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„microRNA expression in learned safety“

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1. Introduction

Fear, an emotion essential for survival, is defined as the reaction to a threatening stimulus and can be categorized into two distinctive forms: instinctive, or unlearned and learned fear. However, the expression of fear can sometimes be unfavorable, if the associated learning processes are inadequate or the resulting behavior inappropriate and therefore give rise to various mental illnesses such as anxiety and depressive disorders. Like fear can be generated and enhanced through learning processes, also inhibitory mechanisms of fear exist. One such inhibitory mechanism is “conditioned inhibition of fear”, which involves learning about signals indicating protection, thus reducing the fear response. These signals are referred to as safety signals and the respective learning process is termed learned safety. A subject’s ability to detect and interpret a safety signal and moreover to react appropriately to safety signals is crucial for mental health. Impairments in perception and reaction to safety signals induce chronic stress and anxiety and subsequently various related psychopathologies.

The first one who described behavioral responses arising from associative learning was Ivan P. Pavlov in his famous conditioning experiments with dogs. Here, he described “conditioned reflexes” elicited by a neutral stimulus (conditioned stimulus (CS)) for example a tone that was previously paired with an excitatory stimulus (unconditioned stimulus (US)) for example food. After presenting the CS paired with the US several times the CS itself became the ability to evoke behavioral responses usually triggered by the US, for example saliva secretion. In addition, he also investigated “conditioned inhibition”, where he introduced not only a CS paired to the US (CS+), but another conditioned stimulus (CS-), which occurs only in absence of the US. Pavlov observed that the CS- induced a reduced behavioral response (saliva secretion) to the CS previously paired with the US (CS+). Furthermore, Pavlov rightly identified conditioned reflexes and conditioned inhibition as associative learning processes.

More than 40 years later, Robert Rescorla revised the work of Ivan Pavlov and developed two tests to assess the inhibitory properties of a CS. In the first test, which he called summation test, a CS- inhibits the response to a CS+
while in the second test, a retardation test, Rescorla observed that the ability of a CS- to elicit behavioral responses when it was paired previously to a US is delayed\textsuperscript{2,3}.

Learned fear has been studied extensively in the past decades and a considerable amount of knowledge has been gained on its underlying neurobiological mechanisms. Compared to learned fear, conditioned inhibition of fear or learned safety has remained less well investigated and its neuronal underpinnings are incompletely understood.

1.1. Learned fear and learned safety

Learned safety is studied in various experimental procedures in rodents and primates. The two most widely used protocols to study the neurobiology of learned safety are explicit unpaired procedures as well as conditional discrimination paradigms. In explicit unpaired procedures, a CS such as a tone is explicitly unpaired with an aversive US, such as a mild electric shock in a training phase. Subsequently, behavioral responses to the auditory conditioned stimulus are assessed\textsuperscript{4–6}. In conditional discrimination procedures (also called AX+/BX- procedures), a stimulus X paired with an additional stimulus A is followed by an aversive event (US) while the same stimulus X paired to another stimulus B predicts safety or the absence of the US. A combination of A with B reduces the fear response elicited by stimulus A\textsuperscript{7}.

However, the behavioral effects of learned safety go beyond the inhibition of learned fear since it has been found that the experience of safety signals are also associated with positive affective conditions\textsuperscript{4,6} and have been shown to reduce long-term effects of anxiety-generating stressors\textsuperscript{8}. Consequently, learned safety in mice also contributes to the reversal of stress-related anhedonia, the inability to experience pleasure in otherwise pleasurable situations, one of the core symptoms of depression in humans\textsuperscript{9}. Similarly, behavioral despair in mice, related to depression in humans, as evaluated in the forced swim test (FST), has been found to be reduced by the presence of the safety signal\textsuperscript{6}.

Taking together the observations described above, the behavioral content of learned safety appears not as the “symmetrical opposite” of learned fear\textsuperscript{10}, but beyond that it facilitates positive emotional conditions, and moreover in
animal models, leads to the reversal of behavioral states associated with depression.

