Technologies solved in 1 x PBS. In other experiments, the animals were injected with 1 mM Poly-L-Lysine (PLL) by Sigma-Aldrich in 1 x PBS or 5 mM Trypaflavine (TPF) by Sigma-Aldrich in 1 x PBS, the concentration was adjusted according to Watashi et al. (2010).

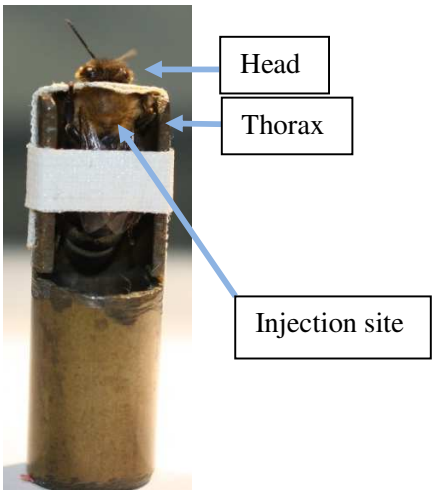

Figure 11 Injection sites on the honeybee thorax

The honeybee fixed in a harnessing tube, the sites for injection are located on the thorax (Source image: Michely).

3.3.8. Brain dissection

The honeybees were immobilised for 2 min on ice. The head of the honeybee was separated from the thorax and fixed on a wax block. The head capsule was opened by slicing from the mandibles to the back of the head (where the ocelli are located) in a straight cut with a scalpel (see figure 12 a). After removing the tracheal membranes, the glands and the ocelli, the optical lobes were separated from the central brain and the central brain was taken out with a forceps (see figure 12 b). The brains of the naive control group were dissected alternating with the brains of the trained group.

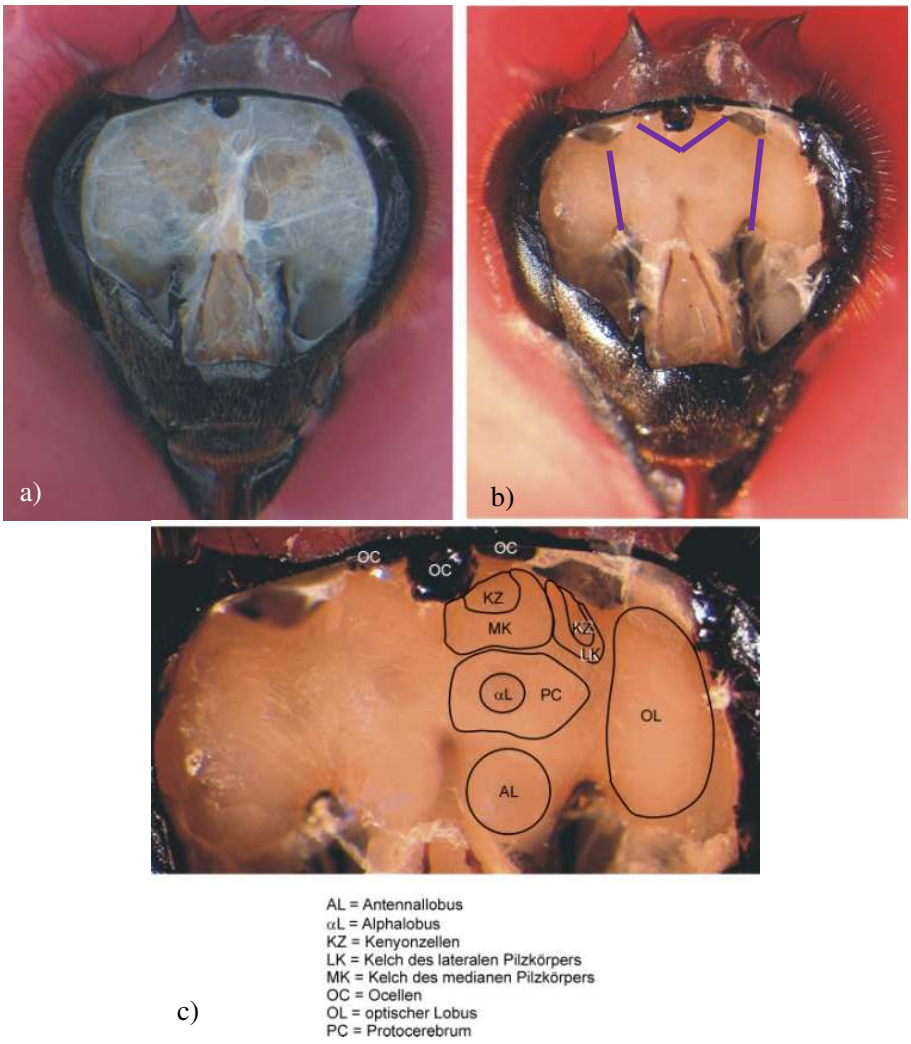

Figure 12 Dissection of a honeybee brain

The upper left picture a) shows a honeybee brain after the opening of the head capsule and the removal of the mandibular glands. Picture b) on the upper right displays the honeybee brain after removal of the tracheal membranes. The violet lines framing the central brain, show the dissection the central part of the brain out with a scalpel, whereby the upper two cuts are necessary to get rid of the ocelli. The picture c) provides an impression of the different brain parts and structure of the honeybee brain. The central part contains the two antennal lobes (AL), the protocerebrum (PC) with its α lobes (αL), the calices of the median (MK) and the lateral mushroom bodies (LK) as well as its kenyon cells (KZ). The ocelli (OC) and the optical lobes (OL) were not used. (Source image: Angelika Gardezi, modified)

3.3.9. RNA-Isolation

RNA was isolated from honeybee brains with TRIzol Reagent Solution purchased from Ambion Life Technologies (Carlsbad, California, USA) or with RNeasy kits (QIAGEN). Per reaction, 3-5 honeybee brains were homogenized in 350 µl TRIzol Reagent with 18 ceramic beads in reagent tubes for 4 min on a shaker, the RNA was isolated according to the manufacturer's protocol. After the homogenisation, 40 µl chloroform were added to each reaction, the samples were mixed for 15 s on a vortex and incubated for 3 min at room temperature. The samples were then centrifuged for 15 min at 4°C and 12000 *g*. After this centrifugation, three phases had separated and the upper aqueous phase was removed and transferred into a new tube. To this aqueous phase, 150 µl isopropyl alcohol were added and the samples were kept at -70°C over night for precipitation of RNA. The samples were thawed the next day and centrifuged for 10 min at 4°C and 12000 *g*. The supernatant was discarded and after removing the supernatant, the RNA pellet was washed with 200 µl 75% ethanol (EtOH) followed by centrifugation for 5 min at 4°C and 7600 *g*. After washing, the pellet was dried for ca. 3 min at room temperature. The dried pellet was now dissolved in 30 µl DEPC-H_2O mixed by pipetting up and down several times and incubated for 5 min at 65°C. After dissolving, the samples were checked for purity and concentration in the Tecan infinite pro reader, using its DNA/RNA measurement program. Afterwards it was divided to 6 parts and cDNA reactions for 5 different miRNAs and 1 mRNA reaction were performed, or the samples were stored at -70°C for later use.

3.3.10. Quality and quantity control of total RNA

To check the concentration of the total isolated RNA, 1 µl RNA was measured using the DNA/RNA quantification function of the Tecan infinite reader. The reference was DEPC-H_2O. The quantity and quality was determined by the measurement of the optical density (OD) at λ= 260 nm (maximum of absorption for nucleic acids) and the OD measurement at λ= 280 nm (maximum of absorption for proteins). The purity was determined by division of the OD_{260} through the $OD_{280.}$ Only RNA samples with a value between 1,8 and 2,0, were used in this work.

3.3.11. Stem-loop primer design

MiRNAs of interest were quantified by the stem-loop RT method as described earlier (Chen et al. 2005). Stem-loop primers for reverse transcription of miRNAs were designed as stated in Chen et al. (2005) and Wu et al. (2007) with a modification for detection by Universal Library Probe #21. The ca. 50 nucleotides long stem-loop RT primer forms a stem-loop with itself whereby 8 nucleotides at the 3'–end are overlapping. The last 6 of those overlapping nucleotides are designed to bind to the miRNA of interest. After reverse transcription, the former stem-loop opens and is a part of the desired cDNA. This cDNA has a binding site for the Universal Library Probe #21 that will bind only to this particular sequence in the Real-Time PCR. The Universal Library Probe consists of a quencher and a fluorescent dye (FAM), these 2 molecules are linked by a nucleotide sequence which binds to the stem-loop primer used for cDNA synthesis. In the state of binding to the cDNA during Real-Time PCR, the quencher and the fluorescent dye are spaced apart from each other, so that the fluorescent dye (FAM) fluoresces. The forward primer for the aforementioned cDNA was specifically designed for the appropriate miRNA-cDNA sequence. The reverse primer for the Real-Time PCR is universally binding to a part of the Stem-loop RT primer-cDNA sequence (see figure 13).

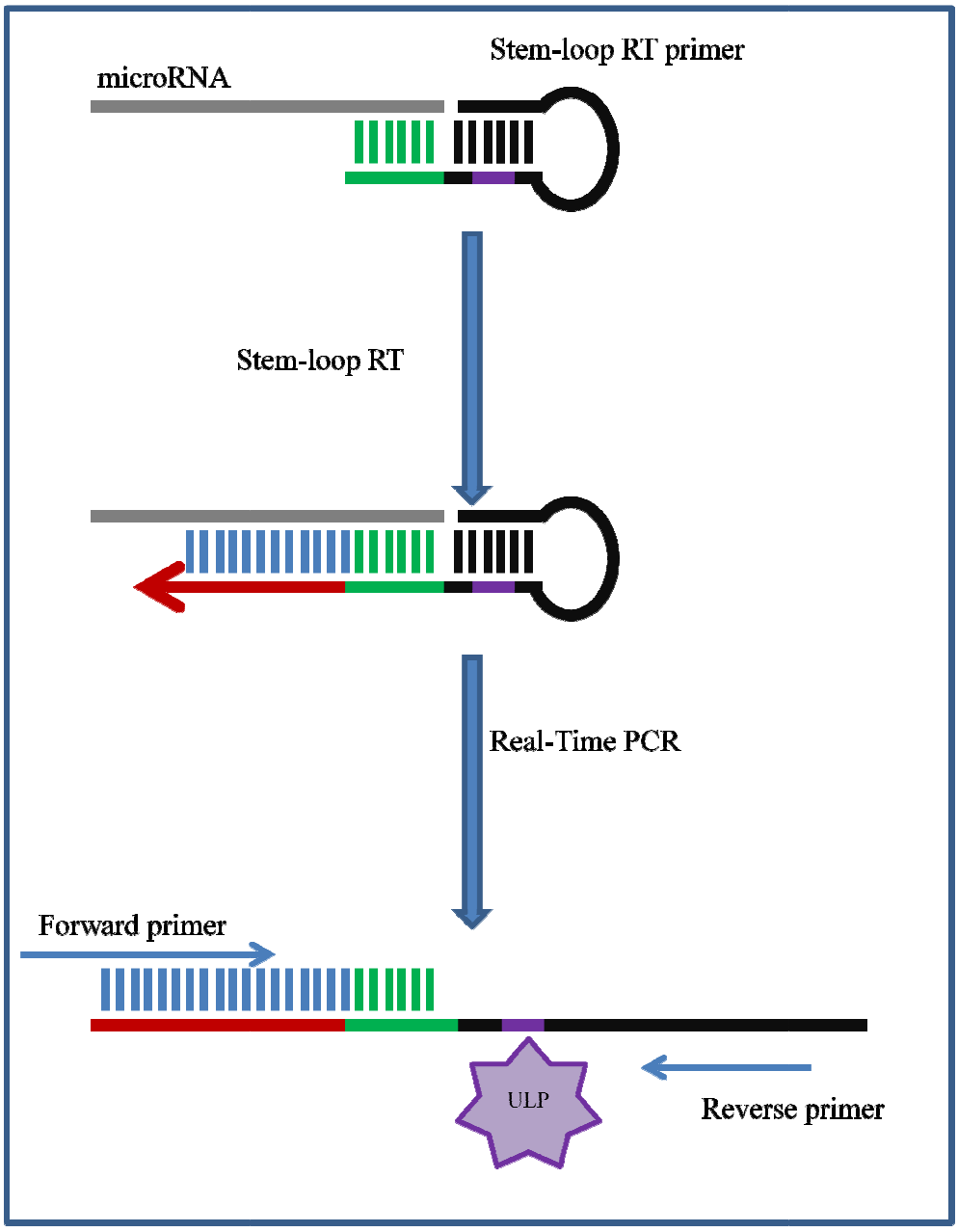

Figure 13 Quantification of miRNAs by stem-loop RT and Real-Time PCR with Universal Library Probe

The isolated miRNA (grey) is reverse transcribed into cDNA with the help of a stem-loop RT primer (black/violet stem-loop) which binds to the last six nucleotides at the 3'-end of a miRNA. In the Real-Time PCR, the stem-loop opens, the reverse primer binds to a part of the former stem-loop. The forward primer is designed to bind to the miRNA specifically. The Universal Library Probe (violet star) binds to its opposite sequence within the former stem-loop and fluoresces (Picture adapted from (Wu et al. 2007).

Table 7 List of honeybee miRNA Sequences, examined in this work

miRNA name	miRNA Sequence	miR-BASE/BLAST ID
ame-miR-12	UGAGUAUUACAUCAGGUACUGGU	>ame-miR-12 MIMAT0001472
ame-miR-124	UAAGGCACGCGGUGAAUGCCAAG	>ame-miR-124 MIMAT0001473
ame-miR-125	CCCCUGAGACCCUAACUUGUGA	>ame-miR-125 MIMAT0001474
ame-miR-989	CGUGAUGUGACGUAGUGGUUCU	>ame-miR-989 MIMAT0018511
ame-miR-3756	UUUCUUUCAUAAGGAGGA	>ame-miR-3756 MI0016157
ame-miR-3769	GGUACCUGAAGAGAGGUUU	>ame-miR-3769 MI0016173
ame-miR-3788	GGGACAGGAGGUAACGG	>ame-miR-3788 MI0016197

Table 8 Stem-loop primer sequences for cDNA synthesis

The binding site for the Universal Library Probe #21 is highlighted in violet. The last 6 nucleotides (highlighted in green) bind to the last 6 bases at the 3'–end of the distinct miRNA.

Stem-loop RT primer	**Sequence**
JR-12-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACCAGT
JR-124-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTTGGC
JR-125-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCACAA
JR-989-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAGAACC
JR-3756-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCCTCC
JR-3769-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCGTCAA
JR-3788-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGAGGGA

3.3.12. cDNA-synthesis

The reverse transcriptase transcribes mRNA with oligo-dT primers or miRNA with specific primers to single stranded cDNA. Revert Aid RT cDNA Synthesis Kit by Thermo Fisher Scientific was used for reverse transcription as follows: 4 µl of RT-Buffer, 1 µl of 10 mM dNTP, 1 µl of primer (2 µM Stem-loop primer for microRNA and oligo-dT or mRNA), 1 µl Ribo Lock and 1 µl Revert Aid Reverse Transcriptase were mixed, total RNA was added and nuclease-free PCR H_2O filled up to 20 µl total followed by 50°C step for 30 min, inactivation at 85°C for 5 min and a 4°C cool-down step. For Real-Time analysis, the cDNA was diluted 1:1 with nuclease-free H_2O.

3.3.13. Real-Time PCR

The quantitative Real-Time PCR is a method for the amplification and quantification of DNA. After reverse transcription of miRNA or mRNA into cDNA, the method can show the relative and quantitative expression of specific genes. Due to fluorescence markers that bind to the cDNA with amplification, the rising product amount can be monitored after each cycle of the reaction. The first significant rise of fluorescence in the exponential phase (C(t) = threshold cycle) correlates directly with the starting quantity of cDNA in the reaction. The miRNAs were detected with the fluorescent Universal Library Probe #21. This detection molecule consists of a quencher and the fluorescent dye FAM (Fluorescein) linked together with a sequence of nucleotides. This sequence binds to the sequence of the stem-loop primer used in the reverse transcription. In the Real-Time PCR for mRNAs, the SYBR fluorescent dye intercalates with double stranded DNA during its amplification in Real-Time PCR. SYBR and FAM can be measured together by Real-Time PCR, because their excitation wavelength is $\lambda = 488$ nm. Real-Time PCR for miRNA samples was carried out using the BioRad CFX cycler with Kapa Probe Fast Universal qPCR mix (Peqlab) and Universal ProbeLibrary Probe #21 (Roche) and for mRNA with Kapa SYBR Fast Universal qPCR mix (Peqlab). The mastermix for miRNA quantification contained 3,8 µl PCR H_2O (nuclease-free), 10 µl 2x qPCR Mastermix (Kapa Probe Fast Universal qPCR mix (Peqlab)), 0,4 µl 10 µM forward primer (Sigma), 0,4 µl 10 µM reverse primer (Sigma), 0,4 µl Universal ProbeLibrary Probe #21 and 5 µl cDNA (diluted 1:1 after reverse transcription). The SYBR

reaction contained 8,2 µl PCR H_2O (nuclease-free), 10 µl 2x qPCR Mastermix (Kapa SYBR Fast Universal qPCR mix (Peqlab)), 0,4 µl 10 µM forward primer (Sigma), 0,4 µl 10 µM reverse primer (Sigma) and 1 µl cDNA (diluted 1:1 after reverse transcription)). Synthetic oligo nucleotides including miRNA oligo nucleotides for standard measurement were purchased from Sigma Aldrich (Munich, Germany). The reaction started at 95°C for 3 min followed by 40 cycles of 1) 95°C for 10 sec, 2) 58 °C for 18 sec, 3) 72°C for 18 sec then followed by 95°C for 10 sec and 58°C for 18 sec and the last step 95°C for 30 sec.

3.3.14. Primer design for the Real-Time PCR primers

The primers were designed to bind the cDNA that was synthesised with the Stem-loop RT Primers. At the 5' end of each forward primer there are 6-7 nucleotides that overlap before the primers bind to the cDNA following the design of Chen et al. (2005) (see table 9). Primers were designed with the help of "Oligonucleotide Properties Calculator". According to the guidelines, the GC amount was chosen between 40-60%, the melting temperature lay between 60°C and 65°C. The Real-Time Primers showed neither self or heterodimers nor stem-loops (except for the Stem-loop RT Primers for cDNA synthesis). The repetition of nucleotides was avoided and BLAST analysis did not show further sequence homologies in the honeybee. In each Real-Time PCR, from every sample I measured also the EF 1α (*Elongation Factor 1α*) mRNA as a control and reference (EF 1α primers designed by Büttner (2011)). The GluA2 primers (GluA2- 1-2 (exon1,2) and GluA2 9-11 (exon 9,11)) were used in one Real-Time experiment as a control additional to the EF 1α (*Elongation Factor 1α*) control and reference (GluA2- 1-2 and GluA2 9-11 primers designed by Kobel (2015)). The mRNA primers were designed using the same GC amount and melting temperature as aforementioned, whereas the difference between two primers should not be more than 1° C, the primer size should range between 17-23 nucleotides and result in an amplicon size between 120 - 150 bp.

Table 9 Real-Time PCR primer sequences

The table displays sequences of the Primers used for Real-Time PCR (5'–end overlap highlighted in blue).

Primer	**Sequence**
JR-124-fwd	CCGGCGTAAGGCACGCGGTG
JR-125-fwd	TCGCGTCCCCTGAGACCCTA
JR-12-fwd	CGCGGCTGAGTATTACATCA
JR-3756-fwd	GCGCGGCCTGATTTCTTTCAT
JR-3769-fwd	GGCGCGGGTAGCTCAAGAGA
JR-3788-fwd	GCGGCGCGTTCCGTTACCTC
JR-989-fwd	TCGCGTCGTGATGTGACGTA
JR-rev-univ	GTGCAGGGTCCGAGGT
Elongation Factor 1α fwd	CCTCCTCAGGACGTATATAAAATCG
Elongation Factor 1α rev	AGCTTCGTGATGCATTTCAACAG
GluA2 1-2 fwd	GCGTCCACCTTTTCGAAAATC
GluA2 1-2 rev	CTGCGCATTTATGAAAGTCTGG
GluA2 9-11 fwd	TGTTAAGGTCGGTGAATGGCG
GluA2 9-11 rev	GAGCCAACAGCCAAATCTGC

3.3.15. Real-Time PCR standard design and setting

The quantitative analysis of each miRNA/cDNA was performed using a standard curve with specific standards and a defined number of copies. As standards for Real-Time PCR, I used RNA oligos purchased from Sigma Aldrich and had the same sequences as the honeybee miRNAs. MiRNA sequences for standards of ame-miR-12, ame-miR-124, ame-miR-125, ame-miR-989, ame-miR-3756, ame-miR-3769 and ame-miR-3788 were used from miRBase. For reverse transcription into cDNA, we used the same stem-loop Primers (2 µM concentration) as for the reverse transcription of the intrinsic miRNAs of the honeybee. For each standard miRNA we used 500 ng for the reverse transcription. After transcription into cDNA the standards were diluted as described in table 11 for Real-Time PCR. The Real-Time PCR was conducted in the same way as the normal Real-Time PCR for the miRNAs as described above. The application of the threshold cycles (C(t)) against the logarithm (log) of the number of copies at the start of the

reaction from each standard dilution (1:10 dilution in nuclease-free H_2O: 10^{-1}-10^{-4} varying table 11) shows the related number of copies of the different samples. The efficiency of the PCR is depending on the slope of the standard curve. The efficiency of the PCR ranged between 90-110 %.

Table 10 Oligo nucleotides for standard q-RT-PCR

The table displays oligo nucleotides for Standard q-RT-PCR (microRNA sequences by miRBase).