1.2. Neuronal circuits involved in learned fear and learned safety

The neurobiological mechanisms of learned fear have been studied extensively by using the fear conditioning paradigm in experimental animals, mainly rodents. One of the protocols most commonly used involves the presentation of a CS, such as a tone, paired to an US, such as an electric shock during a training phase, which is followed by a memory test 24 hours after the last training session. During the memory test, the CS is presented and the behavioral fear response of the animal is evaluated. Indicative of successful fear learning is an increased fear response during the presentation of the CS\textsuperscript{11,12} (Figure 1). A multitude of studies over the past decades have shown that the most relevant brain region in the neuronal circuit of fear conditioning is the amygdala, a subcortical structure of the limbic system\textsuperscript{13–16}. Both conditioned stimulus and stimuli generated from the aversive event are transmitted from sensory processing regions of cortex and thalamus to the lateral amygdala, where synaptic plasticity takes place. Information gained during fear conditioning is further passed on from the lateral amygdala to the central amygdala, which controls regions in hypothalamus and brainstem. Furthermore these structures generate behavioral, autonomic and hormonal fear responses\textsuperscript{11,17} (Figure 2).
Figure 1. Fear and Safety Conditioning. a During the conditioning procedure, the mouse is placed into a conditioning chamber. The electric shock (US) is generated by a shock generator and transmitted by the grid floor. The conditioning chamber is connected to a speaker, which produces the auditory stimulus (CS). b In fear conditioning during the training phase, the US and the CS are presented simultaneously, so the CS becomes threatening itself to the animal. In safety conditioning, CS and US are presented unpaired during the training phase. Therefore the CS acts as a safety signal for the animal. In fear as well as safety conditioning, 24 hours after the last training session memory recall is tested in the conditioning context by presenting the CS to the experimental animals. In fear conditioning the CS implies danger, while in safety conditioning the CS mediates protection from danger.
Figure 2. Neuronal circuit of fear conditioning. Conditioned and unconditioned stimuli are transmitted from processing regions in thalamus and cortex to cells of the lateral amygdala. The lateral amygdala is connected to the central amygdala, which controls fear responses by affecting various brain regions. Subsequently, the central gray regulates the behavioral fear response freezing, the lateral hypothalamus influences the autonomic nervous system and the paraventricular hypothalamus controls the endocrine system\(^\text{18}\).

Paralleling the investigation of learned fear using the fear conditioning paradigm, a behavioral paradigm for learned safety exists, which is referred to as safety conditioning. As in fear conditioning, safety conditioning begins with a training phase- however here the tone CS and the shock US are presented in an unpaired manner, as they do not overlap in time. Again, successful generation of safety memory is tested 24 hours after the last training session and can be measured as the reduction of fear to the conditioning context (Figure 1).
Although still incompletely understood, some advances in the elucidation of the neuronal circuits implicated in learned safety have been recently obtained. An electrophysiological study in 2005\textsuperscript{4} demonstrated that inhibition of neuronal signaling in the lateral amygdala plays a major role in the mechanisms of learned safety. In safety conditioned mice, the auditory CS elicits reduced activity in the lateral amygdala. In contrast, electrical activity measured in fear conditioned mice while presenting the fear signal was increased in the lateral amygdala. In the caudoputamen, augmented electrical activity is observed in response to the auditory stimulus in safety trained mice, which links safety conditioning to a brain region implicated in reward and euphoric responses\textsuperscript{4}. Another way to detect neuronal activity is the evaluation of stress-induced c-Fos immunoreactive cells. Christianson \textit{et al.}\textsuperscript{19} discovered that Fos immunoreactive cells were decreased in the basolateral amygdala and the bed of the stria terminals of rats exposed to safety signals, which means a decrease in activity in these brain regions. In contrast, uncontrollable stressors caused an increase in Fos cells in all regions of the amygdala\textsuperscript{19}.

Similar findings were obtained in functional magnetic resonance imaging (fMRI) studies conducted in healthy humans that were exposed to a safety conditioning protocol comparable to the safety conditioning protocol used in rodents. Although in humans, there was only one training session while in rodents the training phase consists of three days with one training session per day, brain activity measurements demonstrated that in humans the CS elicits reduced activity in the amygdala, while responses in the striatum and the dorsolateral prefrontal cortex (dIPFC) are increased due to the CS\textsuperscript{20}. Diffusion tensor imaging and probabilistic tractography indicated a connection between the dIPFC and the amygdala in response to the safety CS and thereby suggested a model where the activated dIPFC mediates inhibition of the amygdala in safety learning\textsuperscript{20}. Furthermore, another brain imaging study conducted in humans revealed in accordance with the study described above, that activity in the striatum was increased in response to a safety signal, while it was increased in the amygdala when a fear cue was presented\textsuperscript{21}.

The results described above implicate the role of the amygdala, the striatum and the dIPFC in the neural circuit mediating learned safety. Nevertheless,
lesions in the rat central amygdala do not abolish conditioned inhibition. In addition, neonatal lesions of the amygdala of macaques prevent learned safety not in all adult animals\textsuperscript{22,23}. To obtain a complete model of the neuronal circuits involved in learned safety, further studies have to be conducted.

The sensory insular (Si), a brain region receiving sensory inputs from cortex and thalamus and containing efferent amygdala connections has been implicated in learned safety recently. In this study, safety signals have been shown to inhibit fear that occurs during exposure to uncontrollable stressors. Suppression of the Si by a pharmacological GABA agonist disrupts the fear inhibitory properties of the safety signal during stress exposure\textsuperscript{8,19}. Lesions of the posterior intralaminar nucleus after safety conditioning training causes impairments in safety learning, suggesting an additional brain region playing a role in the neurocircuitry of learned safety\textsuperscript{24}.

1.3. Cellular and molecular mechanisms of learned safety
A limited number of studies so far have examined the cellular and molecular mechanisms of learned safety, with a major focus on the amygdala and the hippocampus. In the rat lateral amygdala safety and fear learning affect synapse size differentially. While spine synapses in this particular brain region are increased in size with fear conditioning, their size is reduced in safety-conditioned animals\textsuperscript{25}. Furthermore learned safety caused a decrease in size of synapses with a spine apparatus, a smooth endoplasmatic reticulum found in very large spines, while fear conditioning increased the number of spine apparatuses\textsuperscript{25}.

Gene expression studies in the basolateral amygdala of mice following safety conditioning, have been demonstrating that learned safety causes a specific gene expression pattern\textsuperscript{6}. Despite the fact that learned safety induces an antidepressive effect in mice evaluated in two animal tests for depression (FST, sucrose preference test (SPT)) no change in expression of genes playing a role in mediating the mechanism of pharmacological antidepressants was observed. The prevalent pharmacological antidepressants either act as reuptake inhibitors or block degradation of
monoamines such as serotonin and noradrenalin\textsuperscript{26}. In contrast, learned safety affects dopaminergic and neuropeptidergic signaling by modulating the expression of genes involved in these two signaling systems\textsuperscript{6}. Therefore, although the effects of pharmacological antidepressants and learned safety on behavioral, cellular and molecular level are overlapping, at least parts of the signaling systems mediating these effects seem to be distinct from each other.

In the hippocampus, adult neurogenesis in the dentate gyrus seems to play a crucial role in learned safety. In mice with ablated neurogenesis, safety learning is impaired, while learned safety increases the number of newborn cells surviving in hippocampal dentate gyrus. Levels of the brain-derived neurotrophic factor (BDNF) are augmented in the hippocampus by learned safety what may account for the enhanced survival of newborn neurons\textsuperscript{6}. Interestingly, one mechanism of action of pharmacological antidepressants is facilitating an increase of BDNF levels\textsuperscript{27} and an enhancement of neurogenesis\textsuperscript{28} as it is observed in learned safety. Furthermore, in depressed patients, a reduced volume of the hippocampus and of forebrain structures is observed. These observations led to the hypothesis, that neuronal growth factors play a role in the development of depressive disorders\textsuperscript{29}. It was demonstrated that stress reduces levels of BDNF in the hippocampus\textsuperscript{30}. Moreover, when BDNF is injected into the dentate gyrus of the mouse hippocampus, an antidepressant effect can be observed in the FST and learned helplessness tests\textsuperscript{31}.