Ame-miR-12	5' - UGAGUAUUACAUCAGGUACUGGU
Ame-miR-124	5' - UAAGGCACGCGGUGAAUGCCAAG
Ame-miR-125	5' - CCCCUGAGACCCUAACUUGUGA
Ame-miR-989	5' - CGUGAUGUGACGUAGUGGUUCU
Ame-miR-3756	5' - CUGAUUUCUUUCAUAAGGAGGA
Ame-miR-3769	5' - GGUAGCUCAAGAGAAGGUUGACG
Ame-miR-3788	5' - GUUCCGUUACCUCCUGUCCCUC

Table 11 MiRNA standard dilutions for q-RT-PCR

The table displays miRNA standard dilutions used and developed in this work for q-RT-PCR standard references.

Standard dilution	0,1 ng	0,01 ng	0,001 ng	0,0001 ng
miR-12 Standard	x	x	x	
miR-124 Standard		x	x	x
miR-125 Standard	x	x	x	
miR-989 Standard		x	x	x
miR-3788 Standard		x	x	x

3.3.16. Data evaluation

In every sample (n = 1) the different miRNAs miR-12, miR-124, miR-125, miR-989 and miR-3788 and the house keeping gene *Elongation Factor 1α* (EF 1α) were measured. The Real-Time data were normalised to miRNA standards as described above. The quantity values that were measured by the BioRad cycler in correspondence to the standard straight lines (see figure 14) of each miRNA were exported to Microsoft Office Excel 2007 for evaluation. These Real-Time PCR data were averaged separately for each measured miRNA or cDNA per experiment. The single values were divided through the mean value of all measured samples from one experiment. After the normalisation to every single experiment, the data from all experiments were summarized. The standard deviations were defined and statistics were performed using Student's t-test (independent samples, unequal sample variances, two tailed).

a)

b)

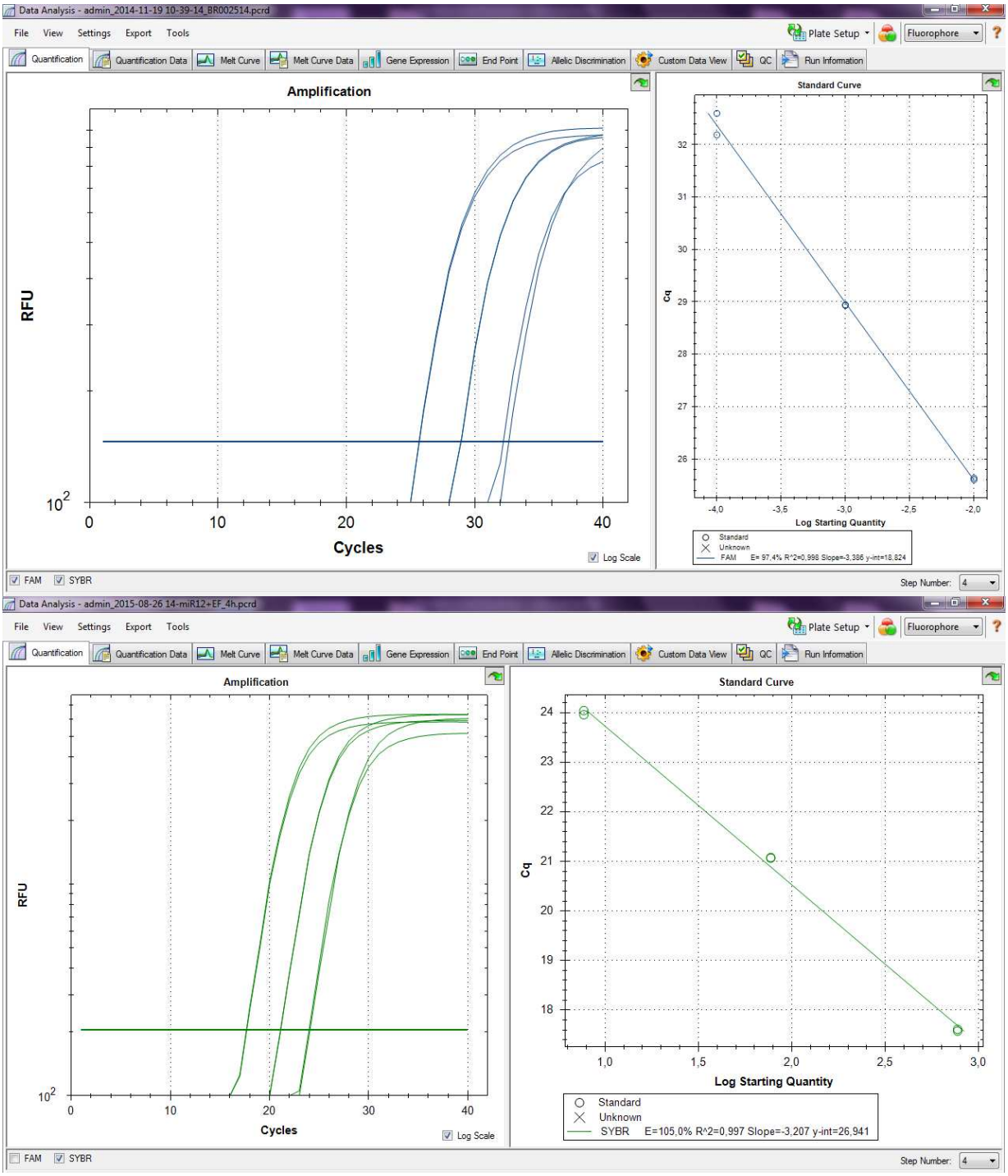

Figure 14 Standard curve for the miR-124 standards and the Elongation Factor 1α standards

a) The diagram a) shows the standard curve for the miR-124 standards. The x-axis shows the logarithm of the starting quantity of cDNA in ng, the y-axis shows the C(t) values. The efficiency of the PCR is 97,4%.

b) The diagram b) shows the standard curve for the EF 1α standards. The x axis shows the logarithm of the starting quantity of cDNA in ng, the y axis shows the C(t) values. The efficiency of the PCR is 105,0%.

The C(t) values of the different standard dilutions have about 3 cycles spacing in between (1:10 dilution). The windows on the left show diagrams in logarithmic scales, the x axis displays the PCR cycles while the y axis shows the fluorescence. The baseline is the horizontal threshold line which gives us the C(t) (threshold cycle) value. This value tells us at which cycle the fluorescence intensity rises exponentially against the baseline background. The windows on the right side show the values that the program determined for the standard samples. The standard dilution was entered to the plate setup before measurement and the C(t) values are determined as described above (Source image: Michely).

3.3.17. Statistical analysis

Statistical analysis was performed with http://vassarstats.net/. MiRNA levels of naive and conditioned animals were compared with Student's t-test (independent samples, unequal sample variances, two tailed). The responsiveness scores were compared by the Mann-Whitney *U*-test. To compare the behavioural data (PER, pairwise), the Chi-Square/Fisher's exact test was used. We demonstrated the Yates value together with the two tailed Fisher's exact probability value for each comparison whereby $p < 0,05$ was considered as significant. The significance of an observed value of r was performed with the raw data from the q-RT-PCR experiments, the correlation coefficient was considered significant, when $p < 0,05$.

4. Results

4.1. MiRNA sequence homologies

The importance of certain miRNAs in neuronal plasticity and learning and memory mechanisms has been clarified in the introduction. In order to find miRNAs in the honeybee, which are homologues to miRNAs known to be important in learning, memory or synaptic plasticity mechanisms from other species, it is necessary to start with a miRNA sequence comparison. The sequences of ame-miR-12, ame-miR-124, ame-miR-125, ame-miR-989, ame-miR-3756, ame-miR-3769 and ame-miR-3788 and their homologues in other species are described in the following section. Those seven miRNAs were chosen according to their homology to miRNAs which are known to play important roles in learning, memory formation processes or synaptic plasticity in other species.

4.1.1. Comparison of miR-12, miR-124 and miR-125 sequences in different species

The miR-12 has been found in different insect species (Lagos-Quintana et al. 2001; Osei-Amo et al. 2012; Greenberg et al. 2012). In *Drosophila melanogaster*, it was associated with olfactory habituation (McCann et al. 2011) and in the honeybee in maze based visual pattern learning (Qin et al. 2014). A comparison of miR-12 sequences in the mosquito *Aedes aegypti*, the European honeybee *Apis mellifera* and the fruit fly *Drosophila melanogaster* shows that the sequences are identical.

Table 12 Comparison of miR-12 sequences in insect species

Species/ miR-BASE ID	**miRNA Sequence**	**BLAST ID**
A. aegypti :>aae-miR-12-5p	UGAGUAUUACAUCAGGUACUGGU	MIMAT0014253
A. mellifera: >ame-miR-12	UGAGUAUUACAUCAGGUACUGGU	MIMAT0001472
D. melanogaster: >dme-miR-12-5p	UGAGUAUUACAUCAGGUACUGGU	MIMAT0000117

The miR-124 is known to be a neuron specific miRNA, which is conserved between vertebrates and invertebrates (Siegel et al. 2011) it has also been described to play a role in associative learning in *Aplysia californica* (Rajasethupathy et al. 2009). The sequences of *Apis mellifera* and *Drosophila melanogaster* miR-124 are identical and at the 3'-end they are 3 bases (AAG) longer

than the sequences of *Mus musculus* and *Homo sapiens* miR-124 which are also identical. The *C. elegans* miR-124 sequence is one base (A) longer than those of the mouse and human miR-124.

Table 13 Comparison of miR-124 sequences

Species/ miR-BASE ID	**miRNA Sequence**	**BLAST ID**
A. mellifera: >ame-miR-124	UAAGGCACGCGGUGAAUGCCAAG	MIMAT0001473
C. elegans: >cel-miR-124-3p	UAAGGCACGCGGUGAAUGCCA	MIMAT0000282
D. melanogaster: >dme-miR-124-3p	UAAGGCACGCGGUGAAUGCCAAG	MIMAT0000351
H. sapiens: >hsa-miR-124-3p	UAAGGCACGCGGUGAAUGCC	MIMAT0000422
M. musculus: >mmu-miR-124-3p	UAAGGCACGCGGUGAAUGCC	MIMAT0000134

The miR-125 plays an important role in vertebrate neuronal differentiation and in synaptic plasticity and function (Le et al. 2009; Edbauer et al. 2010; Boissart et al. 2012). The miR-125 in the honeybee shows a similarity up to one different base in comparison to the miR-125 in *Drosophila* and miR-125b in mouse and human (highlighted in blue). The lin-4 miRNA in *C. elegans* shows a similarity up to 2 different bases to the miR-125 in the other species (highlighted in blue). Sequences of miR-125 in *Drosophila* and miR-125b in *Mus musculus* and *Homo sapiens* are identical.

Table 14 Comparison of miR-125 sequences in different species

Species/ miR-BASE ID	**miRNA sequence**	**BLAST ID**
A. mellifera: >ame-miR-125	CCCCUGAGACCCUAACUUGUGA	MIMAT0001474
C. elegans: >cel-lin-4-5p	UCCCUGAGACCUCAAGUGUGA	MIMAT0000002
D. melanogaster: >dme-miR-125-5p	UCCCUGAGACCCUAACUUGUGA	MIMAT0000397
M. musculus: >mmu-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	MIMAT0000136
H. sapiens: >hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA	MIMAT0000423

4.1.2. Sequence comparisons for the miR-132, miR-138 and miR-329

For the miR-12, miR-124 and miR-125, I compared the mature miRNA sequences of the different species. As there are no sequences for miR-132, miR-138 and miR-329 known in *Apis mellifera,* it was necessary to conduct sequence alignments. Hereby, the stem-loop sequences of the *Homo sapiens* hsa-miR-132, hsa-miR-138 and hsa-miR-329 were compared to

the *Apis mellifera* miRNA database using the BLASTn search tool of miRBASE (http://www.mirbase.org/) homepage.

The miR-132 plays a role in neuronal plasticity and synapse formation (Bicker et al. 2014) and is also known to be conserved through species (Kiezun et al. 2012). With an E-value cut off of E-14, one similar miRNA to the hsa-miR-132, the ame-miR-3788 was found. The stem-loop sequences of hsa-miR-132 and ame-miR-3788 show a similar pattern of 17 nucleotides (the ame-miR-3788 between the 20th and 4th nucleotide of its stem-loop and the hsa-miR-132 between the 48th and the 64th nucleotide of its stem-loop) with 3 nucleotide mismatches highlighted in blue. The role of ame-miR-3788 in learning and memory mechanisms has not yet been described.

Table 15 Comparison of the hsa-miR-132 and ame-miR-3788 stem-loop sequences

Species/ miR-BASE ID	**miRNA sequence match**	**BLAST ID**
A. mellifera: >ame-miR-3788	20- GGGACAGGAGGUAACGG -4	MI0016197
H. sapiens: >hsa-miR-132	48- GGAACUGGAGGUAACAG -64	MI0000449

The miR-138 is another conserved neuronal miRNA (Kiezun et al. 2012; Siegel et al. 2013). High levels of miR-138 in the mouse hippocampus are correlated with better short-term recognition memory performance (Tatro et al. 2013). With an E-value cut off of E-14, I found one similar miRNA, the ame-miR-989 (see table 16).The stem-loop sequences of hsa-miR-138 and ame-miR-989 show a similar pattern of 22 nucleotides (the ame-miR-989 between the 93rd and 72nd nucleotide of its stem-loop and the hsa-miR-138 between the 56th and the 77th nucleotide of its stem-loop) with 4 nucleotide mismatches highlighted in blue. The role of ame-miR-989 in associative olfactory conditioning is unknown. Table 17 shows a sequence comparison between the *Drosophila melanogaster* and the *Apis mellifera* sequences. The sequences are very similar with 3 mismatches highlighted in blue and one nucleotide lacking at the 3' end in *Drosophila*.

Table 16 Comparison of the hsa-miR-138 and ame-miR-989 stem-loop sequences

Species/ miR-BASE ID	**miRNA sequence match**	**BLAST ID**
A. mellifera: >ame-miR-989	93- GAGAACCACUACGUCACAUCAC -72	MI0016141
H. sapiens: >hsa-miR-138-1	56- GAGAACGGCUACUUCACAACAC -77	MI0000476

Table 17 Comparison of mature miRNA sequences of dme-miR-989 and ame-miR-989

Species/ miR-BASE ID	**miRNA sequence**	**BLAST ID**
A. mellifera : >ame-miR-989	CGUGAUGUGACGUAGUGGUUCU	MIMAT0018511
D. melanogaster: >dme-miR-989-3p	UGUGAUGUGACGUAGUGGAAC	MIMAT0005506

The miR-329 (miR-329), which was demonstrated to play several roles in neurons, is conserved through more than 25 species (Kiezun et al. 2012). With an E-value cut off of E-14, two similar miRNAs, the ame-miR-3756 and the ame-miR-3769 were found. The stem-loop sequences of hsa-miR-329 and ame-miR-3756 show a similar pattern of 18 nucleotides (the ame-miR-3756 between the 5th and 22nd nucleotide of its stem-loop and the has-miR-329 between the 32nd and the 49th nucleotide of its stem-loop) with 3 nucleotide mismatches highlighted in blue. The stem-loop sequences of hsa-miR-329 and ame-miR-3769 show 4 nucleotide mismatches (also highlighted in blue), both 19 nucleotides long sequences are located between the 1st and the 19th nucleotide of their corresponding stem-loop sequence. The functions of ame-miR-3756 and ame-miR-3769 in learning and memory mechanisms have not yet been described.

Table 18 Comparison of the hsa-miR-329, the ame-miR-3756 and ame-miR-3769 stem-loop sequences

Species/ miR-BASE ID	**miRNA sequence match**	**BLAST ID**
A. mellifera: >ame-miR-3756	5- UUUCUUUCAUAAGGAGGA -22	MI0016157
H. sapiens: >hsa-miR-329-1	32- UUUCUUUAAUGAGGACGA -49	MI0001725
A. mellifera: >ame-miR-3769	1- GGUACCUGAAGAGAGGUUU -19	MI0016173
H. sapiens: >hsa-miR-329-1	1- GGUAGCUCAAGAGAAGGUU -19	MI0001725

4.2. Learning induced changes of miRNA levels

This work deals with the quantification of the levels of the seven aforementioned miRNAs by q-RT-PCR after weak (single-trial) and strong (three-trial) appetitive olfactory conditioning and at different points in time. As a control group, I tested naive bees that were not conditioned. Weak conditioning with single-trial results in a transient memory, whereas a strong conditioning with three-trials leads to the formation of long-term memory (Wüstenberg et al. 1998). The animals were caught on the previous day, starved over night for at least 16 h and conditioned the next morning with either weak or strong conditioning with an interval of 2 min between each trial. The recall of memory consisting of one CS (clove odour) presentation was performed 2 h and 24 h after conditioning with the same animals. The animals that showed a PER after the 2 h and 24 h odour presentation were chosen for the dissections of the conditioned groups, whereby the central brains (see figure 12) were pooled for one sample.

4.2.1. MiRNA levels after weak conditioning

Figure 16 a) displays the relative amount of the different miRNAs 2 h after single-trial conditioning. The EF 1α, the miR-12, miR-124, miR-125 and the miR-989 did not show significant differences between the naive and the conditioned group, there was a trend to a decrease in the miR-124 conditioned group (Student's t-test: EF 1α: p= 0,59; miR-12: p= 0,57; miR-124: p= 0,06; miR-125: p= 0,32; miR-989: p= 0,10). For the miR-3788, the conditioned group showed a significant increase (Student's t-test: p= 0,039).

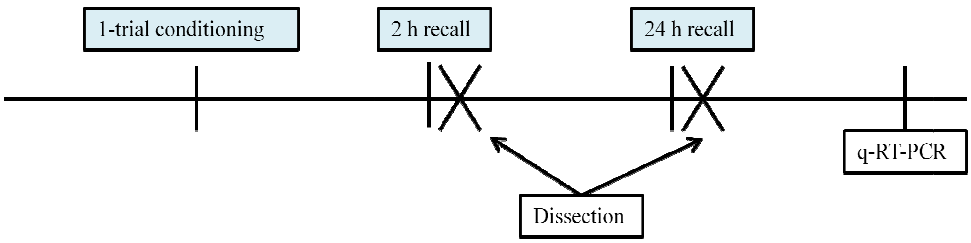

Figure 15 Timeline for the q-RT-PCR experiment after weak conditioning

Honeybees were conditioned with one trial and dissected immediately after the 2 h or the 24 h recall. Then they the q-RT-PCR was conducted with the miRNA and RNA which was isolated from the central brains of the animals.

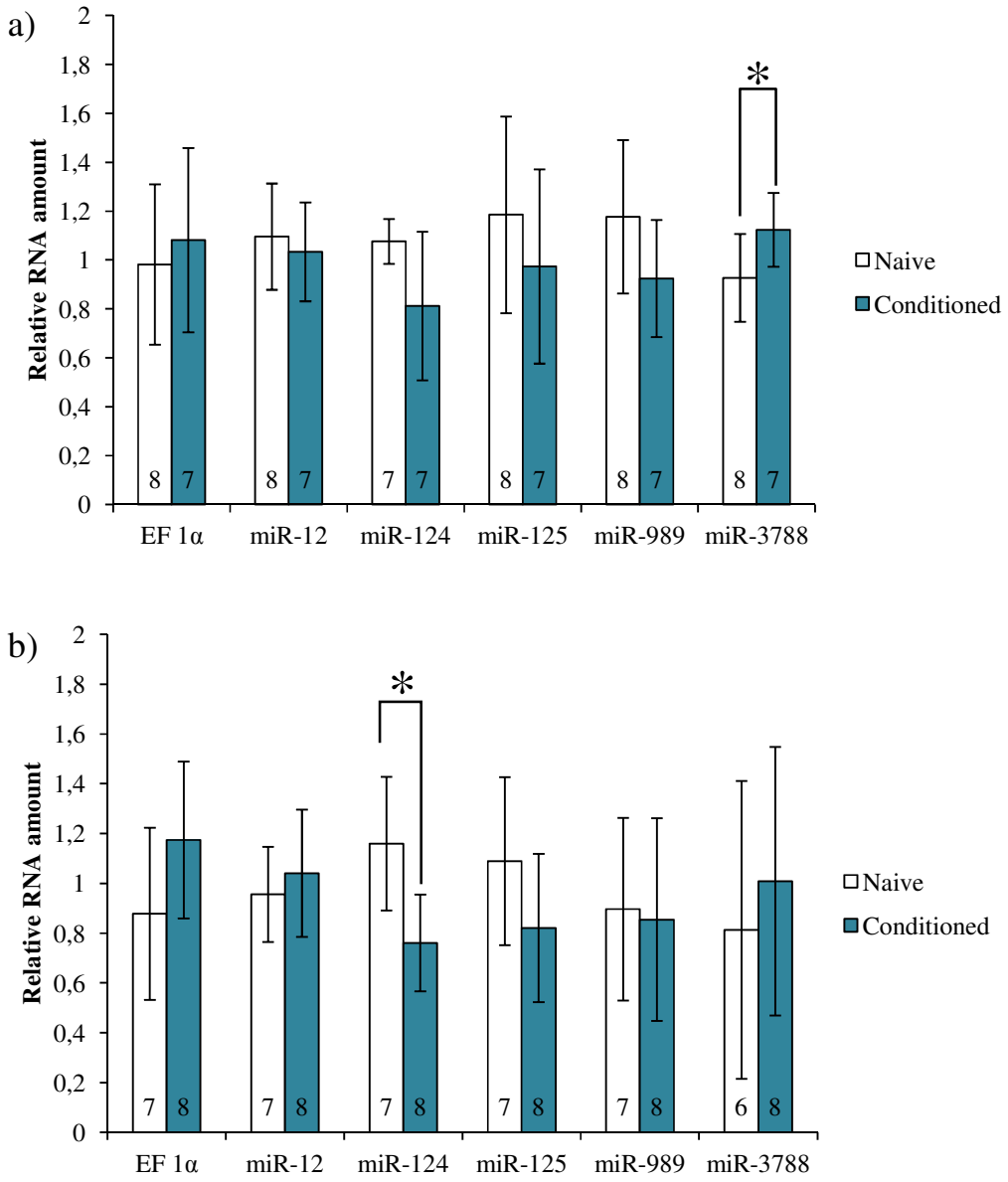

Figure 16 Levels of miRNAs 2 h and 24 h after single-trial conditioning

The columns display the relative amount of RNA (EF 1α) and miRNAs (miR-12, miR-124, miR-125, miR-989 and miR-3788) in the central brain of the honeybee 2 h (a) and 24 h (b) after single-trial conditioning. The diagrams display the relative mean values of the RNA and miRNA amounts and their standard deviation. Significant differences are marked with stars (*p≤ 0,05). The number in the basis of each bar indicates the number of samples.

The next experiment was carried out under the same conditions as the previously described experiment, but the honeybees were tested for both the 2 h and the 24 h memory retrieval and dissected for quantitative Real-Time PCR after the 24 h recall, whereby only the animals that showed a PER after both recalls were selected for the dissection. Figure 16 b) shows a diagram of

the miRNA amounts. The levels of EF 1α, miR-12, miR-125, miR-989 and miR-3788 did not show significant differences between the naive and the conditioned group (Student's t-test: EF 1α: p= 0,1; miR-12: p= 0,4; miR-125: p= 0,13; miR-989: p= 0,8; miR-3788: p= 0,5). The amount of miR-124 was significantly decreased in the conditioned group (Student's t-test: p= 0,007). The miR-3756 and miR-3769, which were also chosen for investigation because of their similarity to the hsa-miR-329 could not be analysed by quantitative Real-Time PCR, as their levels were too low in the honeybee brain.

4.2.2. MiRNA levels after strong conditioning

Figure 18 a) shows a diagram of the miRNA amounts in the central honeybee brain 2 h after three-trial conditioning. The experiment was carried out under the same conditions as the experiments aforementioned. The EF 1α, the miR-12, miR-125, miR-989, miR-3788 levels did not significantly change between the naive and the conditioned group (Student's t-test: EF 1α: p= 0,4; miR-12: p= 0,7; miR-125: p= 0,3; miR-989: p= 0,8; miR-3788: p= 0,7). The amount of miR-124 was significantly decreased in the conditioned group (Student's t-test: p= 0,027).

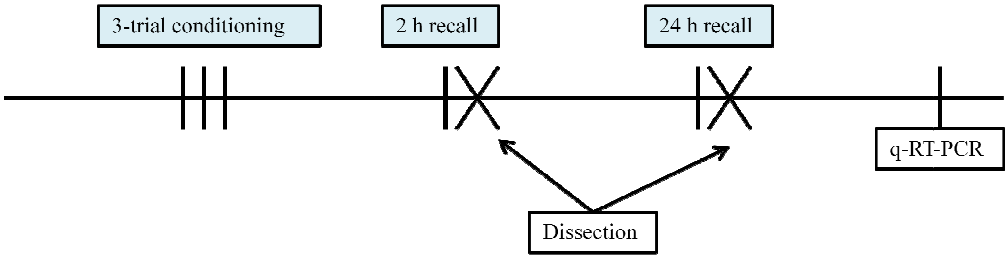

Figure 17 Timeline for the q-RT-PCR experiment after strong conditioning

Honeybees were conditioned with three trials and dissected immediately after the 2 h or the 24 h recall. Then they the q-RT-PCR was conducted with the miRNA and RNA which was isolated from the central brains of the animals.

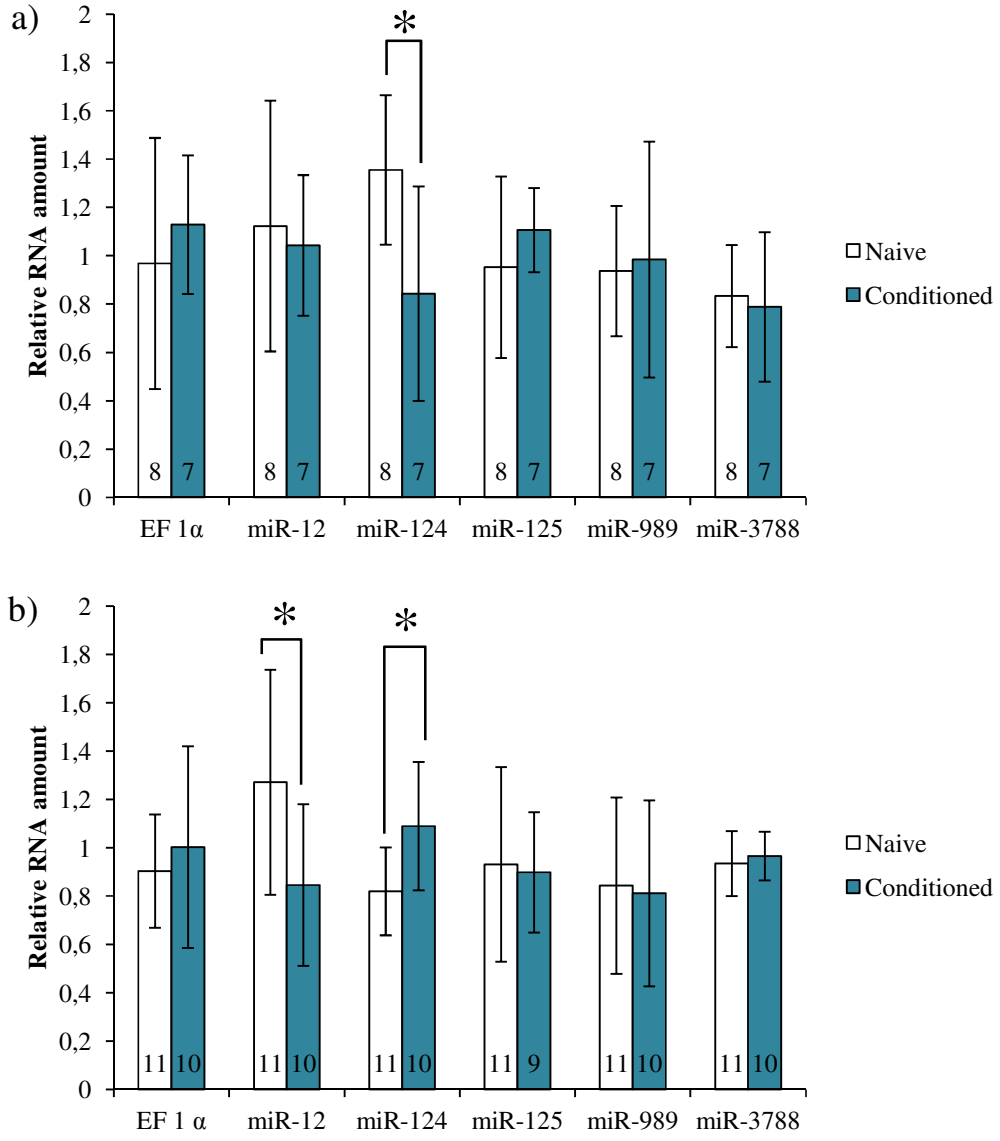

Figure 18 Levels of miRNAs 2 h and 24 h after strong conditioning

The columns show the relative amount of RNA (EF 1α) and miRNAs (miR-12, miR-124, miR-125, miR-989 and miR-3788) in the central brain of the honeybee 2 h (a) and 24 h (b) after three-trial conditioning. The diagrams display the relative mean values of the RNA and miRNA amounts and their standard deviation. Significant differences are marked with stars (*p≤ 0,05). The number in the basis of each bar indicates the number of samples.

Figure 18 b) shows the levels of miRNAs in the central brain of the honeybee 24 h after strong conditioning. The amount of miR-12 shows a significant decrease in the conditioned group compared to the naive group (Student's t-test: p= 0,024). The miR-124 amount was significantly increased in the conditioned group (Student's t-test: p= 0,012). The levels of the housekeeping

gene EF 1α, miR-125, miR-989 and miR-3788 did not show significant changes between the naive and the conditioned group (Student's t-test: EF 1α: p= 0,5; miR-125: p= 0,82; miR-989: p= 0,84; miR-3788: p= 0,54).

Drawing a conclusion from the learning induced analysis of miRNA amounts, the miR-3788 is upregulated 2 h after weak condition, while the miR-12 is downregulated 24 h after strong conditioning. MiR-124 is downregulated 24 h after weak and 2 h after strong conditioning and upregulated 24 h after strong conditioning. Ergo their regulation is not only dependent on the points in time after conditioning but also on the strength of training. The diversity of the miRNA levels leads to the assumption, that they are involved in different aspects of learning and memory formation as acquisition, consolidation and the establishment of different forms of memory. The levels of miR-125, -989 and -3788 did not change in the tested conditions.

4.3. Correlations between the miRNAs quantified by q-RT-PCR

There is evidence, that miRNA genes are not only clustered in families but are also coexpressed and in some cases even coregulated (reviewed in Bartel 2004). Studies in *Drosophila melanogaster* showed, that miR-125 is a putative homologue of the lin-4 miRNA, that miR-100, let-7 and miR-125 are coexpressed and clustered within an 800 bp region on the same chromosome (Sempere et al. 2003; Aravin et al. 2003; Lim et al. 2003). It has also been shown, that the upregulation of miR-100, let-7 and miR-125 and the downregulation of miR-34 is coregulated by the hormone ecdysone and the activity of the ecdysone inducible gene *Broad complex* (Sempere et al. 2003).

In this work I want to investigate, whether the observed changes in the levels of miRNAs are connected among themselves. Therefore a correlation test was performed. The groups (naive (n= 64) and conditioned (n= 64)) and the experiments were pooled for the respective measured RNA/miRNA. The amounts of miR-12, -124 and -125 measured in each of the samples are significantly related to each other. MiR-989 is correlated only with miR-12 and miR-124, while miR-125 correlates with miR-3788. All other correlations are not significant as indicated in the appendix (table 34) in greater detail. The significant correlations are listed in table 19. Thus, the strong relation between miR-12, -124 and -125 prompted me to investigate their specific roles in

associative learning and memory formation, especially in acquisition, consolidation and after weak or strong conditioning in more detail.

Table 19 Significant correlations

The table depicts the significance of a correlation coefficient.

	R^2	r	t	df	p
miR-124 vs. miR-12	0,1061	0,32573	2,71	62	0,008632
miR-3788 vs. miR-125	0,1552	0,39395	3,375	62	0,001276
miR-12 vs. miR-125	0,1769	0,42059	3,65	62	0,000539
miR-12 vs. miR-989	0,4683	0,68432	7,39	62	<0,0001
miR-124 vs. miR-125	0,2373	0,48713	4,392	62	<0,0001
miR-124 vs. miR-989	0,3112	0,55785	5,293	62	<0,0001

4.4. Transient manipulation of miRNA function *in vivo*

To determine, whether a partial inhibition of the miRNA machinery has an effect on learning and memory formation, honeybees were treated with 1 mM Poly-L-Lysine (PLL) which reduces DICER-mediated RNA processing or with 5 mM Trypaflavine (TPF) which reduces the association of miRNA with AGO2 (Watashi et al. 2010). The treatment with those substances in combination with learning and memory mechanisms in the honeybee had never been tested before. The PLL or TPF treatment at this point in time had no significant effect on acquisition, learning and memory formation, the data of these experiments can be found in the appendix in table 35-39. As revealed by the analysis of the learning induced changes, the different miRNAs show differential levels after conditioning and also may have different roles in developing memory formation processes. The two compounds PLL and TPF interfere with processes in miRNA biogenesis in general and not with one specific miRNA. To identify the role of single miRNAs in learning and memory formation processes, it is necessary to inhibit the single miRNAs specifically.

4.4.1. MicroRNA-Inhibitor design

The miRNA-Inhibitors (Integrated DNA Technologies, USA) were designed after Lennox et al. (2013) and inhibit the miRNA function by hybridising to the mature strand and impeding its function through sterical blocking. Due to that sterical block, the miRNA incorporated into the RISC complex is not able to bind its target any longer. They were designed antisense to the miRBase (http://www.mirbase.org/) mature miRNA sequences by using the tool from the Integrated DNA Technologies website for the design of miRNA-Inhibitors (http://eu.idtdna.com/site/order/mirna). The miRNA-Inhibitor, a single stranded oligo nucleotide sequence is one nucleotide shorter than the mature miRNA and has 2'-O-Me residues with ZEN™ chemical modifications at the ends. The 2'-O-Me residues protect the miRNA-Inhibitor from endonuclease degradation and increase its binding affinity to the miRNA targets whereas the ZEN™ modification impedes exonuclease degradation and also increases the binding affinity to the target miRNA (Lennox et al. 2013). The miRNA-Inhibitor was used for injection into the thorax of the honeybees. The use of AMOs provides a method to transiently knock-down miRNA function *in vivo* in a sequence specific manner (Krützfeldt et al. 2005; Lennox et al. 2013).

4.5. Defining the role of miR-12, miR-124 and miR-125 in memory formation processes in the honeybee

In q-RT-PCR experiments I showed that miR-124, miR-12 and miR-3788 levels were changed after conditioning the miR-125 and miR-989 levels did not change. The miR-3756 and miR-3769 amounts were too low to be measured by q-RT-PCR.

The miR-3788 was significantly increased 2 h after weak conditioning and for the miR-124 I found a trend of decrease 2 h after weak conditioning. The miR-124 levels were significantly reduced 24 h after weak conditioning and 2 h after strong conditioning. In contrast to the decreased levels mentioned before, 24 h after strong conditioning, the miR-124 amount is significantly upregulated. The amount of miR-12 was significantly decreased 24 h after strong training.

To specify the role of miR-12, miR-124 and miR-125 in learning and memory I wanted to create a loss of function situation. In contrast to knock-out models, where a whole gene can be knocked out, the use of a transient manipulator can be of greater benefit in studying dynamic processes. MiRNA-Inhibitors provide a perfect tool for a transient manipulation of the miRNA of interest. Therewith an inhibition, specific to one special miRNA and applicable at different points in time is possible and is used in this study.

4.5.1. Appetitive olfactory conditioning and points in time for miRNA-Inhibitor treatment

To define the role of miR-12, -124 and -125 in acquisition and consolidation, different points in time for the treatment with miRNA-Inhibitor were tested. As there are no studies addressing the time dependent on and offset effects of miRNA-Inhibitors *in vivo* in detail, I decided to test five different points in time and their individual effects on acquisition and memory consolidation. Cristino et al. 2014 showed, that miRNA-Inhibitor treatment 1 d before conditioning had effects on the memory of honeybees 24 h after strong conditioning. Shaw et al. 2001 described the negative effects of lipopolysaccharide (LPS) on spatial learning in mice, when applied 4 h before the training. Gong et al. 2016 applied DNA methyltransferase inhibitor 1 h before conditioning to honeybees, to test the effects on acquisition. Thus, the treatment with miRNA-Inhibitors 1 d, 4 h and 1 h prior to conditioning were chosen to test their effects on acquisition in this work. The

application of miR-125-Inhibitor (Backer 2015) 1 h after strong conditioning resulted in improved memory 2 h and 24 h after conditioning. The use of translation blocking reagent anisomycin (ANI) (Felsenberg 2011) 1 h after conditioning and the transcription blocker actinomycin D (ACT-D) 1 h and 6 h after conditioning (Wüstenberg et al. 1998) has been studied in honeybees to test their effects on the consolidation phase. Hence, the points in time 1 h and 6 h after conditioning were chosen to reveal the early and late effects of miRNA-Inhibitor treatment on the consolidation phase. The inhibitors were applied by thorax injection into the hemolymph of the animal and the memory recall was performed 2 h, 24 h and in some cases also 48 h after conditioning. The points in time for the miRNA-Inhibitor treatments were chosen according to the following criteria.

4.5.1.1. Time line for miRNA-Inhibitor treatment

a. 1 d before conditioning, based on other studies (Martin 2011; Cristino et al. 2014)
b. 4 h before conditioning to test the effects on the acquisition phase (Shaw et al. 2001)
c. 1 h before conditioning to test effects on the acquisition phase and on the early consolidation phase (Gong et al. 2016; Heidtmann 2010)
d. 1 h after conditioning (Felsenberg 2011; Backer 2015; Wüstenberg et al. 1998) to test the effects on the consolidation phase exclusively
e. 6 h after conditioning (Wüstenberg et al. 1998) to study the effects on the late consolidation phase

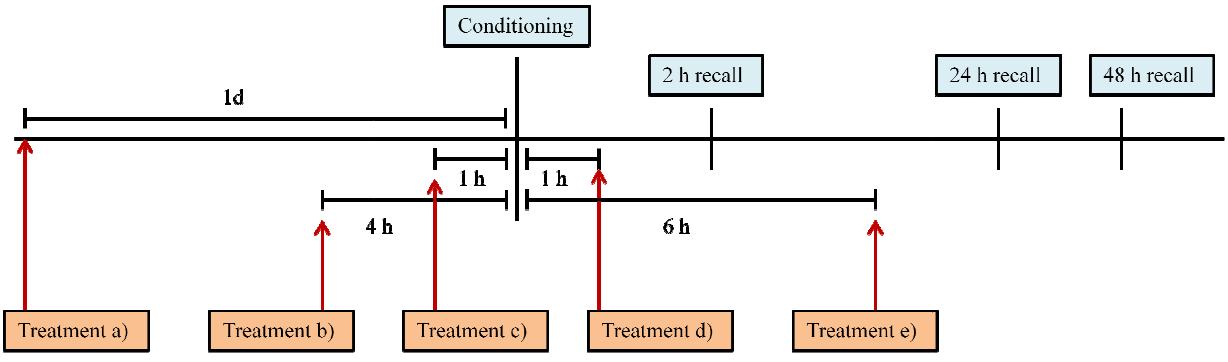

Figure 19 Time line for miR-Inhibitor treatment

Honeybees were treated with miR-Inhibitor either 1 d, 4 h or 1 h before the conditioning, or 1 h or 6 h after the conditioning. The memory recall was then carried out 2 h, 24 h and in some cases also 48 h after the conditioning.

4.5.2. The effects of miR-12 on the acquisition phase in the honeybee

4.5.2.1. MiR-12-Inhibitor treatment a): 1 d before strong conditioning

Based on (Cristino et al. 2014), who reported effects on learning and memory formation by applying miRNA-Inhibitor 1 d before conditioning, I also tested the effects of the miR-12-Inhibitor 1 d before conditioning. The bees were conditioned with three-trials of CS-US pairing and the memory was retrieved after 2 h and 24 h. The miR-12-Inhibitor treatment at this point in time had no significant effect on acquisition, learning and memory formation as indicated in table 20.

Table 20 MiR-12-Inhibitor treatment a): 1 d before strong conditioning

Injection of miR-12-Inhibitor 1 d before three-trial Training (recall 2 h, 24 h). Bees (numbers in parentheses) were injected with miR-12-Inhibitor (0,5µM) or PBS. The 2. and the 3. trial describe the PER [%] to the clove odour.

Time of injection: 1 d before strong (three-trial) conditioning	2. trial:	3. trial:	Recall: 2 h	Recall: 24 h
PBS [PER %] (n= 39)	17,9%	38,5%	64,1%	46,2%
MiR-12-Inhibitor [PER %] (n= 29)	6,9%	31%	44,8%	27,6%
Chi Square, Fisher's exact	p= 0,28	p= 0,61	p= 0,14	p= 0,13

4.5.2.2. MiR-12-Inhibitor treatment b): 4 h before weak and strong conditioning

To test, whether the miR-12 is implicated in the acquisition phase, the bees were treated with miR-12-Inhibitor 4 h before the conditioning. As compared to the control group, the miR-12-Inhibitor treated group shows a decrease of memory 2 h (PER 2 h: p= 0,0009 Chi Square, Fisher's exact test) and 24 h (PER 24 h: p= 0,03 Chi Square, Fisher's exact test) after weak

conditioning. For the strong training we can see a similar pattern after 24 h, but not after 2 h (see figure 20). The miR-12-Inhibitor treated group shows impaired memory after 24 h (PER 2. trial: p= 0,55; PER 3. trial: p= 0,78; PER 2 h: p= 0,33; PER 24 h: p= 0,0007 Chi Square, Fisher's exact test).

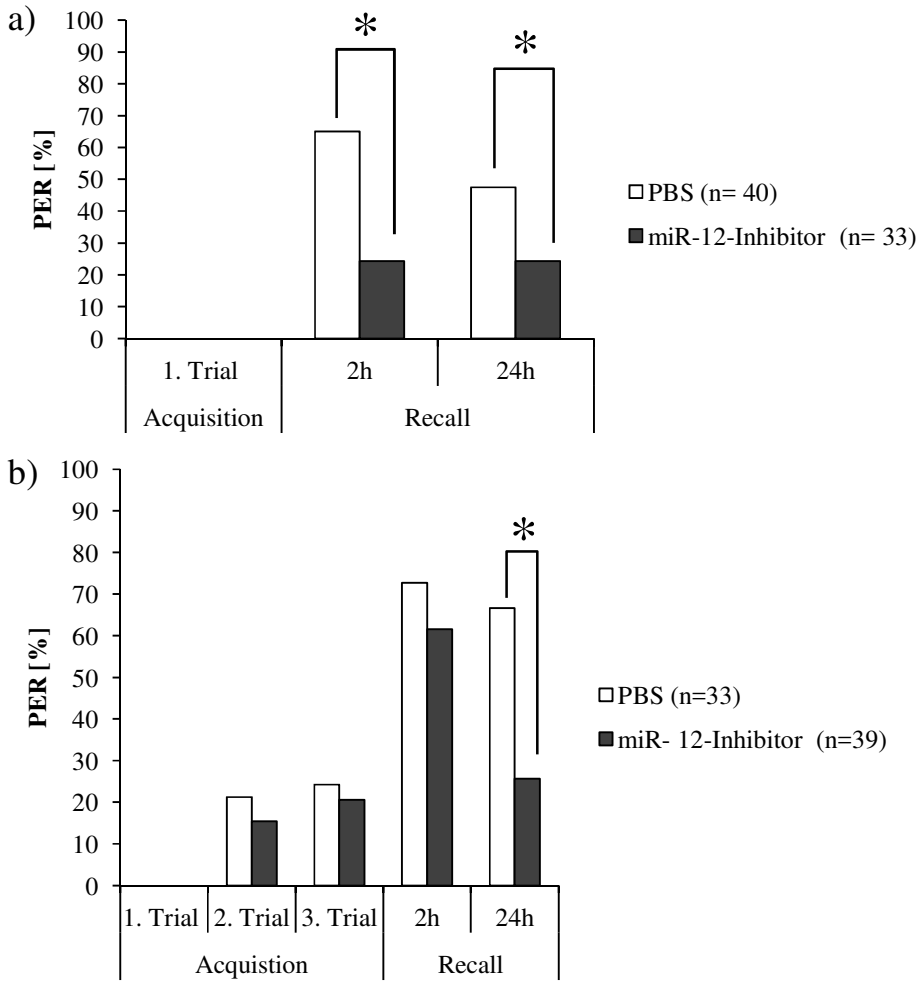

Figure 20 MiR-12-Inhibitor treatment 4 h before weak and strong conditioning

Percentage of animals that showed a PER during weak (a) or strong (b) conditioning and memory recall. Significant differences (details in text) are marked with stars (*p≤ 0,05). The bees (numbers in parentheses) were injected with either PBS or miR-12-Inhibitor into the thorax 4 h before the conditioning.