With regards to the observation that learned safety triggers the same mechanisms in the adult hippocampus as pharmacological antidepressants namely an increase in BDNF levels and in neurogenesis and reverses symptoms of depression, learned safety can be considered as an animal model for a behaviorally-induced antidepressant.
1.4. Additional factors influencing learned safety

Not all individuals that experience exposure to adverse environmental conditions, such as trauma or stress, develop the associated psychopathologies, including post traumatic stress disorder (PTSD) and depression. Thus, a distinct set of individual conditions may therefore exist which additionally contribute to the development of mental illnesses. In the learned safety animal model, differences between two genetically distinct inbred mouse strains Svlmj (SI) and the C57BL/6J (B6) were found. In SI mice safety signals failed to inhibit contextual fear, while in B6 mice safety learning was accomplished well\(^3\). These results indicate that there is a genetic basis for the ability to detect and respond properly to safety signals. It has to be determined in future studies, if there is an association between the genetic factors determining the ability for safety learning and the development of mood and anxiety disorders.

With regards to its translational potential, only few studies investigating learned safety in humans exist. The first ones who examined safety learning in humans was Christian Grillon who analyzed the effect of safety signals on anxiety, by evaluating the fear potentiated startle response, a physiological response in animals and humans elicited by a threatening stimulus \(^3\). In 2005, Jovanovic et al.\(^3\) translated a conditional discrimination paradigm previously used only in rodents to a humans study and demonstrated that safety learning is impaired in patients suffering from PTSD\(^3\). Indeed the inability to identify and react to safety signals directly corresponds to a symptom frequently occurring in PTSD namely hyperarrousal or hypervigilance\(^3\). In one particular study examining patients suffering from major depressive disorder (MDD), no impairment of safety learning was observed \(^3\). Nevertheless, the fact that an animal model of learned safety induces an antidepressive state on behavioral, cellular and molecular level\(^6\) suggest that research using the animal model of learned safety may provide further insights into the mechanisms mediating positive affective conditions in the brain and moreover provide evidence for the neurobiological basis of behaviorally active antidepressant therapeutic strategies.
1.5. microRNAs (miRNAs)

1.5.1. miRNA biogenesis

miRNAs are small noncoding RNAs, which regulate gene expression at the posttranscriptional level in metazoans and plants. They play a role in almost all physiological functions currently investigated and aberrant expression of miRNAs has been linked to various disease states\textsuperscript{38}. There are around 800 known miRNAs in humans, which show a differential expression pattern in various cell types and developmental grades, thereby fine-tuning cell-type and developmental specific processes\textsuperscript{38}. An explanation for their ubiquitous function is that one miRNA can have hundreds of different gene targets. On the other hand, 50 % of all protein coding genes in mammals are regulated by miRNAs, which explains their importance for proper biological functioning\textsuperscript{38,39}. miRNA genes are first being transcribed by RNA Polymerase II into pri-miRNAs, which are further processed in the nucleus by the RNase Drosha into 70 nucleotide long pre-miRNA hairpins. After this first processing step, pre-miRNAs are exported out of the nucleus, into the cytoplasm by Exportin 5. Next the pre-miRNAs are further processed by the enzyme Dicer into a 20 bp miRNA/miRNA* duplex. One strand of the duplex is incorporated into a multiprotein complex called RISC, which targets mRNAs complementary in sequence to its incorporated miRNA strand. Depending on the level of complementarity of the miRNA seed region to the bound mRNA, the mRNA is either degraded by the endonuclease activity of RISC, or its translation is blocked reversibly\textsuperscript{40} (Figure 3).
Figure 3. microRNA biogenesis. miRNAs are transcribed by RNA Polymerase II into pri-miRNAs. Inside the nucleus Drosha further processes them into pre-miRNAs, which are exported out of the nucleus by Exportin5. In the cytoplasm, Dicer converts the pre-miRNA into a miR:miR* duplex. One strand of this duplex is incorporated into the RISC complex, which binds to mRNA targets with complementary sequence. Further, the mRNA is either degraded or translation is repressed\textsuperscript{40}.

1.5.2. miRNAs in the nervous system and the link to psychiatric diseases

miRNAs have been reported to be essential for correct neuronal development in vertebrates with particular roles in neuronal differentiation\textsuperscript{41} and also adult neurogenesis\textsuperscript{42,43}, impairments of which have been linked to stress\textsuperscript{44} and depressive disorders\textsuperscript{45}. Furthermore, miRNAs control synaptic plasticity\textsuperscript{46}, a process fundamental in learning and memory\textsuperscript{47}. These observations demonstrate the importance of miRNAs and their regulation in the physiology
of the central nervous system. The question of how miRNA expression can be modulated by exogenous stimuli has been recently frequently addressed with the association between stress, miRNA expression levels and consequently the expression of microRNA target genes being one major focus of interest\textsuperscript{48}. In this regard it has been shown that acute and chronic restraint stress lead to a change in miRNA expression profiles in amygdala\textsuperscript{49} and frontal cortex\textsuperscript{50} in mice. Furthermore it was shown that miR-34c, a miRNA specifically upregulated after acute restraint stress, regulates the expression of the corticotropin releasing factor type 1 receptor, which is involved in modulating stress responses\textsuperscript{49}. Moreover, glucocorticoid receptors (GR) which are decisively involved in the negative feedback loop for synthesis of glucocorticoids (GC) have been found to be targets of miR-18a and miR-124a\textsuperscript{51,52}.

Since stress is one of the predisposing factors for developing depressive disorder, it seems likely, that miRNAs may also be involved in the pathophysiology of depression and indeed, dysregulation of the miRNA system has been described to contribute to the development of the disease\textsuperscript{53,54}. An additional link between miRNAs and depression is provided by the fact that psychoactive pharmacological agents are proposed to also exert their function by affecting miRNA levels. Chronic treatment with lithium and sodium valproate (VPA) reduced expression levels of several miRNAs in rat hippocampus. The genes targeted by these miRNAs play roles in neurogenesis and cell signaling and are known genetic risk factors for the development of mood disorders\textsuperscript{55}. Additionally, miRNAs seem to be involved in fear learning and memory and the associated anxiety states, as it has been shown that that the expression of some miRNAs is regulated in the amygdala of experimental animals after fear conditioning\textsuperscript{56}. Also, specific changes in miRNA expression related to the extinction of fear have been reported, with a selective effect of miR-128b expression in the mouse prefrontal cortex\textsuperscript{57}.

Taken together these findings propose a pivotal role for miRNAs in the regulation of the stress response and the pathophysiology of mood and anxiety disorders. Moreover, an understanding of the regulation of miRNAs in animal models of these diseases may not only contribute to the elucidation of the underlying neurobiological mechanisms but may also identify specific
miRNAs as possible therapeutic targets in treatment of these psychopathologies. Considering the role of learned safety as animal model related to depression and PTSD outlined above, an investigation of the involvement of miRNAs in the associated molecular underpinnings and their regulation by safety learning may shed new light on the pathophysiology of mood and anxiety disorders and forms the rationale for the present study.
2. Aims and Hypothesis

The aim of this thesis was to investigate the mechanisms of learned safety specific modulation of gene expression in the amygdala focusing on the role of miRNAs, small non-coding RNAs regulating gene expression at the post-transcriptional level.

Based upon the fact that miRNAs have been shown to play an important role in various neurobiological processes starting from guidance of neuronal development\textsuperscript{58} to regulation of stress responses\textsuperscript{44,59} and that dysregulation of miRNA function is associated with various psychopathologies including depression\textsuperscript{59,60}, we proposed to test the hypothesis that miRNAs are differentially expressed in amygdala of mice trained for learned safety and hereby modulate learned safety-specific gene expression.

3. Experimental design

To test whether safety learning changes the expression of miRNAs, amygdalar expression of 11 miRNAs (sense and antisense) that were chosen due to their previous association with functions in amygdala and stress response or with their deregulation in depressive disorders (Table 1) was compared between mice trained for learned safety and learned fear (Figure 4). In this experiment, the fear conditioning protocol was adapted to the safety conditioning protocol, therefore mice of both groups received the same amount of shock and tone presentations and effects due to the experimental setup can be excluded.
Figure 4. Experiment 1. In the first experiment of this thesis, it was evaluated if there is a change in amygdalar miRNA expression between mice trained for learned safety and mice trained for learned fear.