Non-associative learning and gustatory sensitivity 4 h after miR-12-Inhibitor treatment were also tested and there was no detectable significant difference between the miR-12-Inhibitor injected and the control bees (see table 21). Thus miR-12-Inhibitor specifically affects processes

implicated in associative learning but is not affecting the acquisition phase. There was no effect on non-associative learning and gustatory sensitivity by injection of the miR-12-Inhibitor.

Table 21 Non-associative learning and gustatory sensitivity 4 h after miR-12-Inhibitor treatment

Bees (numbers in parentheses) were injected with miR-12-Inhibitor or PBS 4 h before testing gustatory sensitivity, habituation and sensitisation. The gustatory sensitivity scores were compared with Mann Whitney *U*-Test. The numbers of stimuli during habituation were statistically analysed using the Student's t-test: (two tailed). The percentages of sensitised bees during sensitisation were analysed by Chi Square Fisher's exact test (two tailed).

Behavioral Test	PBS	miR-12-Inhibitor	Statistic data
Gustatory sensitivity	1,68 (n= 68)	1,69 (n= 66)	U= 2257, P= 0,95
Habituation	1±0,69 (n= 22)	0,95±0,83 (n= 23)	df= 43, t= 0,21, P= 0,83
Sensitisation	3,8% (n= 26)	0% (n= 27)	X^2= 0,14, P= 0,49

4.5.2.3. MiR-12-Inhibitor treatment c): 1 h before strong conditioning

To investigate, whether the treatment with miR-12-Inhibitor has a direct effect on the acquisition phase during learning, the miR-12-Inhibitor was injected 1 h before the strong conditioning and the memory was retrieved 2 h, 24 h and 48 h after training. The injection of miR-12-Inhibitor 1 h before the three-trial training did not have significant effects on acquisition, learning or memory (see table 22).

Table 22 MiR-12-Inhibitor treatment c): 1 h before strong conditioning

Bees (numbers in parentheses) were injected with miR-12-Inhibitor or PBS 1 h before conditioning. The 2. and the 3. trial describe the PER [%] in response to the clove odour.

Time of injection: 1 h before strong (three-trial) conditioning	2. trial:	3. trial:	Recall: 2 h	Recall: 24 h	Recall: 48 h
PBS [PER %] (n= 53)	32,1%	47,2%	79,2%	49,1%	52,8%
MiR-12-Inhibitor [PER %] (n= 62)	46,8%	53,2%	74,2%	37,1%	41,9%
Chi Square, Fisher's exact	p= 0,12	p= 0,57	p= 0,65	p= 0,25	p= 0,26

Gustatory sensitivity 0,5 h and 1,5 h after miR-12-Inhibitor treatment was also tested. There was no significant difference between the miR-12-Inhibitor injected and the control bees (see table 23). Hence, miR-12-Inhibitor treatment does not affect the acquisition phase and the early consolidation phase and has no influence on the gustatory sensitivity.

Table 23 MiR-12-Inhibitor treatment does not affect gustatory sensitivity

Bees (numbers in parentheses) were injected with miR-12-Inhibitor (0,5 µM) or PBS before testing gustatory sensitivity. The gustatory sensitivity scores were compared with Mann Whitney *U*-Test.

Behavioral Test	PBS	miR-12-Inhibitor	Statistic data
Gustatory sensitivity 0,5 h after treatment	1,73 (n= 42)	1,9 (n= 40)	*U*= 840, *P*= 1
Gustatory sensitivity 1,5 h after treatment	1,64 (n= 42)	1,67 (n= 40)	*U*= 844, *P*= 0,97

Injection of miR-12-Inhibitor 1 d prior to conditioning did not affect acquisition, learning and memory formation. The treatment 4 h before learning was conducted to test the effects of the miR-12-Inhibitor on the acquisition phase; it does not change acquisition but impairs memory after weak as well as after strong conditioning while gustatory sensitivity, habituation and sensitisation are not affected. To test the effects on the acquisition phase and on the early consolidation phase, the miR-12-Inhibitor was injected 1 h before conditioning. MiR-12-Inhibitor treatment does not affect the acquisition phase and the early consolidation phase after strong conditioning and has also no influence on the gustatory sensitivity at this point in time.

4.5.3. The effects of miR-12 on the consolidation phase in the honeybee

4.5.3.1. MiR-12-Inhibitor treatment d): 1 h after weak and strong conditioning

To test, whether the miR-12-Inhibitor treatment affects the early consolidation phase after learning, the treatment was conducted 1 h after weak and strong conditioning. The memory recalls were carried out 2 h, 24 h and 48 h after training.

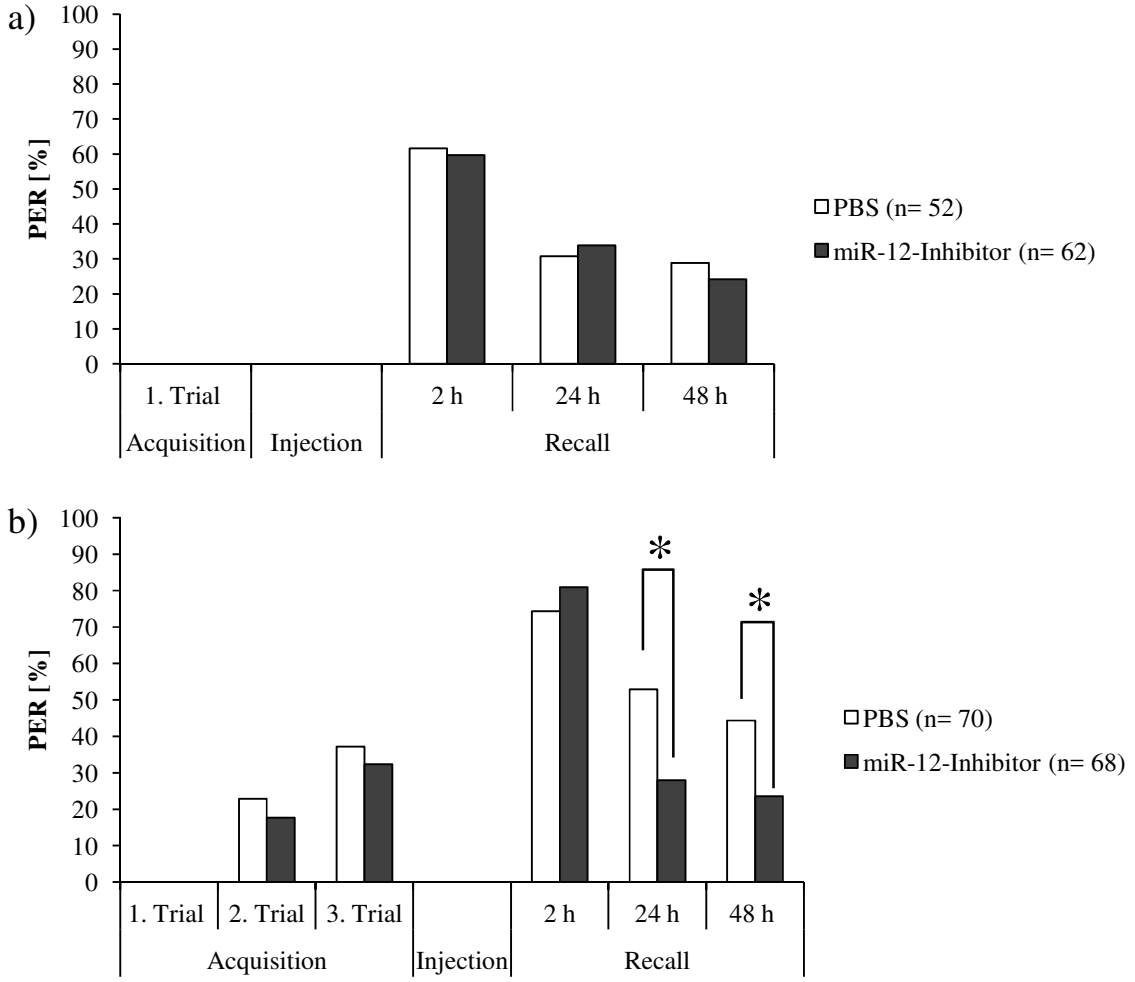

Figure 21 MiR-12-Inhibitor treatment 1 h after weak and strong conditioning

The figure displays the percentage of honeybees showing a PER during weak (a) or strong (b) conditioning and memory recall. Significant differences (details in the text) are marked with stars (*p≤ 0,05). The bees (numbers in parentheses) were injected with PBS or miR-12-Inhibitor 1 h after the conditioning.

The treatment with miR-12-Inhibitor 1 h after weak (single-trial) conditioning had no significant effects on memory formation (PER 2 h: p= 0,85; PER 24 h : p= 0,84; PER 48 h : p= 0,67 Chi Square, Fisher's exact test). The miR-12-Inhibitor-treated bees showed significantly impaired memory 24 h (PER 2 h: p= 0,41; PER 24 h: p= 0,003 Chi Square, Fisher's exact test) and 48 h (PER 48 h: p= 0,012 Chi Square, Fisher's exact test) after strong conditioning (see figure 21 b)). In conclusion, the miR-12-Inhibitor does affect the consolidation phase exclusively after strong conditioning.

4.5.3.2. MiR-12-Inhibitor treatment e): 6 h after strong conditioning

The miR-12-Inhibitor was injected 6 h after strong conditioning to test its effects on the late consolidation phase. The memory recall was carried out 2 h, 24 h and 48 h after training. The treatment with miR-12-Inhibitor 6 h after strong conditioning (see table 24) had no significant effects on memory formation.

Table 24 MiR-12-Inhibitor treatment e): 6 h after strong conditioning

Bees were injected with PBS or miR-12-Inhibitor 6 h after strong conditioning (recall 2 h, 24 h, 48 h). PBS treated animals (n= 58) and miR-12-Inhibitor treated animals (n= 54) did not show significant differences.

Time of injection: 6 h after strong (three-trial) conditioning	Recall: 2 h	Recall: 24 h	Recall: 48 h
PBS [PER %] (n= 58)	67,2%	50%	50%
MiR-12-Inhibitor [PER %] (n= 54)	70,4%	57,4%	57,4%
Chi Square, Fisher's exact	p= 0,83	p= 0,45	p= 0,45

When applied 1 h after strong conditioning, the miR-12-Inhibitor does affect the consolidation phase exclusively. There were no effects of miR-12-Inhibitor treatment 1 h after weak conditioning and no effects on the late consolidation phase when applied 6 h after conditioning. Summarised, the miR-12-Inhibitor specifically affects processes implicated in associative learning and consolidation.

Since the miR-12-Inhibitor treatment 4 h before conditioning resulted in impaired memory 2 h and 24 h after weak training and 24 h after strong training, I can conclude that the miR-12 is involved in the positive regulation of processes during acquisition. The effects of miR-12-Inhibitor injection 1 h after strong conditioning led to impaired memory after 24 h and 48 h. This reveals a positive function of miR-12 in the consolidation phase.

4.5.4. The effects of miR-124 on the acquisition in the honeybee

In q-RT-PCR experiments we showed that the level of miR-124 was downregulated one day after weak training and 2 h after strong training but upregulated one day after strong training. This points to potential different functions of miR-124 with respect to training strength and memory phase.

4.5.4.1. MiR-124-Inhibitor treatment a): 1 d before weak and strong conditioning

Cristino et al. 2014 reported effects on learning and memory formation by applying miRNA-Inhibitor 1 d before conditioning. To test the effects with miR-124-Inhibitor at this point in time, the treatment was conducted 1 d before the weak and strong conditioning experiments. Weak conditioning 1 d after miR-124-Inhibitor treatment had no significant effect on memory (PER: 2 h p= 1; 24h p= 0,089; Chi Square, Fisher's exact test) (see figure 22 a).

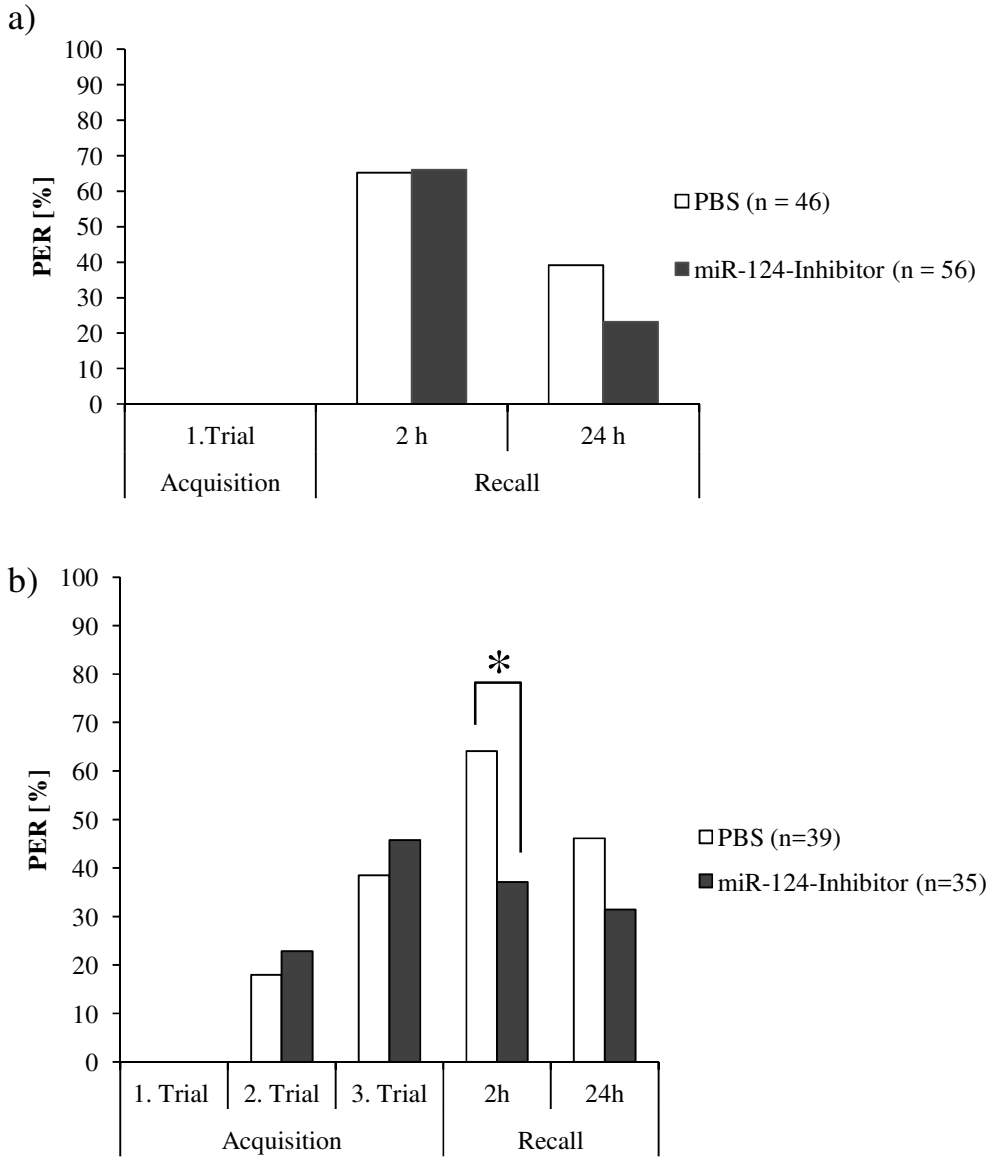

Figure 22 Weak and strong conditioning 1 day after treatment with miR-124- Inhibitor

The figure displays the percentage of honeybees showing a PER during weak (a) and strong (b) conditioning and memory recall. Differences (details in text) are marked with stars (*p≤ 0,05). Bees (numbers in parentheses) were injected 1 d before the conditioning with miR-124-Inhibitor or PBS.

Treatment with miR-124-Inhibitor 1 d before strong conditioning resulted in impaired 2 h memory for the treated animals. As figure 22 b) shows, there is no difference in acquisition for the 2. and the 3. trial. (PER 2. trial: p= 0,77; PER 3. trial: p= 0,63, Chi Square, Fisher's exact test). The PER after the 2 h recall but not the 24 h recall is significantly lower in the miRNA-Inhibitor treated group (PER 2 h: p= 0,035; PER 24 h: p= 0,23 Chi Square, Fisher's exact test).

There was no significant effect of miR-124-Inhibitor treatment 1 d before habituation, sensitisation and gustatory sensitivity see table 25.

Table 25 MiR-124-Inhibitor treatment a): 1 d before gustatory sensitivity, habituation and sensitisation

Bees (numbers in parentheses) were injected with miR-124-Inhibitor (0,5µM) or PBS 1d before testing gustatory sensitivity, habituation and sensitisation. The gustatory sensitivity scores were compared with Mann Whitney *U*-Test. The numbers of stimuli during habituation were statistically analysed using the Student's t-test. The percentages of sensitised bees during sensitisation were analysed by Chi Square Fisher's exact test.

Behavioral Test	PBS	miR-124-Inhibitor	Statistic data
Gustatory sensitivity	3,00 (n= 43)	2,53 (n= 43)	U= 800,5,, P= 0,28
Habituation	1±0,7 (n= 69)	1,02±0,7 (n= 53)	df= 120, t= -0,17, P= 0,51
Sensitisation	3,9% (n= 51)	1,9% (n= 51)	X^2= 0, P= 1

4.5.4.2. MiR-124-Inhibitor treatment b): 4 h before weak and strong conditioning

The miR-124-Inhibitor treatment was carried out 4 h before conditioning to test the effects on the acquisition phase. The miR-124-Inhibitor was injected 4 h before weak and strong conditioning and the memory recall was performed 2 h and 24 h after conditioning. The treatment with inhibitor impairs the memory 2 h after weak conditioning (PER 2 h: p= 0,03 Chi Square, Fisher's exact test). There is no significant change between the treated and the control group after 24 h (PER 24 h: p= 1, Chi Square, Fisher's exact test).

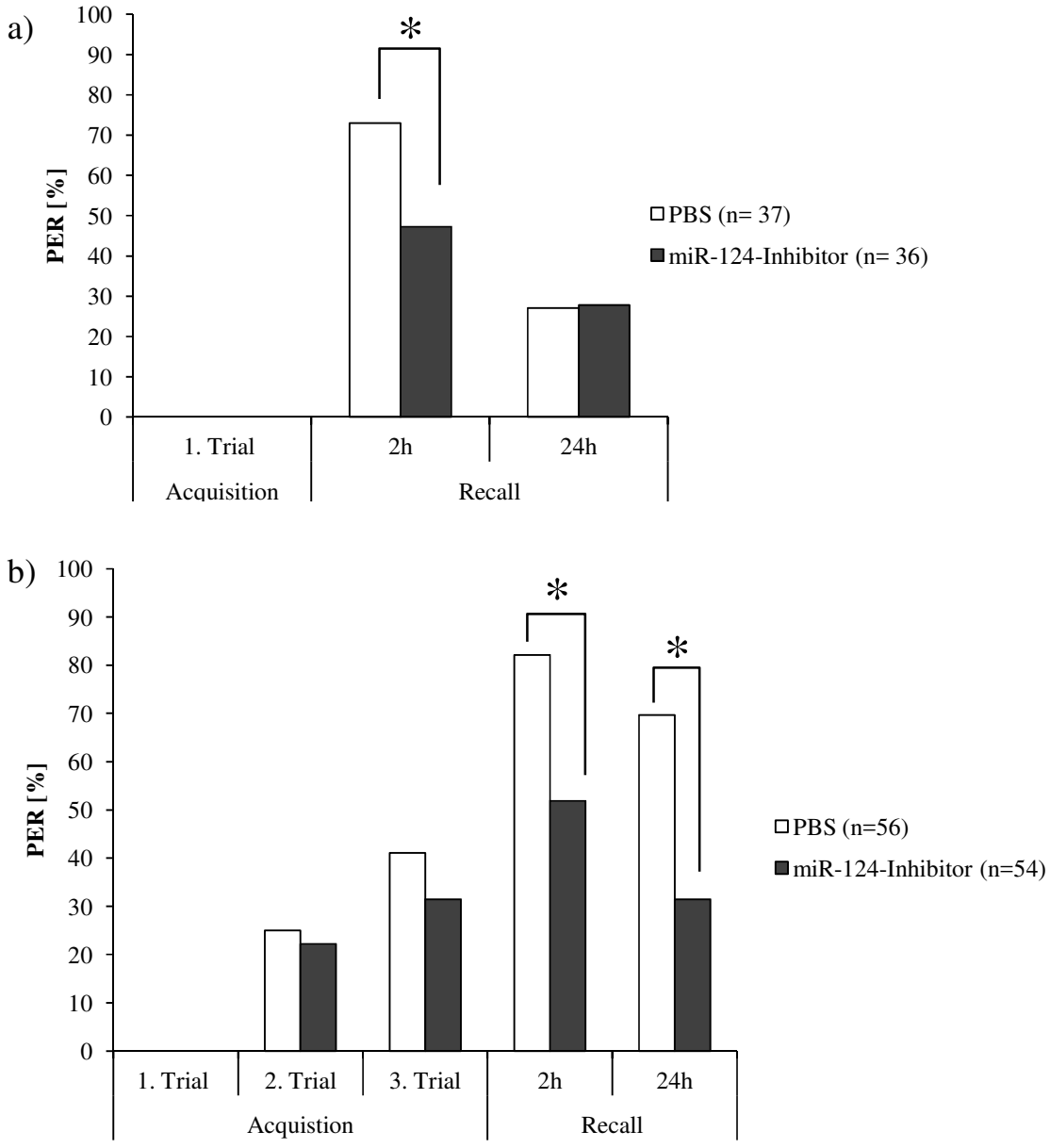

Figure 23 Weak and strong conditioning 4 h after treatment with miR-124-Inhibitor

The figure displays the percentage of honeybees showing a PER during weak (a) and strong (b) conditioning and memory recall. Differences (details in text) are marked with stars (*p≤ 0,05). Bees (numbers in parentheses) were injected 4 h before the conditioning with miR-124-Inhibitor or PBS.