To further test if a change in expression of miRNAs in amygdala between learned safety and learned fear trained mice is specific for learned safety the expression levels of the same 11 miRNAs were compared in mice trained for learned fear with shock and tone alone controls. Since miRNAs regulate the expression of target mRNAs, a bioinformatical target search was performed, to obtain possible candidate genes for miRNAs that show a learned safety specific expression.
Table 1. List of miRNAs including their function in the nervous system that were investigated in the context of this thesis.

<table>
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<tr>
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<th>Effect</th>
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<tr>
<td>miR-15a</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49</td>
</tr>
<tr>
<td>miR-15b</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49</td>
</tr>
<tr>
<td>miR-34c</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49</td>
</tr>
<tr>
<td>miR-34a</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49, Zhou et al. 55</td>
</tr>
<tr>
<td>miR-92a</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49</td>
</tr>
<tr>
<td>miR-100</td>
<td>expression level changes after acute stress in mouse amygdala</td>
<td>Haramati et al. 49</td>
</tr>
<tr>
<td>miR-134</td>
<td>downregulates CREB and BDNF, SIRT1 inhibits expression of miR134</td>
<td>Gao et al. 61, Rong et al. 62, Meerson et al. 63</td>
</tr>
<tr>
<td></td>
<td>plasma levels of miR134 decreased in manic patients</td>
<td></td>
</tr>
<tr>
<td></td>
<td>increased expression level after acute stress in rat amygdala</td>
<td></td>
</tr>
<tr>
<td>miR-132</td>
<td>targets BDNF and MeCP2 is regulated by the CREB pathway</td>
<td>Klein et al. 64, Kawashima et al. 65</td>
</tr>
<tr>
<td>miR-18a</td>
<td>reduces glucocorticoid receptor levels in neuronal cell culture</td>
<td>Vreugdenhil et al. 52</td>
</tr>
<tr>
<td>miR-183</td>
<td>increased expression level after acute stress in rat amygdala</td>
<td>Meerson et al. 53</td>
</tr>
<tr>
<td>miR-212</td>
<td>most highly expressed in hippocampus and amygdala</td>
<td>Olsen et al. 66</td>
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compared to various brain regions
4. Materials and Methods

4.1. Animals

9-10 week old male C57BL6/N mice were used for all experiments. Mice were single housed with *ad libitum* food and water under standard conditions. All experiments were designed to reduce animal suffering and keep the number of animals used at the minimum level. Animal experiments described in this study were approved by the national ethical committee on animal care and use (Bundesministerium für Wissenschaft und Forschung) and carried out according to international laws and policies.

4.2. Behavioral Procedures

Mice were handled two days before the start of behavioral training with one handling session of approximately 1 minute per day during which training for all animals occurred in behavioral chambers (MED Associates, Vermont, USA) housed within a sound-proof box. The first experiment consisted of a 3 days training phase for safety and fear conditioning with one training session per day. Safety conditioning consisted of four explicitly unpaired US and CS presentations (one session per day for 3 days). The fear conditioning protocol was matched to the number of auditory CS and shock US presentations of the safety conditioning paradigm and thus constituted 4 paired CS-US presentations per day\(^6\). The time points of CS and US presentations were varied in all 3 training days (Figure 5). The US was an electric foot shock of 0.6 mA for 2s delivered through the bars of the floor of the conditioning chamber for all experiments. The CS was a tone at 75 dB that lasts for 20s in the safety conditioning and fear conditioning adapted to safety conditioning protocol and 30s in the typical fear conditioning protocol.

The second experiment comprised a typical fear conditioning protocol, consisting of a 2 days training phase with one training session per day during which 2 paired CS and US (using the specifications described above) were presented. The fear conditioning group was compared to shock and tone alone control groups. In shock alone controls 2 US but no CS were presented at same time points as in the fear conditioning group. In tone alone controls 2
CS but no US were presented at the same time points as in the fear conditioning group.