The animals were conditioned with three-trials (strong training) (see figure 23). The miR-124-Inhibitor was injected 4 h before conditioning and the memory recall was performed 2 h and 24 h after conditioning. There is no difference in acquisition for the 2. trial (PER 2. trial: Chi Square, Fisher's exact test, p= 0,82) and the 3. trial (PER 3.Trial: Chi Square, Fisher's exact test, p= 0,33)

and the PER after the 2 h and the 24 h recall is significantly lower in the treated group (PER: 2 h: p= 0,00005; PER 24 h: p= 0,0001 Chi Square, Fisher's exact test). The miR-124-Inhibitor treatment had no significant effects on acquisition. It also had no effects on other behavioural tests like habituation, sensitization or gustatory sensitivity (see table 26).

Table 26 MiR-124-Inhibitor does not affect non-associative learning and gustatory sensitivity

Bees (numbers in parentheses) were injected with miR-124-Inhibitor or PBS 4 h before testing gustatory sensitivity, habituation and sensitisation. The gustatory sensitivity scores were compared with Mann Whitney *U*-Test. The numbers of stimuli during habituation were statistically analysed using the Student's t-test. The percentages of sensitised bees during sensitisation were analysed by Chi Square Fisher's exact test.

Behavioral Test	PBS	miR-124-Inhibitor	Statistic data
Gustatory sensitivity	1,68 (n= 68)	1,88 (n= 62)	U= 2211,, P= 0,51
Habituation	1±0,69 (n= 22)	1,15±0,90 (n= 23)	df= 43, t= 0,65, P= 0,51
Sensitisation	3,8% (n= 26)	3,8% (n= 26)	X^2= 0, P= 1

The treatment with miR-124-Inhibitor 1 d prior to weak conditioning did not have effects on acquisition and memory formation. However, the treatment with miR-124-Inhibitor 1 d prior to strong conditioning resulted in an impaired memory 2 h after but had no effect on the acquisition. The effects of miR-124-Inhibitor 4 h before weak and strong conditioning did not affect the acquisition significantly but impaired the 2 h memory and additionally the 24 h memory after strong conditioning. I conclude, that miR-124 has a positive function in the acquisition phase.

4.5.5. The effects of miR-124 on the consolidation phase in the honeybee

4.5.5.1. MiR-124-Inhibitor treatment 1 h after conditioning

The animals were conditioned with one trial (weak conditioning) or with three-trials (strong training) (see table 27). The treatment with miR-124-Inhbitor or PBS was carried out 1 h after the conditioning to test the effects on the consolidation phase. There were no significant effects between the two groups, neither after weak nor after strong conditioning.

Table 27 Weak and strong conditioning 1 h after miR-124-Inhibitor treatment

Bees (numbers in parentheses) were injected with miR-124-Inhibitor or PBS 1 h after weak or strong conditioning (recall 2 h, 24 h, 48 h).

Time of injection: 1 h after strong (three-trial) conditioning	Recall: 2 h	Recall: 24 h	Recall: 48 h
PBS [PER %] (n= 51)	68,6%	64,7%	52,9%
MiR-124-Inhibitor [PER %] (n= 60)	53,3%	63,3%	36,7%
Chi Square, Fisher's exact test	p= 0,12	p= 1	p= 0,12
1 h after weak (single-trial) conditioning			
PBS [PER %] (n= 54)	59,1%	24,3%	/
MiR-124-Inhibitor [PER %] (n= 55)	63,6%	40%	/
Chi Square, Fisher's exact test	p= 0,69	p= 0,10	/

The treatment with miR-124-Inhibitor 1 h after conditioning had no significant effects on memory formation. Therefore, I suppose, that the miR-124 is not affecting the consolidation phase. Because of its positive function in the acquisition phase, I define it as a positive regulator of learning processes.

4.5.6. The effects of miR-125 on the acquisition in the honeybee

4.5.6.1. MiR-125-Inhibitor treatment b) 4 h before conditioning

Although no learning induced changes in miR-125 levels were observed, I tested if miR-125 function is required for memory acquisition. To test the effect of miR-125-Inhibitor on the acquisition phase, the bees were treated 4 h before conditioning.

Treatments with miR-125-Inhibitor 4 h before weak and strong conditioning show no significant effects. For the weak training we can see a pattern similar to the strong training after 2 h but not after 24 h (2 h PER: p= 0,31 Chi Square, Fisher's exact test; 24 h PER: p= 0,64 Chi Square, Fisher's exact test) and a trend to improved memory in the miRNA-Inhibitor treated group after 48 h (48 h PER: p= 0,065 Chi Square, Fisher's exact test)(see figure 24 a)).

There is no difference in acquisition for the 2. trial (PER 2. trial: p= 0,82 Chi Square, Fisher's exact test) and the 3. trial (PER 3.trial: p= 0,3 Chi Square, Fisher's exact test). The miRNA-Inhibitor treated group shows a trend to decreased memory 24 h after strong conditioning (PER 2 h: p= 1; PER 24 h: p= 0,09 Chi Square, Fisher's exact test)(see figure 24 b)).

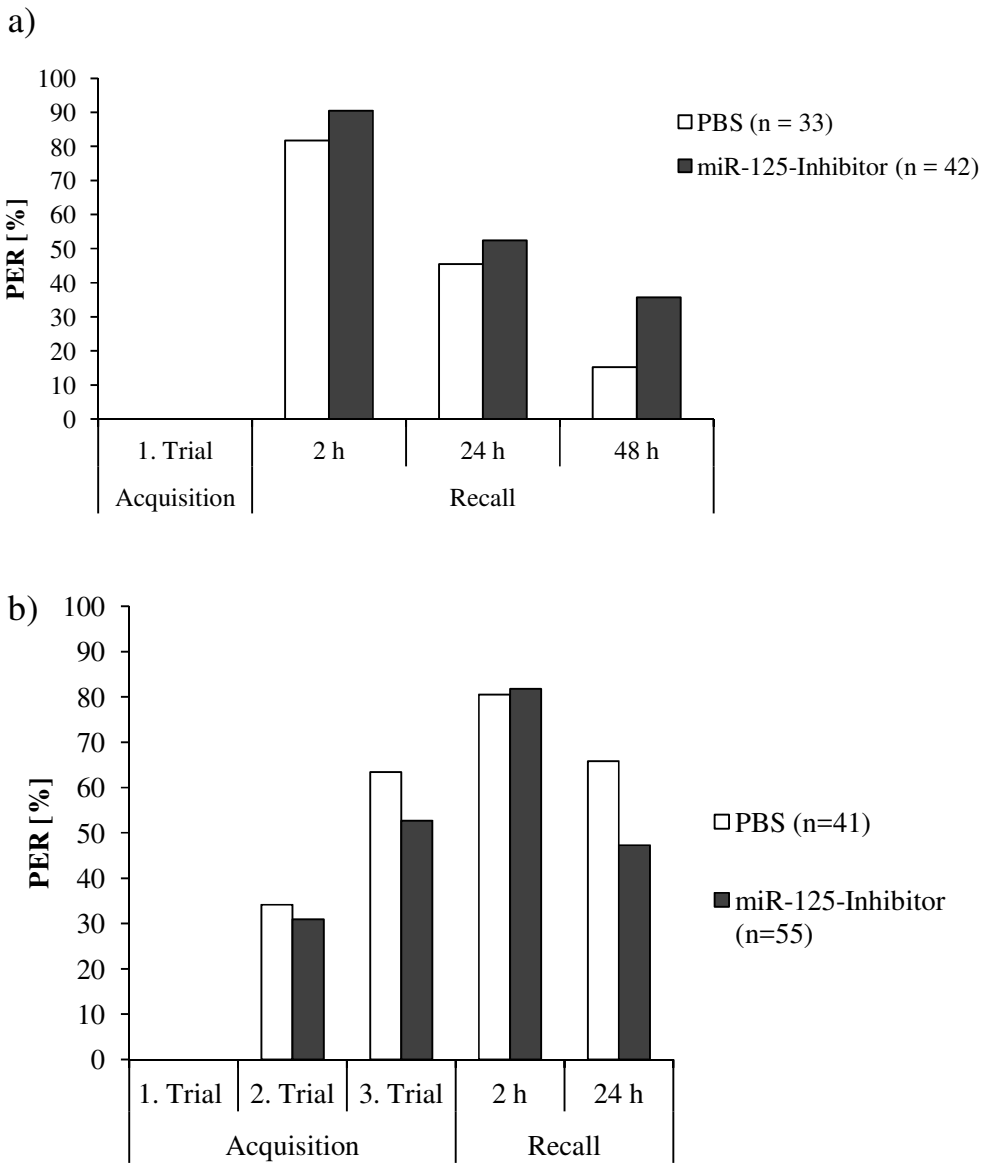

Figure 24 Weak and strong conditioning 4 h after miR-125-Inhibitor treatment

The figure displays the percentage of honeybees showing a PER during weak (a) and strong (b) conditioning and memory recall. Bees (numbers in parentheses) were injected 4 h before the conditioning with miR-125-Inhibitor or PBS.

There were no significant changes in responsiveness and sensitisation 4 h after miR-125-Inhibitor treatment (see table 28).

Table 28 MiR-125-Inhibitor does not affect non-associative learning and gustatory sensitivity

Bees (numbers in parentheses) were injected with miR-125-Inhibitor or PBS 4 h before testing gustatory sensitivity, habituation and sensitisation. The gustatory sensitivity scores were compared with Mann Whitney *U*-Test. The numbers of stimuli during habituation were statistically analysed using the Student's t-test. The percentages of sensitised bees during sensitisation were analysed by Chi Square Fisher's exact test.

Behavioral Test	PBS	miR-125-Inhibitor	Statistic data
Gustatory sensitivity	3,28 (n= 43)	4 (n= 43)	U= 1035,5, P= 0,34
Habituation	1±0,71 (n= 26)	1,16±0,60 (n= 21)	Df= 45, t= 0,84, P= 0,41
Sensitisation	23% (n= 30)	22% (n= 27)	X^2= -0,01, P= 1

The acquisition phase was not affected by miR-125-Inhibitor treatment, neither were the gustatory sensitivity, habituation and sensitisation. The treatment with miR-125-Inhibitior 4 h prior to conditioning had also no influence on the 2 h, 24 h or 48 h memory, neither after weak, nor after strong conditioning (2 h, 24 h were tested). Therefore I conclude that the miR-125 does not have a regulatory influence the acquisition phase.

4.6. Interaction between miRNAs are revealed by inhibition of single miRNAs

4.6.1. Analysis of miRNA amount in the central brain 2 h after miR-12-Inhibitor treatment

The correlation analysis performed with the q-RT-PCR data showed, that miR-124, miR-12 and miR-125 are connected. To determine how treatment with single miRNA-Inhibitors affect the levels of other miRNAs in the central brain of the honeybee, a quantification of EF 1 α-, miR-12-, miR-124-, miR-125-, miR-989-, and miR-3788- levels was carried out by q-RT-PCR 2 h after treatment with miR-12-Inhibitor.

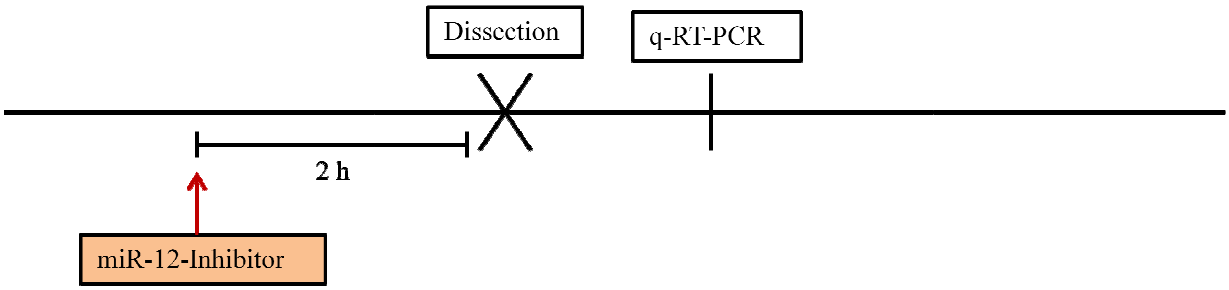

Figure 25 MiR-Inhibitor treatment 2 h before central brain dissection and q-RT-PCR

Honeybees were treated with miR-12-Inhibitor 2 h before the dissection of the central brain with subsequent quantification of isolated miRNA and mRNA by q-RT-PCR.

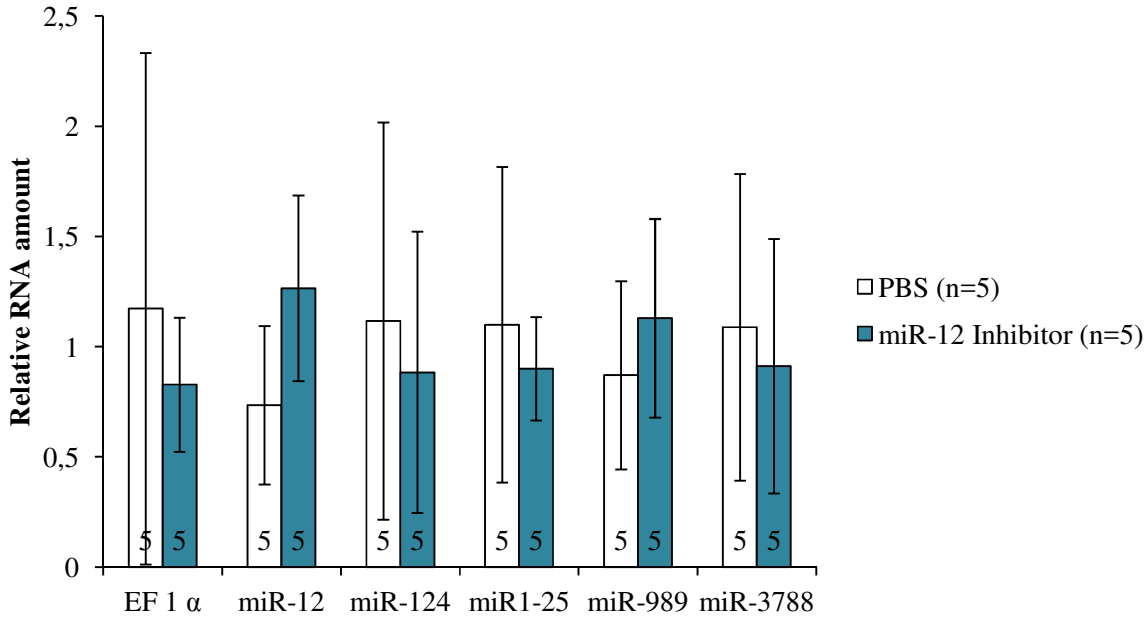

Figure 26 MiR-12-Inhibitor treatment, analysis of central brain miR-12, miR-124, miR-125, miR-989, miR-3788-levels after 2 h by q-RT-PCR

The columns display the relative amount of RNA (EF 1α) and miRNAs (miR-12, miR-124, miR-125, miR-989 and miR-3788) in the central brain of the honeybee. Animals were treated with either miR-12-Inhibitor or PBS 2 h before dissection. The diagrams display the relative mean values of the RNA and miRNA amounts and their standard deviation. Significant differences are marked with stars (*p≤ 0,05). The number in the basis of each bar indicates the number of samples.

The miR-12 amount shows a trend to an increase compared to the control group (miR-12-Inhibitor: p= 0,065, Student's t-test). There were no significant effects detected (Control group (1x PBS) vs. miR-12-Inhibitor treated group (in 1x PBS) Student's t-test: EF 1 α: p= 0,55, miR-124: p= 0,65, miR-125: p= 0,57, miR-989: p= 0,37, miR-3788: p= 0,67). Thus, single treatment with miR-12-Inhibitor does not interfere with the levels of miR-12, -124, -125, -989, -3788 or EF 1 α 2 h after injection.

4.6.2. MiR-12- or miR-124-Inhibitor treatment, affects the levels of miR-12, miR-124 and miR-125 after 4 h

Since treatment with miR-12-Inhibitor showed no effect on other miRNAs 2 h later, a quantification of miR-12-, miR-124-, miR-125- levels was carried out by q-RT-PCR 4 h after treatment with miR-12-Inhibitor or miR-124-Inhibitor. As a control and reference in comparison to the miRNA amounts the housekeeping gene EF 1 α and another gene GLuA2 -which is frequently measured in our lab-, were also tested. The levels (see figure 28) of miR-12, miR-124 and miR-125 is significantly increased in the miR-124-Inhibitor treated group (black bars, n= 5). Although it is not appropriate to perform multiple statistical comparisons with small numbers, I used the Student's t-test to compare the obvious differences between the groups. Significant differences are marked by *p<0,005.

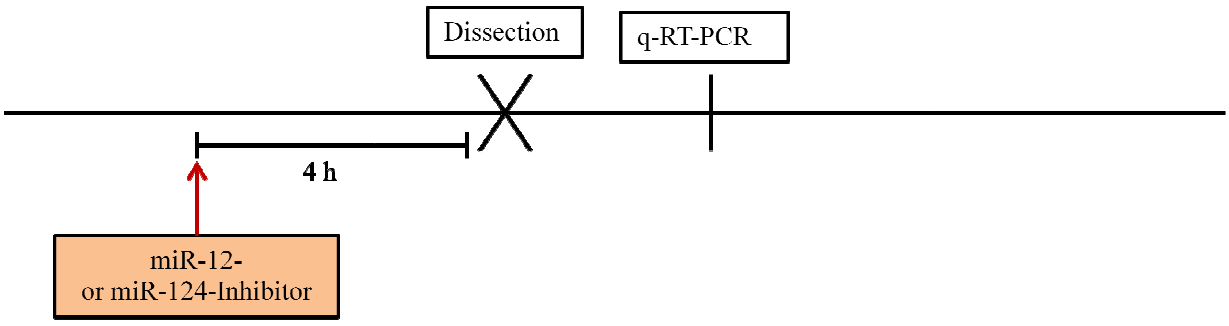

Figure 27 MiR-Inhibitor treatment 4 h before central brain dissection and q-RT-PCR

Honeybees were treated with miR-Inhibitor 4 h before the dissection of the central brain with subsequent quantification of isolated miRNA and mRNA by q-RT-PCR.

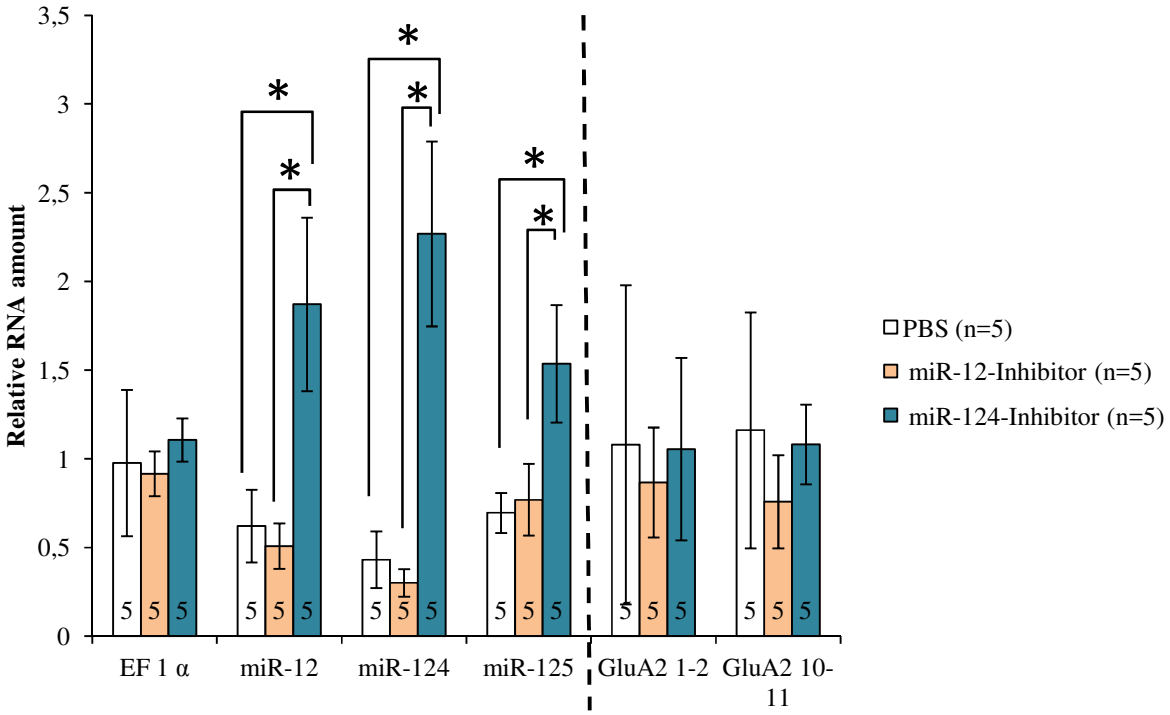

Figure 28 Relative amount of EF 1α, miR-12, miR-124, miR-125 and Glu A2 1-2 and 10-11, 4 h after treatment with miR-12- or miR-124-Inhibitor

The columns display the relative amount of miRNAs (miR-12, miR-124 and miR-125) and RNAs (EF 1α; Glu A2 1-2 and 10-11) in the central brain of the honeybee. Animals were treated with either miR-12-Inhibitor, miR-124-Inhibitor or PBS 4 h before dissection. The data is presented as the relative mean values of the RNA and miRNA amounts and their standard deviations. Significant differences are marked with stars (*p≤ 0,005). The number in the basis of each bar indicates the number of samples. The amount of miR-12, miR-124 and miR-125 is significantly increased in the miR-124-Inhibitor treated group (Student's t-test: miR-12 amount: PBS vs. miR-124-Inhibitor treated group: p= 0,0026; miR-12-Inhibitor treated vs. miR-124-Inhibitor treated group p= 0,0024; miR-124 amount: PBS vs. miR-124-Inhibitor treated group p= 0,0008; miR-12-Inhibitor treated vs. miR-124-Inhibitor treated group: p= 0,0009; miR-125 amount: p= 0,5; PBS vs. miR-124-Inhibitor treated group p= 0,0031; miR-12-Inhibitor treated vs. miR-124-Inhibitor treated group p= 0,0035).

All three miRNAs were upregulated after miR-124-Inhibitor treatment. These results support the results from the correlation analysis, which implicated a connection between miR-12, -124 and -125.

4.6.3. MiR-12-Inhibitor treatment 1h after strong conditioning, analysis of central brain miRNA-levels 24 h and 48 h after conditioning by q-RT-PCR

The treatment with miR-12-Inhibitor 1 h after strong conditioning with three-trials resulted in an impaired long-term memory 24 h and 48 h after the conditioning (see table 29 and 30). To check for altered miR-12, miR-124, miR-125, miR-989, miR-3788 levels in the central brain of the honeybee, I quantified their amounts by q-RT-PCR 24 h and 48 h after strong conditioning with three-trials and treatment with miR-12-Inhibitor 1 h after the training.

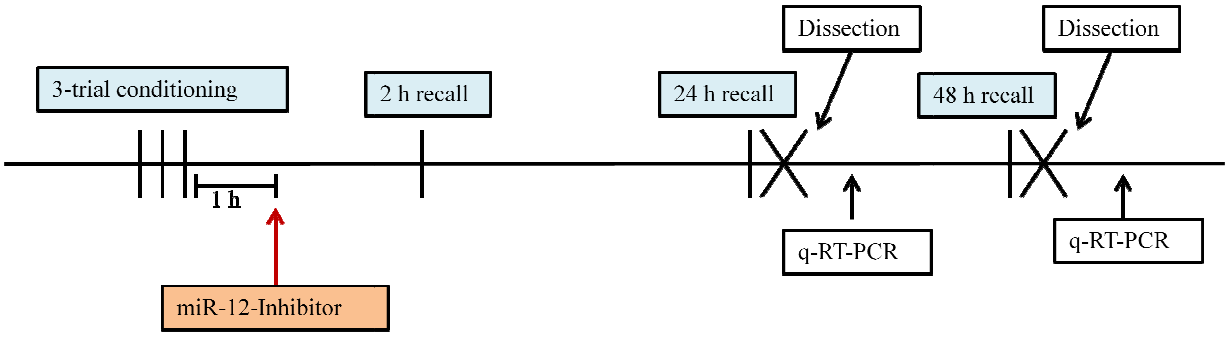

Figure 29 MiR-Inhibitor treatment 1 h after strong conditioning, after 24 h or 48 h memory recall, central brain dissection and q-RT-PCR

Honeybees were treated with miR-Inhibitor either 1 h after strong condition, a memory recall was performed at 2 h, 24 h and 48 h. The dissection of the central brain was performed either after the 24 h or after the 48 h recall with subsequent quantification of isolated miRNA and mRNA by q-RT-PCR.

The animals that responded to the recalls (2 h and 24 h) were chosen for central brain dissection and q-RT-PCR analysis in the control group (PBS n= 11). In the miR-12-Inhibitor group responders (miR-12-Inhibitor (+) n= 10) and non-responders (miR-12-Inhibitor (-) n= 7) were chosen separately. There were no significant changes in the miRNA levels (see table 29).

Table 29 MiR-12-Inhibitor treatment 1 h after strong conditioning, analysis of central brain miR-amount 24 h after conditioning by q-RT-PCR

The table displays the relative RNA/miRNA amount of strongly conditioned and miR-12-Inhibitor treated bees (numbers of bees per group are in parentheses) after the 24 h recall.

Relative RNA amount	EF 1 α	miR-12	miR-124	miR-125	miR-989	miR-3788
PBS (n= 11)	0,81±0,44	0,96±0,37	0,93±0,51	1,14±0,42	1,09±0,58	1,18±0,67
miR-12-Inhibitor (-) (n= 7)	0,6±0,45	0,88±0,56	1,07±1,32	0,97±0,57	0,92±0,69	0,96±0,25
miR-12-Inhibitor (+) (n= 10)	0,96±0,54	1,01±0,5	1,07±0,52	1,05±0,41	1,1±0,43	0,84±0,25
Statistical data						
Student's t-test: PBS vs. miR-12-Inhibitor (-)	0,34	0,72	0,79	0,52	0,60	0,35
Student's t-test: PBS vs. miR-12-Inhibitor (+)	0,52	0,82	0,54	0,61	0,97	0,14

The animals that responded to the recalls (2 h, 24 h and 48 h) were chosen for central brain dissection and q-RT-PCR analysis in the control group (n= 3). In the miR-12-Inhibitor group (n= 6), responders and non-responders (PER) were mixed.

Table 30 MiR-12-Inhibitor treatment 1 h after strong conditioning, analysis of central brain miR-levels 48 h after conditioning by q-RT-PCR

The table displays the relative RNA/miRNA amount of strongly conditioned and miR-12-Inhibitor treated bees (numbers of bees per group are in parentheses) after the 48 h recall.

Relative RNA levels	EF 1 α	miR-12	miR-124	miR-125	miR-989	miR-3788
PBS (n= 3)	0,6±0,2	0,74±0,11	0,29±0,07	0,58±0,18	0,81±0,36	0,87±0,21
miR-12-Inhibitor (+/-) (n= 6)	0,94±0,4	0,72±0,24	0,62±0,50	0,85±0,46	0,72±0,44	1,17±0,45

The combination of strong conditioning, miR-12-Inhibitor treatment and quantification of miRNA amount via q-RT-PCR did not show significant effects after the 24 h recall. The number of animals after the 48 h recall was too low to perform statistical analysis.

5. Discussion

The unravelling of highly complex cellular protein cascades and pathways which are involved in synaptic plasticity, learning and memory formation processes took us a big step towards the understanding of our brain and its functions. One remarkable aspect of miRNAs is that they influence the processes of synaptic plasticity, learning and memory formation on a posttranscriptional level. Preceding investigations characterised the relevance of miRNAs in synaptic plasticity and in the mechanisms generating memory (Vo et al. 2005; Ashraf and Kunes 2006; Ashraf et al. 2006; Schratt et al. 2006).

This work shows, that through the combination of different behavioural and molecular biological studies, the role of different honeybee miRNAs can be revealed.

In this work it has been proven for the first time, that there are learning induced changes of miR-124 and miR-3788 after weak conditioning. In addition it has been demonstrated that the miR-12 amount changes after strong conditioning.

It has also been shown that there are correlations between certain miRNAs. For example the miR-12, the miR-124 and the miR-125 were correlated and miR-989 was correlated with miR-12 and miR-124, while miR-125 was correlated with miR-3788.

Furthermore it has been demonstrated in this study that transient inhibition of miR-12- and miR-124-function affects the formation of STM and LTM in the honeybee

5.1. Weak and strong conditioning induce changes in ame-miR-12, ame-miR-124 and ame-miR-3788 levels

The analysis and quantification of the learning induced changes of the honeybee miRNAs ame-miR-12, ame-miR-124, ame-miR-125, ame-miR-989, ame-miR-3756, ame-miR-3769 and ame-miR-3788 in naive and conditioned honeybees were the first part of this work. Their levels were examined 2 h and 24 h after weak as well as after strong conditioning (see table 31). So far no other work did investigate changes in the amount of the above mentioned miRNAs after weak and strong appetitive olfactory conditioning in the honeybee *Apis mellifera.*

Qin et al. 2014 described by next generation small RNA sequencing analysis that the levels of miR-12, miR-124, miR-125 and miR-989 were increased in honeybees after maze-based visual pattern learning. They conditioned honeybees with Y-maze experiments, dissected the brains after the acquisition and analysed the levels of miRNAs by small RNA sequencing. The study of Qin et al. 2014 did not investigate the levels of miRNAs at different points in time after learning and they also did not mention the time parameters they used, while I examined the miRNA levels by q-RT-PCR, at 2 h and 24 h after weak and strong conditioning. I found an upregulation of miR-124 24 h after strong conditioning (see table 31) but no other accordance with the study of Qin et al. 2014 for the other miRNAs examined in this work. There are no works comparing the two aforementioned learning paradigms regarding the phases of learning and memory, the duration of training and the molecular mechanisms which are triggered by the different stimuli (visual and odour perception). Thus, a comparison between the study of Qin et al. 2014 and this work remains unclear and has to be scrutinised critically.

Table 31 Learning induced changes in miRNA levels

The levels of miR-12, miR-124 and miR-3788 after single trial or three trial conditioning and different points in time are displayed in this table. The arrows pointing downwards show a downregulation while the arrows pointing upwards show an upregulation of miRNA levels. The dotted arrow indicates a trend.

	1 trial 2 h	1 trial 24 h	3 trial 2 h	3 trial 24 h
miR-124	⇣	↓	↓	↑
miR-12				↓
miR-3788	↑			

5.2. The miR-12 is a positive regulator in acquisition and consolidation

The miR-12 was shown to be clustered with miR-283 and miR-304 within one single intron in *Drosophila melanogaster* (Aravin et al. 2003; Ruby et al. 2007). Lagos-Quintana et al. 2001 stated that levels of miR-12 can vary in between different developmental stages in *Drosophila melanogaster*. Another study showed, that the expression levels of miR-12 is age dependent and upregulated in old forager bees (Behura and Whitfield 2010). An upregulation of miR-12 in inactive ovaries of *Apis mellifera* virgin queens (compared to mated queens) has been described

by (Macedo et al. 2016). The miR-12 was shown to be upregulated after infection with bacteria (*Wolbachia pipientis)* in a cell line of *Aedes aegypti* (Osei-Amo et al. 2012). Those findings indicate that increase and degradation of miR-12 can change through various influences.

This work confirms that learning induces a change of miR-12 amounts. No other study investigated the levels of miR-12 at different points in time after weak and strong appetitive olfactory conditioning.A stable form of memory, also called long-term memory, is induced through the strong conditioning with three trials (Müller 2013). The formation of LTM can be divided into different phases. After the acquisition phase where the honeybees learn to associate the odour with a reward, follows a phase of memory consolidation where transcriptional processes are initiated. The resulting memory phase which is called mid-term memory lasts for 1 d and is then replaced by LTM (Müller 2013). Strong conditioning with three trials resulted in reduced miR-12 levels 24 h after the training (see table 31).

A transient manipulation of miRNA function was the next step in this work to further analyse the function of miR-12 in acquisition and consolidation. So far, no other work investigated the role of miR-12 in different memory phases in the honeybee and the transient inhibition of miR-12 was never tested before to study the effects on learning and memory formation.

The miR-12 is a positive regulator in the consolidation of LTM

Davis and Squire (1984) stated that the protein synthesis, which is necessary for an establishment of LTM, is restricted to one or two hours after training. The point in time for the miR-12-Inhibitor treatment 1 h after strong conditioning was selected to test the effects on consolidation exclusively (Wüstenberg et al. 1998; Backer 2015). This work showed that due to the loss of function of miR-12 during the consolidation phase after strong conditioning, the memory was significantly impaired after 24 h and after 48 h (see table 32). The decrease in memory due to the loss of function of the miR-12 shows, that miR-12 is essential for development of LTM in the honeybee.

Both findings - the reduced levels of miR-12 1 d after strong conditioning and the impaired memory at 1 d and 2 d after strong conditioning and transient inhibition of miR-12 - confirm its role as a positive regulator in the consolidation of LTM.

There were no other studies investigating the transient inhibition of miR-12 in learning and only few studies examined the role of miRNAs in consolidation. A study by Dias et al. (2014) did not

inhibit the miR-34a but studied the effects of miR-34a overexpression on consolidation. Dias et al. 2014 overexpressed the miR-34a by the infusion of lentiviral miR-34a-sponges into the basolateral amygdala of mice 2 weeks before fear conditioning or habituation whereby the consolidation of fear conditioning was then tested after 24 h. They could show that the miR-34a is necessary for the consolidation of cued fear memory (Dias et al. 2014). The only parallel between this work and the work of Dias et al. (2014) is the memory retrieval test at 24 h after conditioning, whereby both studies conclude that the corresponding miRNAs are necessary for the consolidation of memory.

The decrease of miR-12 amount after strong conditioning might be caused by a regulation mechanism which can control the levels of miRNA through initiating their increase or their decay. So far, there has been no mechanism identified to be involved in the regulation of miR-12 in learning and memory formation, but it has been demonstrated, that miR-132 is regulated by the Brain-derived neurotrophic factor (BDNF) through the transcription factor CREB by increasing the miR-132 precursor expression (Vo et al. 2005).

A plausible explanation for the downregulation of miR-12 at 24 h after strong conditioning would be the interaction with a target mRNA and subsequent degradation of the miR-12- mRNA-target complex to ensure the formation of LTM. The general mechanism which leads to the formation of LTM involves the adenylyl cyclase, which is activated by Ca^{2+} or modulatory inputs, and mobilises amongst others PKA and CREB (Abel and Lattal 2001). It is likely, that miR-12 is involved in this mechanism.

The revelation of the role of miRNAs in different signalling cascades will be an important step in understanding their function in learning and memory formation. The confirmation of miRNA-mRNA target interactions would take us a bit closer to achieve that goal. Until now, there were no target interactions for the miR-12 described in the honeybee.

Table 32 Regulation of acquisition and consolidation by miR-12

Depicted in the tables are the acquisition and consolidation experimental schemes showing different points in time and strength of training. The table shows that miR-12 regulates the acquisition positively at 2 h and 24 h after weak conditioning and at 24 h after strong conditioning. It also regulates the consolidation positively after 24 h and 48 h.

miR-12	Recall 2 h	Recall 24 h	Recall 48 h
Acquisition 1 d strong	No effect	No effect	/
Acquisition 4 h weak	**Positive regulation**	**Positive regulation**	/
Acquisition 4 h strong	No effect	**Positive regulation**	/
Consolidation 1 h weak	No effect	No effect	No effect
Consolidation 1 h strong	No effect	**Positive regulation**	**Positive regulation**

MiR-12 is a positive regulator of acquisition

As described earlier, the weak conditioning with one trial leads to the formation of STM whereas the strong conditioning with three trials results in the formation of LTM and both initiate different signalling cascades (Müller 2012). The effects of a transient miR-12 inhibition on both weak and strong conditioning were carried out to specify the roles of miR-12 in different pathways that lead to the formation of either STM or LTM. To further examine the effects of transient miRNA manipulation on acquisition miR-12-Inhibitor treatment was performed 4 h before weak and strong conditioning (additionally to the treatment 1 d before conditioning which had no effects on learning and memory formation as indicated in table 32). Only one study by Shaw et al. (2001) described the negative effects of lipopolysaccharide (LPS) on spatial learning in mice, when applied 4 h before the training (Shaw et al. 2001) which is not comparable to my research because the did not investigate miRNA inhibition. The transient inhibition of miR-12 function 4 h before weak conditioning had strong effects on the 2 h memory (see table 32). Thus I can classify the miR-12 as a positive regulator in the acquisition of STM. The miR-12 inhibition 4 h before strong conditioning affected the 24 h memory recall (see table 32); therefore it can be defined as a positive regulator of acquisition in MTM.

Targets of miR-12

Eligible mechanisms for a participation of miR-12 regarding its role in the positive regulation of acquisition in STM and LTM would be the general mechanisms leading to STM or LTM. The

STM initiating mechanism starts with a stimulation of AMPA or NMDA receptors that allow Ca^{2+} to enter the postsynaptic neuron and hereby activates immediate effects of PKC, NOS, CaMKII and calcineurin amongst others (Abel and Lattal 2001). The mechanism triggering LTM involving the PKA-CREB signalling pathway was already described above.

So far, the study by Osei-Amo et al. 2012 is the only work which confirmed targets of the miR-12. They provided evidence for two target genes of the miR-12 in *Aedes aegypti,* the MCT1 and the MCM6 gene. MCM6 is a DNA replication licensing factor and MCT1 a monocarboxylate transporter. They predicted the targets for the miR-12 using the bioinformatical tool *RNA hybrid*, performed expression analysis for MCM6 and MCT1 in two cell lines derived from *Aedes aegypti* (one of the cell lines showed high miR-12 levels due to infection with the bacterium *Wolbachia pipientis*) via q-RT-PCR. They could show that MCM6 and MCT1 were downregulated in the miR-12-rich cell line and confirmed the miRNA-mRNA target interactions in cells cotransfected with GFP reporter constructs containing the target genes and miR-12 mimics.
MCT1 was described to play a role in contextual fear memory (Xu et al. 2016). Xu et al. (2016) found out, that rats which were exposed to early bisphenol A exposure showed deficits in contextual fear memory, which correlatated with decreased MCT1 protein expression and oligodentrocyte loss in the hippocampus.
As mentioned before, the targets of miR-12 in the honeybee have to be confirmed to further specify the role of those miRNAs in learning and memory formation. It seems very likely, that the miR-12 targets are involved in the regulation of acquisition of STM and in the consolidation of LTM.

5.3. The miR-124 is a positive regulator of acquisition

The neuronal miR-124 was described to be conserved from worm to human (Conaco et al. 2006; Li et al. 2010). A study by Lim et al. (2005) proved, that overexpression of miR-124 in HeLa cells shifted the mRNA expression towards a neuronal pattern. The transcription of miR-124 in the rat brain was shown to be controlled by the inhibiting transcription factor EVI1 in association

with the deacetylase HDAC1 (Hou et al. 2015). Among the miR-124 targets that were already identified are laminin γ 1 and integrin β 1which were both expressed in neuronal progenitors in the chick neural tube (Cao et al. 2007), PTBP1 which revealed a role of miR-124 in pre-mRNA splicing (Makeyev et al. 2007), the SRY-box transcription factor (Sox9) (Cheng et al. 2009) and *anachronism* in neuroblasts of *Drosophila melanogaster* (Weng and Cohen 2012). These studies indicate that the miR-124 is involved in various neuronal mechanisms and functions.

I found a downregulation of miR-124 2 h after strong appetitive conditioning followed by an upregulation of miR-124 measured after 24 h (see table 31). The ame-miR-124 levels in this work are comparable to the findings of Rajasethupathy et al. (2009) in neuronal cells of *Aplysia californica*. They found, a downregulation of miR-124 levels 2 h after 5-HT treatment and an upregulation 12 h after 5-HT treatment (Rajasethupathy et al. 2009).

Like in their study in *Aplysia* and in my work in the honeybee, Cristino et al. (2014) also showed an upregulation of the levels of miR-124 after strong conditioning in honeybees. In contrast to my work, where I dissected the central honeybee brains after the memory recalls (2 h and 24 h after conditioning), they dissected the honeybee brains after the last conditioning trial. Their conditioning method does also differ from the method used in this work. They conditioned honeybees with 12 trials over 2 days (6 trials with an inter trial interval of 10 min per day) with a mixture of 14 common floral odorants and linalool without performing a 2 h memory retrieval. In contrast I conditioned the honeybees with either one trial to trigger the formation of STM or with three trials to activate the formation of LTM, in both cases combined with a 2 h and a 24 h memory recall (Müller 2013). Despite the methodical differences, the results of the work of Rajasethupathy et al. (2009) and Cristino et al. (2014) are comparable to this study.

In this work, the ame-miR-124 showed a trend to decreased levels two hours after weak conditioning and q-RT-PCR. Additionally, the ame-miR-124 amount was significantly lower after weak conditioning and 24 h (see table 31). No other study investigated the levels of miR-124 after weak conditioning or examined the effects on short-term memory. The changes of miR-124 amount, which I found in this work, imply its function in learning and memory formation processes like acquisition, consolidation or the establishment of different forms of memory.

The learning induced changes initiated the further analysis of miR-124 functions concerning acquisition and consolidation phases of learning and memory formation. This work was the first to examine the transient inhibition of miR-124 in different memory phases in the honeybee.

The miR-124 is not involved in positive regulation of consolidation

Rajasethupathy et al. (2009) found that miR-124 impairs the formation of LTF in *Aplysia californica* by targeting CREB. They applied miR-Inhibitors to cultured neurons of *Aplysia californica* at 24 h before harvesting RNA or protein and found that miR-124 inhibition increases CREB1 levels (Rajasethupathy et al. 2009). As described above, the miR-124 levels in this work and in the studies of Rajasethupathy et al. (2009) and Cristino et al. (2014) were elevated at 24 h after strong conditioning. Comparing the observations from Rajasethupathy et al. (2009) with my findings, I suggest that miR-124 is not involved in the positive regulation of LTM. The transient inhibition of miR-124 in the consolidation phase after strong conditioning did not affect memory in this work.

I can conclude from the transient inhibition that unlike the miR-12, the miR-124 is no positive regulator of consolidation in LTM (see table 33). Consistent with this discovery are also the levels of miR-124 at 24 h after strong conditioning which were elevated contrarily to the miR-12 levels at this point in time.

The miR-124 is a positive regulator of acquisition

A study by Cristino et al. (2014) tested the effects of miR-210 and miR-932 inhibition on the acquisition phase in honeybees. The miR-932 which is conserved only in insect species (Cristino et al. 2014), was chosen for their study, because of its gene location within the intron 2 of neuroligin 2 in the honeybee (Biswas et al. 2008). Neuroligin and neurexin proteins form complexes that bridge post- and presynaptic compartments of synapses (Biswas et al. 2008). In a microarray analysis of odour conditioned bees followed by q-RT-PCR analysis, Cristino et al. (2014) revealed a connection of miR-124 and 6 other miRNAs: miR-210, miR-932, miR-34, miR-278, miR-275 and miR-928. The study of Cristino et al. (2014) cannot be directly compared to this work, because of the different experimental procedure and the other miRNAs used. They fed the animals with cholesterol conjugated AMOs against miR-210 and miR-932 1 d before strong conditioning, they strongly conditioned the honeybees with 6-trials and performed a memory recall only after 24 h. The memory of miR-932-Inhibitor treated bees was significantly decreased, while the memory of miR-210-Inhibitor treated bees was not changed significantly. The learning performance was not affected by treatment.

In this study, the inhibition of miR-124 function 1 d before strong conditioning resulted in an impaired 2 h memory while the 24 h memory was not affected (see table 33). Like in the study by Cristino et al. (2014), there was no direct effect on the learning performance. But both studies show that the learning performance was unaffected after miR-Inhibitor treatment, while the memory can be affected by inhibition of different miRNAs.

A study in mice investigated the role of the hippocampal miR-132 in memory acquisition of trace fear conditioning. The infusion of a lentivector that expressed anti-miR-132 5 days before trace fear conditioning into the hippocampus impaired acquisition, while the locomotor activity was normal before the training (R. Y. Wang et al. 2013). As in this work, Wang et al. (2013) found that the acquisition was impaired and concluded that the corresponding miRNA was essential to build it.

To further examine the effects of transient miRNA manipulation on acquisition I decided to treat the honeybees 4 h before weak and strong conditioning additionally to the treatment 1 d before conditioning which was described earlier. To my knowledge, the miR-124 inhibition 4 h before conditioning was never tested before to study the effects on learning and memory formation. Only one study by Shaw et al. (2001) described the negative effects of lipopolysaccharide (LPS) on spatial learning in mice, when applied 4 h before the training (Shaw et al. 2001) which is not comparable to my research. The miR-124-Inhibitor treatment 4 h before weak conditioning resulted in an impaired memory after 2 h (see table 33). The animals that were treated 4 h before strong conditioning showed an impaired memory after 2 h and 24 h (see table 33). For this reason I define the miR-124 as a positive regulator of acquisition. Additionally in other works miR-124 has been described to be a positive regulator of neuronal maturation, connectivity and synaptic plasticity (reviewed by McNeill and Van Vactor 2012).

The memory phases following strong conditioning in the honeybee were described as MTM (mid-term memory) which lasts for 1 d and is followed by a phase called eLTM (early long-term memory), lasting until day 3 after the conditioning, followed by lLTM (late long-term memory) (Müller 2012).

The observations from the learning induced changes in miR-124 levels and from the transient miR-124-Inhibition analysis found in this work imply that miR-124 plays a role in the early mechanisms inducing STM and LTM formation. The low miR-124 levels 2 h after weak and strong conditioning and the impaired memory 2 h after miR-124 inhibition and weak and strong conditioning suggest a role in the formation of STM and in the early mechanisms that lead to the

formation of MTM, later followed by LTM. As described earlier, the low miRNA levels suggest miRNA-mRNA target interactions and through this decay of miRNAs.

Table 33 Regulation of acquisition and consolidation by miR-124

Depicted in the tables are the acquisition and consolidation experimental schemes showing different points in time and strength of training. The table shows that miR-124 regulates acquisition positively 2 h after weak and at 2 h and 24 h after strong conditioning.

miR-124	**Recall 2 h**	**Recall 24 h**	**Recall 48 h**
Acquisition 1 d strong	**Positive regulation**	No effect	/
Acquisition 4 h weak	**Positive regulation**	No effect	/
Acquisition 4 h strong	**Positive regulation**	**Positive regulation**	/
Consolidation 1 h weak	No effect	No effect	/
Consolidation 1 h strong	No effect	No effect	No effect

Targets of miR-124

As mentioned before, targets of miR-124 in the honeybee have to be confirmed to further specify its role in learning and memory formation. For the miR-124, targets which could be involved in the acquisition of STM and MTM are conceivable interaction partners

It has been described that the transcriptional repressor REST (RE1 silencing transcription factor) which regulates a family of mouse miRNA genes is targeted by miR-124a (Conaco et al. 2006). The miRNA family regulated by REST includes the miR-9, the miR124a and the miR-132 (Conaco et al. 2006). This study implicates, that miRNAs can be regulated by their own targets.

It is conceivable that the regulation of miR-124 could be operated by signalling mechanisms which are involved in the generation of learning and memory. These interactions have to be revealed by examining the target interactions of miR-124. Until now, there were no target interactions for the miR-124 confirmed in the honeybee.

Several targets of miR-124 were confirmed in other species, some of them known to be involved in the formation of learning memory. MiR-124 was previously described to target GluA2 (AMPA-type glutamate receptor) in the hippocampus of mice (Ho et al. 2014). Ho et al. (2014) showed that miR-124 regulates GluA2 in the cell-bodies before the GluA2 protein is transported to synapses and dendrites. They were also able to locate the miR-124 in cell-bodies and dendrites

and the GluA2 mRNA in the somata (Ho et al. 2014). Kraft (2015) showed in *in situ* hybridisation experiments, that the ame-miR-124 is also localised in the somata and in the dendrites of the honeybee brain. Furthermore, it has been demonstrated, that demyelination in the hippocampus as well as impaired memory are combined with elevated miR-124 expression and low AMPA receptor levels (Dutta et al. 2014). In addition, miR-124 was described to constrain synaptic plasticity in absence of serotonin through regulation of CREB (cAMP response element-binding protein) in *Aplysia californica* (see figure 30) by binding to its 3'UTR (Rajasethupathy et al. 2009).

AMPA receptors are involved in the short-term mechanism, while CREB is a transcription factor which is important for the formation of LTM (Alberini 1999). Both could also be possible target candidates for miRNAs in the honeybee. There were eight isoforms of CREB described to exist in the honeybee (Eisenhardt et al. 2003; Eisenhardt et al. 2006). Anyway, this hypothesis at first has to be proven by different target prediction and validation methods which will be discussed later in more detail.

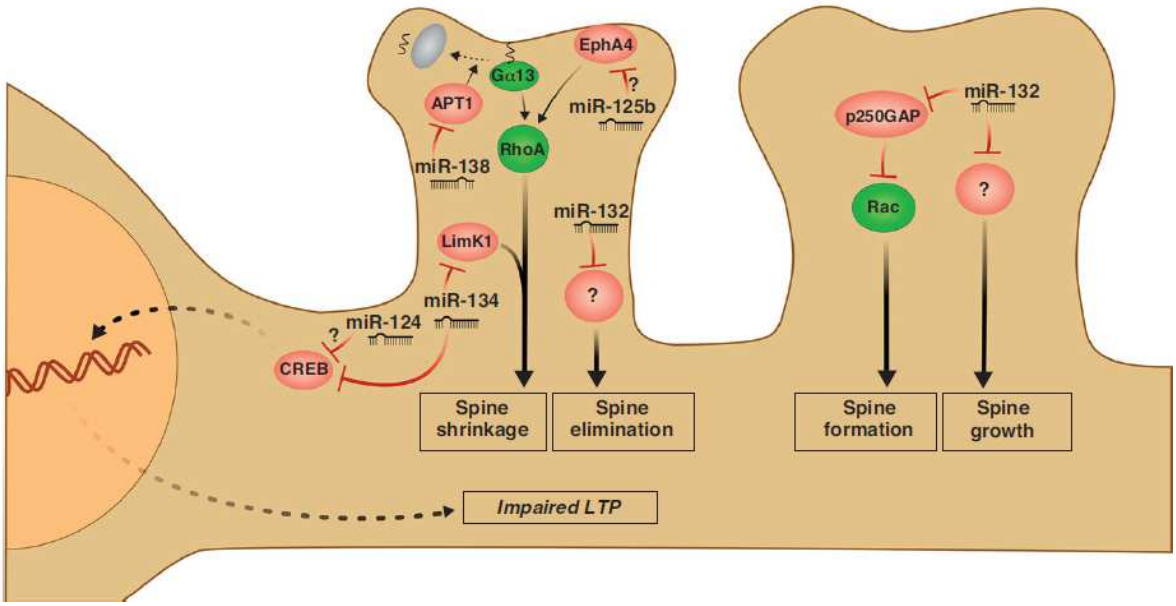

Figure 30 The roles of different miRNAs in dendrites

The number and size of dendritic spines is regulated by miRNAs such as miR-124, which can control targets like CREB that initiates long-term potentiation. The role of miR-132 in spine morphogenesis is still not clear. The miR-125b targets the Eph receptor A4 (EphA4) and through this regulates spine shrinkage. The interactions, marked with question marks still have to be validated. (Modified after Siegel et al. 2011)

5.4. The miR-125 does not affect acquisition

The honeybee miR-125 was found to be a homologue of the miR-125b. There is a differentiation in mammals between the miR-125a and the miRNA-125b. While the miR-125a regulates synaptic plasticity positively (Muddashetty et al. 2011), the miR-125b has been described as a negative regulator of maturation in neurons (McNeill and Van Vactor 2012), as a negative regulator of p53 in zebra fish and humans (Le, Teh, et al. 2009) and as a promoter of neuronal differentiation in human cells (Le, Xie, et al. 2009).

In this work I inhibited the miR-125 function 4 h before weak and strong conditioning. The transient blocking of miR-125 did not affect learning and memory formation at this point in time. Therefore I conclude that the miR-125 does not have a regulatory influence on the acquisition phase.

Studies on the consolidation phase were done by Backer (2015) in our group. By use of the same miR-125-Inhibitor 1 h after weak appetitive olfactory conditioning, a trend to increased memory 24 h after conditioning was found while injection 1 h after strong appetitive olfactory conditioning resulted in increased memory 2 h and 24 h after conditioning (Backer 2015). The findings of Backer (2015) assume that the miR-125 is a negative regulator of memory formation in honeybees.

As shown in figure 30 it was described in mice, that overexpression of miR-125b resulted in longer and thinner processes and thereby leads to spine shrinkage of hippocampal neurons via targeting the Eph receptor A4 (EphA4) (Edbauer et al. 2010). A loss of EphA4 leads to filopodia-like protrusions in neuronal cells of the hippocampus (Edbauer et al. 2010). McNeill and Van Vactor (2012) described the miR-125b to negatively regulate maturation in neurons. So far, there were no targets for the ame-miR-125 identified.

5.5. Elevated levels of miR-3788 after weak conditioning

The sequence comparisons of the human miR-132 sequence with the honeybee miRNA database resulted in one possible homologue: the miR-3788. The miR-132 was described to be a positive regulator of maturation, connectivity and synaptic plasticity in neurons (reviewed by McNeill and Van Vactor 2012). Its expression is regulated by the Brain-derived neurotrophic factor (BDNF)

through the transcription factor CREB by increasing the miR-132 precursor expression (Vo et al. 2005). Studies that investigated the overexpression of miR-132 show controversial effects on memory (Scott et al. 2012; Hansen et al. 2013). One study found, that a specific overexpression of miR-132 in the perirhinal cortex of the rat resulted in impaired short-term recognition memory associated with reduced long-term depression and long-term potentiation (Scott et al. 2012). In contrast another study by Hansen et al. (2013) found enhanced cognitive capacity while sensitively over-expressing miR-132 in the hippocampi of doxycycline regulated miR-132 transgenic mice. Peixoto et al. (2015) investigated miRNA levels before and after fear conditioning and showed downregulation of miR-219 in the mouse brain 30 min after retrieval while the miR-132, miR-212 and miR-410 were shown to be upregulated by q-PCR analysis at 30 min after acquisition and retrieval. Another study in mice investigated the role of the hippocampal miR-132 in memory acquisition of trace fear conditioning (Wang et al. 2013). They showed that the levels of miR-132 were elevated at 30 min after trace fear conditioning. The elevated miR-132 levels that were shown in these studies after conditioning are in accordance with my findings for the miR-3788 levels.

Examining the learning induced changes of ame-miR-3788 levels, I found an increase 2 h after weak conditioning (see table 31). As described in Müller (2013), the weak conditioning with 1 Trial results in the development of STM. The upregulation of miR-3788 points to a possible participation in the molecular development of short-term memory. It seems also possible, that the lower levels of miR-3788 after strong conditioning and at 2 h and at 24 h (see table 31) are caused by miR-3788-mRNA target interactions. This would suggest that the miR-3788 is involved in the formation of LTM. There are no other works addressing the ame-miR-3788 levels after learning.

Targets of miR-132, the ame-miR-3788 homologue

MiR-132 was shown to be required for spine formation and dendritic growth in the hippocampus (Magill et al. 2010). Addressing the evidence for target interactions, the miR-132 has been described to regulate spine formation positively by repressing the translation of p250GHP (see figure 30) a member of the Rho family GTPase-activating protein (Vo et al. 2005; Wayman et al. 2008). As p250GHP was shown to interact with the NMDA NR2B receptor subunit (Wayman et al. 2008) and the NMDA receptor is involved in the initiation of STM which can indirectly lead

to LTM via the adenylyl cyclase (Abel and Lattal 2001), a role of miR-132 in those mechanisms seems possible also due to its regulation through CREB (Vo et al. 2005). MiR-132 was also shown to target MeCP2 (Klein et al. 2007) which was described to be a regulator of transcription in the central nervous system and to promote hippocampal synaptic plasticity (Na et al. 2013).
To my knowledge, there are no studies that investigated target interactions for the ame-miR-3788.

5.6. Connections between miRNAs

Studies have demonstrated that miRNA-genes are clustered in families and that those can be coexpressed and also coregulated (Lau et al. 2001; Lim et al. 2003; Bartel 2004; Baskerville and Bartel 2005). The coregulation of several miRNA genes through a protein or hormone has been shown in the mouse as well as in the fruit fly. The hormone ecdysone coregulates the upregulation of miR-100, let-7 and miR-125 and the downregulation of miR-34 via the activity of the gene *Broad complex* (Sempere et al. 2003). The miR-100, let-7 and miR-125b are also clustered in mammals and the expression of let-7 family members and the miR-125b was induced in the brain of mammals in neuronal differentiation (Sempere et al. 2004). The miR-12 gene is clustered with the miR-283 and miR-304 genes in *Drosophila melanogaster* (Aravin et al. 2003) and the expression patterns of miR-12 and miR-304 are correlated very closely to each other (Ruby et al. 2007). These studies describe the coexpression and coregulation of miRNAs on the gene expression level. Coregulation and correlation on the mature miRNA levels still have to be revealed.
A correlation experiment with mature miRNA levels in this work revealed a connection between the miR-12, the miR-124 and the miR-125. The miR-125 was correlated with the miR-132 homologue miR-3788. MiR-989 the honeybee homologue I found for the miR-138 was correlated with miR-12 and miR-124. The connections between the miRNAs in this work were found by correlation analysis, using the amounts of miRNA samples quantified by q-RT-PCR. To my knowledge, there were no other studies investigating the correlations of those mature miRNAs until now.

An interaction analysis in this work revealed, that miR-12, -124 and -125 levels were elevated 4 h after single treatment with miR-124-Inhibitor (without subsequent conditioning). So far, there are no other studies addressing the changes of miRNA amounts after inhibition of other miRNAs. Regarding the results it seems conceivable, that miR-12 and miR-125 amounts are dependent on the miR-124 amount maybe because all three miRNAs are involved in the same signalling cascades in learning and memory formation. Furthermore the miR-12, miR-124 and miR-125 could be regulated by the same mechanisms. But this hypothesis has to be proven by further experimental strategies.

Beneath the analysis of target mRNAs, one approach to analyse the interactions of miRNAs would be an immunoprecipitation of miRNA associated proteins, as the Fragile-X-mental-retardation-1-protein (FMRP). FMRP has been shown to be a part of the RISC complex in *Drosophila melanogaster* as well as in mammals and to repress translation through the miRNA pathway (Y. Yang et al. 2009). Xu et al. (2008) described that the *Drosophila melanogaster* miR-124a is regulated by FMRP. As described in a study by Edbauer et al. (2010) in the mouse brain, the miR-132, miR-125b and miR-124 among other miRNAs are associated with FMRP which is working as a translational repressor. As mentioned before, the miR-124 is highly conserved through species, the miR-125b is comparable with the miR-125 in the honeybee and the search for a homologue of miR-132 in the honeybee revealed the miR-3788 as a possible candidate. Edbauer et al. (2010) confirmed the coregulation of miRNAs by coimmunoprecipitation of FMRP and associated mature miRNAs with anti-FMRP antibodies and subsequent q-RT-PCR, using FMR1 knockout mice as controls. Coimmunoprecipitation could also be used in the honeybee to further study the coregulation of miRNAs.

5.7. Outlook

In this work, AMOs were used for the transient *in vivo* inhibition of specific miRNAs. Their functionality had already been confirmed in cell cultural experiments (Lennox et al. 2013). In another *in vivo* study, the inhibition of miRNAs with AMOs that were dissolved in saline and injected into mice provided evidence for the functionality of this method (Davis et al. 2009). The use of cholesterol-conjugated (Cristino et al. 2014) or penetratin-conjugated (Rajasethupathy et al. 2009) miRNA-Inhibitors is also possible and can be conducted to ensure an improved uptake of miR-Inhibitors.

The blocking of miRNAs with AMOs depicts one possible method to define the function of the corresponding miRNA, but it also raises further questions. In this work, the quantification of miRNA levels after miRNA-Inhibition did not result in decreased miRNA levels but turned out to be unchanged or even partially increased. This problem has also been discussed in other studies, the interference of miRNA detection could for example be relativised by running the miRNA samples on a denaturing 20% formamide gel prior to quantification (Krützfeldt et al. 2005; Davis et al. 2009). A method for the quantification of free miRNA and/or bound miRNA would be necessary to gain reliable results. Another possibility is the quantification of the miRNA precursor levels at several points in time before and after the miRNA inhibition. This approach could also be helpful to gain an overview for the effectiveness of the miRNA inhibition regarding the time of effect. Rajasethupathy et al. (2009) investigated the levels of miR-124 precursors by q-RT-PCR after 5-HT treatment and found no changes but they did not inhibit the miR-124 before or after the 5-HT treatment. They concluded that regulation of miR-124 takes place at a later step of miRNA biogenesis (Rajasethupathy et al. 2009).

The loss of function is one possible method to study the role of miRNAs. To gain a better understanding of the whole functionality of one miRNA, overexpression can be another method of choice. Overexpression of specific miRNAs through use of miRNA mimics provides a method to reach this goal (Thomson et al. 2011; Rajasethupathy et al. 2009). The combination of those two methods (miR-mimics and miR-Inhibitors) would provide a further step in the process of analysing miRNA function. Griggs et al. (2013) investigated overexpression by *in vivo* transfection with miR-182 mimic in the lateral amygdala of *Rattus norvegicus*. They treated the animals 48 h prior to auditory fear conditioning and found an impairment of LTM 24 h after

conditioning. The miR-182-mimic was also tested on short-term memory without affecting it 90 min after conditioning. Additionally, the miR-182 expression which was quantified by q-RT-PCR was significantly decreased 1 h and 24 h after auditory fear conditioning (Griggs et al. 2013). A study by Rajasethupathy et al. (2009) combined two techniques and designed a miR-124 mimic duplex to increase the levels of miR-124 and a miR-124-Inhibitor to reduce its levels in sensory neurons of *Aplysia californica.* They showed that after injection of miR-124 mimic, LTF was significantly impaired at 24 h and 48 h after exposure to five pulses of 5-HT. They also confirmed the functionality of miR-124-Inhibitors and miR-124-mimics by *in situ* hybridisation experiments in sensory neurons (Rajasethupathy et al. 2009).

To reveal the full function of miRNAs, it is absolutely necessary to find their mRNA targets. The task is not easy to perform because there are several steps to be taken before a miRNA target can be validated. The target prediction with bioinformatical tools as RNA-Hybrid (Rehmsmeier et al. 2004; Krüger and Rehmsmeier 2006) would be the first step to search for possible targets for the miRNA of choice. These tools perform complex analysis of sequence alignment between the miRNA sequence and the genome of the corresponding model organism by using specific algorithms. The tools can predict many different targets or a few, depending on the parameters of the search and the miRNA itself. After the investigation of bioinformatical target predictions, the resulting outcome has to be validated. As one miRNA can target many different mRNAs with different functions, the selection of a few possible candidates for further analysis can be challenging. Methods for the prediction and validation of miRNA targets were reviewed by Kuhn et al. (2009); Thomson et al. (2011). One possible method for the validation of a miRNA-mRNA target interaction is the immunoprecipitation of RISC components like for example the AGO protein. After the immunoprecipitation, using antibodies against the AGO protein and gaining all the miRNA-mRNA which are bound to the AGO, a deep sequencing or microarray analysis can be performed to identify the sequences (Thomson, et al. 2011). This combination of methods would ideally reveal more than a few possible miRNA-mRNA target interactions but is relying on the stability of the complexes (Thomson, et al. 2011). An example for this approach was delivered by Hendrickson et al. (2009), who investigated a target search for the miR-124 in the human genome. They performed immunoprecipitation of AGO complexes in miR-124 transfected human embryonic kidney (HEK) 293T cells and subsequent microarray analysis and found over 600 mRNAs to be recruited by miR-124 (Hendrickson et al. 2009).

MiRNA expression is very different with respect to the cell type or tissue and the type of miRNA. Some are expressed at a very low amount, while others are expressed in a great abundance (Bartel 2004). In *C. elegans*, the miR-124 average level counts about 800 molecules per cell (Lim et al. 2003). Regarding the stability of miRNAs, their half-lives range from 28 to 220 h, also depending on the cell type and miRNA (which is 2-20 times longer than the half-live of typical mRNAs (average about 10 h)) (Zhang et al. 2012). The miRNA degradation and turnover has been reviewed by Zhang et al. (2012), who described, that cells control miRNA function either by regulating the activity of the miRNA or by turnover, which can be modulated by miRNA-mRNA target interactions. These studies show that the miRNA expression, stability and degradation are manifold and can also depend on the cell types and the specific miRNA.

The interpretation of experiments which show changes in miRNA amounts is challenging and can be caused by a variety of regulatory mechanisms. By using transient miR-Inhibitors I showed that the learning induced changes of miRNA levels in this work were related to acquisition and consolidation in weak and strong conditioning phases and at different points in time. The identification of miRNA interaction partners and miRNA targets is necessary to define their specific place in the signalling pathways of learning and memory formation.

5.8. Conclusion

The findings in this work gave new insights in the role of miRNAs in associative learning. The learning induced changes of miR-3788 levels imply a role in the formation of STM. The miR-3788 could hereby represent a honeybee homologue for the miR-132. Through the comparison of data from the analysis of learning induced changes and the transient inhibition of miRNAs, I can conclude that miR-12 and miR-124 are involved in the positive regulation of memory acquisition and influence the establishment of STM as well as the formation of MTM and LTM.

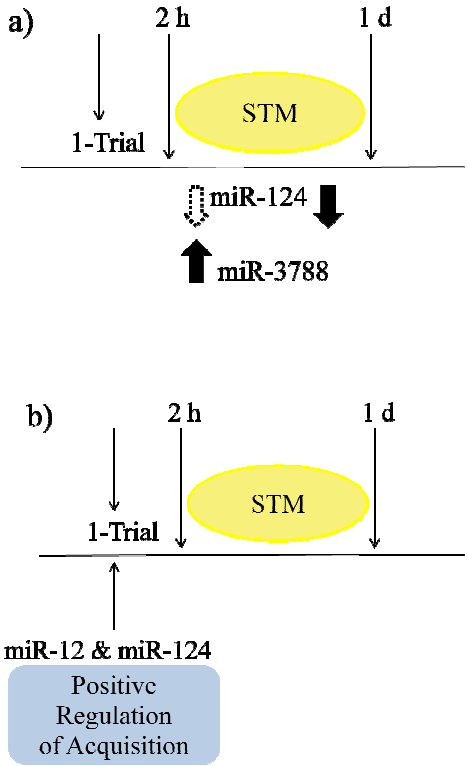

Figure 31 Learning induced changes and regulation of STM

The figure

a) Displays the regulation of miR-124 and miR-3788 levels after weak conditioning and q-RT-PCR

b) Shows the positive regulatory roles of miR-12 and miR-124 in acquisition of weak conditioning that were revealed by transient inhibition of miRNA function. The memory phase resulting from weak conditioning is termed short-term memory (STM) (Müller 2012).

Additionally, the combination of learning induced changes and transient inhibition of miR-12 revealed its positive regulating role in consolidation of LTM. In contrast to other works, this work provides an overview of learning induced changes of miR-12, miR-124 and miR-3788 levels for different points in time after weak and strong conditioning. A correlation analysis revealed a connection between miR-12, miR-124, miR-125 and miR-3788, implying their shared role in the processes of learning and memory formation. MiR-132, miR-125 and miR-124 targets

that are important players in neuronal pathways were already described in other organisms (Siegel et al. 2011). In the future, a target analysis in the honeybee could link the findings in this work to classify the miRNA-mRNA interactions in the signalling pathways of learning and memory formation processes.

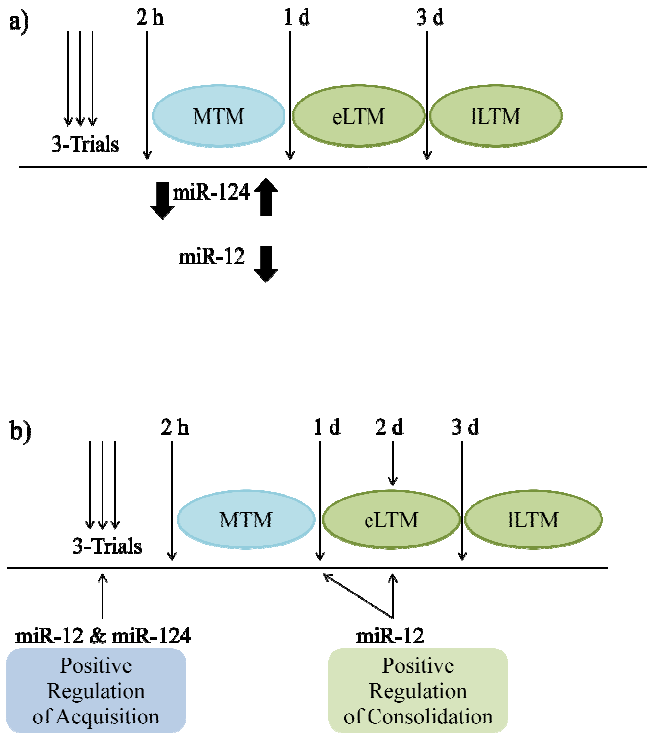

Figure 32 Learning induced changes and regulation of LTM

The figure

a) shows the regulation of miR-12 and miR-124 levels after strong conditioning and q-RT-PCR

b) displays the positive regulatory roles of miR-12 and miR-124 in acquisition and consolidation of strong conditioning that were revealed by transient inhibition of miRNA function. The memory phases resulting from strong conditioning are depicted as MTM (mid-term memory), eLTM (early long-term memory) and lLTM (late long-term memory) and were described by (Müller 2012).

The aim of the work, to identify the roles of certain miRNAs in associative learning, was completed. Summarised, this work took us a major step towards understanding the role of miRNAs in learning and memory formation processes, especially regarding their functions in acquisition and consolidation of memory.

6. Zusammenfassung

MiRNAs sind kleine, hochkonservierte, kodierende RNAs. Sie regulieren die posttranskriptionelle Genexpression durch Inhibition der Translation und Destabilisierung ihrer Ziel-mRNAs. MiRNAs sind zu interessanten Elementen in der Entschlüsselung molekularer Mechanismen geworden, so könnten sie für viele das fehlende Puzzlestück darstellen. Um die molekularen Mechanismen, die hinter Lernen und Gedächtnisbildung (L&G) stehen, zu enthüllen, ist es entscheidend, die Rollen dieser Puzzlestücke zu identifizieren. Ich habe in dieser Arbeit die lerninduzierten Änderungen der zu der Honigbiene homologen miRNAs (miR-12, -124, -125b, -132, -138 und miR-329) analysiert, welche wegen ihrer bereits beschriebenen Rollen in synaptischer Plastizität und L&G ausgesucht wurden. Ich zeigte, dass die Mengen von miR-124, -12 und miR-3788 (Homologe von miR-132) abhängig von der Stärke und der Zeit nach der Konditionierung sind. Eine Korrelationsanalyse zeigte eine Verbindung zwischen miR-12, -124, -125 und miR-3788. Weiterhin habe ich die spezifische Rolle von miR-12 und miR-124 in L&G mit Hilfe von transienter Inhibition durch AMOs untersucht. MiR-12 stellte sich als positiver Regulator der Konsolidierung von Langzeitgedächtnis heraus, wobei miR-124 eine positiv regulierende Rolle in der Akquisition zeigte, die essentiell für die Bildung von Kurzzeit- und Mittelzeitgedächtnis war. Zusammengefasst, konnte ich eine spezifische Rolle für miRNAs in Akquisition und Konsolidierung von L&G aufzeigen.

7. Summary

MiRNAs are small non-coding RNAs, which are highly conserved throughout species. They regulate posttranscriptional gene expression through inhibition of translation and destabilisation of their targets such as mRNAs. MiRNAs have become interesting elements in the decoding of cellular mechanisms, as for many they might be the missing piece in their puzzles. To understand the molecular mechanisms behind learning and memory (L&M) it is pivotal to uncover those missing pieces and define their roles in the machinery. In this work I analysed the learning induced changes of the honeybee miRNA homologues of miR-12, miR-124, miR-125b, miR-132, miR-138 and miR-329, which were selected for their previously described roles in relation to synaptic plasticity and L&M. I showed that the levels of miR-124, miR-12 and miR-3788 (a miR-132 homologue) were depending on the time after– and on the strength of conditioning. I revealed a connection between miR-12, -124, -125 and miR-3788 by correlation analysis. Furthermore I addressed the specific role of miR-12 and miR-124 in L&M phases by transient inhibition of their function with AMOs. I showed that the miR-12 is a positive regulator of consolidation in the formation of long-term memory whereas the miR-124 is a positive regulator of acquisition essentially for the formation of short-term and mid-term memory. Summarised, I described specific roles for miRNAs in acquisition and consolidation of L&M formation.

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9. Appendix

Table 34 Correlations between the miRNAs quantified by q-RT-PCR

There were no significant correlations between the EF 1 α and the miR-124,-125,-989 and -3788. There were no significant correlations for miR-12 versus miR-3788, for miR-124 versus miR-3788 and for miR-989 versus miR-3788. The groups (naive (n= 64) and conditioned (n= 64)) and the experiments were pooled for the respective measured RNA/miRNA.

	R^2	r	t	df	p
EF 1 α vs. miR-12	0,0454	0,21307	1,717	62	0,090898
EF 1 α vs. miR-124	0,0049	0,07	0,553	62	0,582568
EF 1 α vs. miR-125	0,0205	0,14318	1,139	62	0,258961
EF 1 α vs. miR-989	0,022	0,14832	1,181	62	0,242203
EF 1 α vs. miR-3788	0,0278	0,16673	1,221	62	0,18799
miR-12 vs. miR-3788	0,0065	0,08062	0,637	62	0,526653
miR-124 vs. miR-3788	0,0022	0,0469	0,37	62	0,712867
miR-125 vs. miR-989	0,0819	0,28618	2,352	62	0,021868
miR-989 vs. miR-3788	0,0069	0,08307	0,657	62	0,5138676

9.1. The influence of non-specific inhibitors on the miRNA machinery

9.1.1. Poly-L-Lysine and Trypaflavine treatment 1 h before strong conditioning

To determine, whether a partial inhibition of the miRNA machinery has an effect on learning and memory formation, honeybees were treated with 1 mM Poly-L-Lysine (PLL) which reduces DICER-mediated RNA processing or with 5 mM Trypaflavine (TPF) which reduces the association of miRNA with AGO2 (Watashi et al. 2010). The treatment with those substances in combination with learning and memory mechanisms in the honeybee had never been tested before. Honeybees were injected into the thorax with 1 mM PLL, or 5 mM TPF conditioned with three-trials of CS-US pairing, and retrieved 2 h and 24 h after conditioning. The PLL or TPF treatment at this point in time had no significant effect on acquisition, learning and memory formation (Chi Square, Fisher's exact test, two tailed PBS vs. PLL: PER 2. trial: p= 0,56; PER 3. trial: p= 0,32; Recall: PER 2 h: p= 1; PER 24 h : p= 0,69; PBS vs. TPF: PER 2. trial: p= 0,84; PER 3. trial: p= 1; Recall: PER 2 h: p= 1; PER 24 h : p= 1).

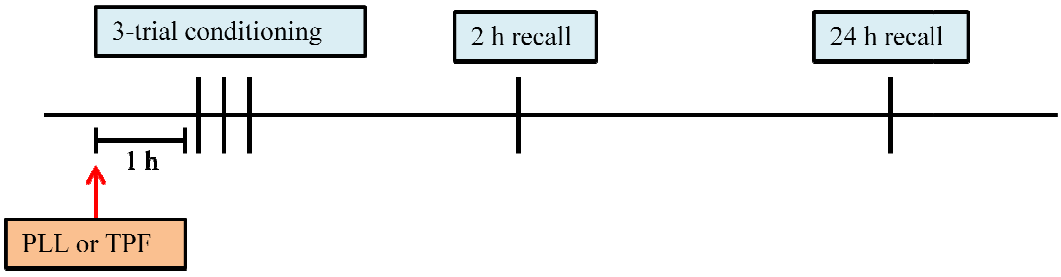

Figure 33 Poly-L-Lysine and Trypaflavine treatment 1 h before strong conditioning

Honeybees were treated with Poly-L-Lysine and Trypaflavine, conditioned with three trials and the memory was retrieved after 2 h and 24 h.

Table 35 Poly-L-Lysine and Trypaflavine treatment 1 h before strong conditioning

Percentage of honeybees (numbers in parentheses) showing a PER after three-trial conditioning. The bees were injected with PBS, 1 mM Poly-L-Lysine (PLL) or 5 mM Trypaflavine (TPF) 1 h before the conditioning.

Group	2. trial	3. trial	2 h	24 h
PBS (n= 54)	46,3%	59,2%	85,2%	68,5%
PLL (n= 58)	39,6%	69%	86,2 %	63,8%
TPF (n= 57)	49,1%	60%	84,2%	68,4%

9.1.2. Poly-L-Lysine and Trypaflavine treatment 1 h before weak conditioning

Table 36 Poly-L-Lysine and Trypaflavine treatment 1 h before weak conditioning

This experiment was conducted under my supervision in the practical course for the Master Student's in May 2014. Percentage of honeybees (numbers in parentheses) showing a PER after single-trial conditioning. The bees were injected with PBS, 1 mM Poly-L-Lysine (PLL) or 5 mM Trypaflavine (TPF) or both combined (PLL+TPF) 1 h before the conditioning.

Group	2 h	24 h
PBS (n= 15)	60%	47%
PLL (n= 13)	69%	15%
TPF (n= 16)	56%	31%
PLL +TPF (n= 10)	50%	40%

9.1.3. Poly-L-Lysine and Trypaflavine treatment 1 h after weak conditioning

Table 37 Poly-L-Lysine and Trypaflavine treatment 1 h after weak conditioning

This experiment was conducted under my supervision in the practical course for the Master Student's in May 2014. Percentage of honeybees (numbers in parentheses) showing a PER after single-trial conditioning. The bees were injected with PBS, 1 mM Poly-L-Lysine (PLL) or 5 mM Trypaflavine (TPF) or both combined (PLL +TPF) 1 h after the conditioning.

Group	2 h	24 h
PBS (n= 25)	68%	36%
PLL (n= 19)	84%	37%
TPF (n= 22)	68%	27%
PLL +TPF (n= 23)	83%	26%

Table 38 Gustatory sensitivity 1 h after treatment with PLL or TPF

This experiment was conducted under my supervision in the practical course for the Master Student's in May 2014. The treatment did not influence gustatory sensitivity. The bees were injected with PBS, 1 mM Poly-L-Lysine (PLL) or 5 mM Trypaflavine (TPF) or both combined (PLL +TPF). The gustatory sensitivity scores were compared with Mann Whitney *U*-Test.

Behavioral Test	PBS	PLL	TPF	
Gustatory sensitivity	3,7 (n= 20)	3,5 (n= 20)	3,6 (n= 20)	*PBS vs. PLL:* *U*= 189, *P*= 0,77 *PBS vs. TPF:* *U*= 196, *P*= 0,92

9.1.4. Treatment with Poly-L-Lysine 1 h before strong conditioning, analysis of miR-levels in the central brain after 2 h

The inhibition of parts of the miRNA machinery with PLL or TPF did not result in any effects on the learning and memory of strongly conditioned honeybees after 2 h. I wanted to test, whether changes in the levels of miRNAs can be determined 3 h after the treatment with PLL.

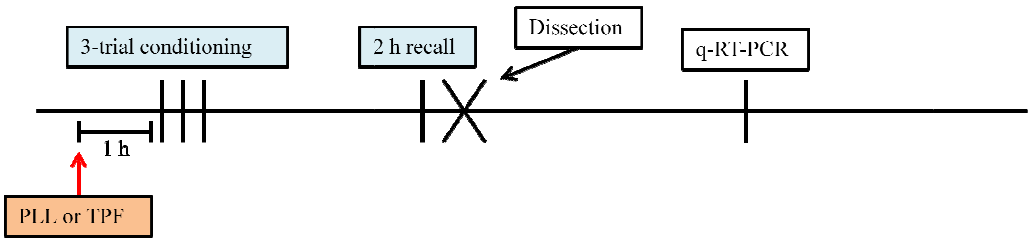

Figure 34 Poly-L-Lysine and Trypaflavine treatment 1 h before strong conditioning and quantification of miRNA amount

Honeybees were treated with Poly-L-Lysine and Trypaflavine, conditioned with three trials and the memory was retrieved after 2 h with subsequent dissection of the central brain and quantification of miRNA and mRNA by q-RT-PCR.

Table 39 Relative amount of miR-12, miR-124, miR-125, miR-989, miR-3788 in the central honeybee brain 2 h after strong training

Bees (numbers in parentheses) were injected with PLL and conditioned with three-trials. The PLL injected group was divided into a non-responding and a responding group, meaning the PER at the 2 h memory retrieval.

Relative miRNA amount					
Group	miR-12	miR-124	miR-125	miR-989	miR-3788
PLL (–) (n= 4)	1,34±1,3	0,62±0,41	0,92±0,24	1,13±0,26	1,07±0,21
PLL (+) (n= 4)	1,01±0,78	1,05±1,59	0,9±0,12	1,32±0,43	0,93±0,32
PBS (n= 4)	0,65±0,66	1,33±2,0	1,18±0,75	0,54±0,29	1,00 ±0,08

The honeybees were treated with 5 mM PLL (in 1x PBS) and strongly conditioned with three-trials 1 h after the treatment. The animals that responded to the 2 h recall were chosen for central brain dissection. They were dissected for central brain miRNA quantification by q-RT-PCR immediately after the 2 h recall. The honeybees treated with PLL were divided into two groups: responder (PLL (+) (n= 4)) and non-responder (PLL (–) (n= 4)). Animals that were treated with PBS (n= 4)) 1 h before strong conditioning were tested as a control group. The number of animals was too low to perform statistical analysis.

10.List of abbreviations

µ	Micro 10^{-6}
2'-O-Me	2' –O-methyl
3' UTR	untranslated region
5-HT	serotonin
ACT-D	Actinomycin D
AF	Activating factor
AGO	Argonaute family protein
AL	Antennal lobe
ame	*Apis mellifera*
AMO	Anti miRNA oligonucleotide
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANI	Anisomycin
APT1	acyl protein thioesterase1
Atx-2	Ataxin-2 protein
BDNF	Brain-derived neurotrophic factor
bp	Base pair
C(t)	Cycle threshold
C/EBP	CCAAT-box-enhanced binding protein
cDNA	Complementary DNA
cGMP	cyclic guanosine monophosphate
CREB	cAMP response element binding protein
CS	Conditioned stimulus
d	Day
dme	*Drosophila melanogaster*
DNA	Desoxy ribonucleic acid
EF 1α	Elongation factor 1 alpha
EtOH	Ethanol
Fig.	Figure
FMRP	Fragile-X-mental-retardation-1- protein

fwd	Forward primer
g	gram
g	9,81 m/s^2 earth rotation rate and gravity
GFP	Green fluorescent protein
GluA2	Apis mellifera ionotropic glutamate receptor
GluCl	glutamate-gated chloride channels
h	Hours
H_2O dest.	Distilled water
H_2O_2	hydrogen peroxide
KCl	potassium chloride
KDM1A	lysine-specific demethylase 1
l	Litre
LNA	Locked nucleic acid
LPS	lipopolysaccharide
LTD	Long-term depression
LTF	Long-term facilitation
LTM	Long-term memory
LTP	Long-term potentiation
M	Molar
m	Milli 10^{-3}
MBs	Mushroombodies
MCM6	DNA replication licensing factor
MCT1	Monocarboxylate transporter 1
MeCP2	methyl CpG binding protein 2
Mef2	myocyte enhancing factor 2
min	Minutes
miR	microRNA
miRNA	Micro RNA
mRNA	Messenger RNA
MTM	Mid-term memory
n	Nano 10^{-9}
NaCl	sodium chloride

NCBI	National Centre for Biotechnology Information
NMDA	*N*-methyl-*D*-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nt	Nucleotide
NTC	No template control
OC	Ocelli
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PER	Proboscis extension response
PKA	Protein kinase A
pre-miRNA	precursor-miRNA
pri-miRNA	primary-miRNA
q-RT-PCR	Quantitative Real-Time Polymerase Chain Reaction
rev	Reverse primer
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
s	Seconds
sGC	soluble guanylyl cyclase
siRNA	Small interfering RNA
STF	Short-term facilitation
STM	Short-term memory
TE	Tris-EDTA
US	Unconditioned stimulus
ZEN	N,N-diethyl-4-(4-nitronaphtalen-1-ylazo)-phenylamine
λ	Wavelength

11. List of figures

12. List of tables

13. Acknowledgement

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14. Education and work experience

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School education:

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October 2005- April 2012: Study of Biology at the University of Freiburg

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September 2012- Present: Ph.D. student, research assistant at the Saarland University (Department of Neurobiology http://neurobiologie.uni-saarland.de/)

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January 2008- January 2012: Student research assistant in the lab of Prof. Dr. Michael Reth (Molecular immunology)

April- October 2009: Participation in **iGEM- i**nternational**G**enetically**E**ngineered**M**achine competition (http://2009.igem.org/Team:Freiburg_bioware)

January- October 2014: Instructor of the first iGEM team (**iGEM- i**nternational**G**enetically**E**ngineered**M**achine competition) of the Saarland University (http://2014.igem.org/Team:Saarland)

Publications:

1. Michely, J. & Müller, U., (2015): *Effects of MicroRNAs in Memory Formation Processes in the Honeybee (Apis mellifera)* Guest talk in the group of Prof.Dr. Rössler at the Würzburg University

2. Michely, J. & Müller, U., (2015): *The Impact of MicroRNAs in Memory Formation Processes in the Honeybee (Apis mellifera)* 11th Göttingen Meeting of the German Neuroscience society (Talk)

3. Ehrhardt, M., Hartz, P., Loehfelm, A., Ecker, E., Finkler, M., Jarzembowski, L., Pirritano, D., Riske, A., Riske F., Strehl, A., Folz, J., Michely, J., Mueller, U. (2014): *Project RUFUS or the Force of the naked mole rat*. iGEM 2014 Boston Jamboree (Poster, Talk)

4. Michely, J. & Müller, U. (2014): *The Role of microRNAs in memory formation processes in the honeybee (Apis mellifera).* The LXXIX Cold Spring Harbor Symposium on Quantitative Biology: Cognition (Poster)

5. Rennertz, J. & Müller, U. (2013): *The Role of microRNAs in Learning and Memory in the Honeybee Brain.* Neurodowo Göttingen Meeting (Poster)

6. Rennertz, J. & Müller, U. (2013): *The Role of microRNAs in Learning and Memory in the Honeybee Brain.* 10th Göttingen Meeting of the German Neuroscience society (Poster)