Freezing behavior was tested 24 hours after the last training session. In the first experiment, contextual fear was tested by placing each mouse into the conditioning chamber and evaluating freezing behavior before and during presentation of the CS (Figure 5). In the second experiment, mice were tested for memory of the CS by placing the animals into a new context and measuring the freezing behavior before and during the CS presentation. Time spent freezing was determined by software-assisted analysis of digital video recordings using the FreezeFrame software (Actimetrics, Evanston, IL).

**Figure 5. Safety and fear conditioning protocol.** In all training sessions (day1-day3), CS and US are presented 4 times. In safety conditioning CS and US are unpaired, while in fear conditioning they are delivered in a paired manner. The time points of CS and US presentations varied in all 3 training days. 24 hours after the last
training session memory is tested, where freezing behavior is evaluated before and during the CS presentation.

4.3. Dissection
All animals were sacrificed by neck dislocation 2 hours after memory recall testing. Brains were dissected out, placed into optimal cutting temperature compound (O.C.T.), frozen in liquid nitrogen and stored at -80°C.

4.4. Micropunch Procedure
Brains were sliced into 300 µm thick sections with a cryostate (Microtom) set at -8°C. 3 coronal sections through the amygdala (bregma -1.22 mm, bregma -1.58 mm, bregma -1.82 mm) were placed on RNA-free laser membranes. The sections were frozen on dry ice, amygdala were punched out using cannula of 0.69 mm diameter and released immediately into an Eppendorf tube filled with 200 µl lysis buffer (Qiagen). Samples were homogenized by a vortex mixer, frozen in dry ice and stored at -80°C until further usage.

4.5. RNA extraction
RNA extraction from amygdala samples was carried out using the Qiagen miRNA Micro Kit (cat.no. 217084) as described in the protocol supplied (miRNeasy Micro Handbook67).
Briefly, amygdala lysates were thawed and transferred into 1000 µl Phenol/guanidine-based QIAzol® Lysis Reagent. 240 µL Chloroform were added, samples were mixed and centrifuged at 12000 rpm (revolutions per minute) and 4°C for 15 minutes. The upper aqueous phases were transferred into new Eppendorf tubes and 1.5 volumes of 100% ethanol were added. The samples were applied to RNeasy MinElute spin columns and centrifuged at 10000 rpm and room temperature (RT) for 15 seconds. The flow-through was discarded and 700 µL RWT buffer was added. Samples were centrifuged at 10000 rpm and RT for 15 seconds and the flow through was discarded. Next, 500 µl RPE buffer was added, samples were centrifuged at 10000 rpm and RT for 15 seconds and the flow through was discarded. Further, 500 µl 80% ethanol was added, samples were centrifuged at 10000 rpm and RT for 2
minutes and the flow through was discarded. Then, the RNeasy MinElute spin columns were placed into a new 2mL collection tube, the lids of the columns were opened and they were centrifuged at 16000 rpm and RT for 5 minutes. The RNeasy MinElute spin columns were transferred into new 1.5 mL Eppendorf tubes, RNA was eluted with 14 µL RNase-free H2O from the columns and they were centrifuged at 16000 rpm and RT for 1 minute. The RNA concentration and purity was measured photometrical by a Nanodrop instrument. After the measurement, samples were frozen in -80°C until further usage.

4.6. cDNA synthesis

cDNA synthesis was carried out using the Qiagen miScript II RT Kit (cat.no. 218161) following the supplier’s instructions miScript PCR System Handbook.

500 ng of RNA per reaction was required for cDNA synthesis. Depending on the concentration of RNA dissolved in RNase-free H2O, required volumes were calculated for each sample. The procedure was conducted on ice. First, samples were thawed and the required volume for each sample was transferred into a new Eppendorf tube. Samples were diluted up to a volume of 12 µL with RNase-free H2O. A mastermix with reagents from Qiagen miScript II RT Kit was prepared according to Table 2. 8 µL of mastermix was added to each sample. Next, samples were placed into a thermocycler, and incubated for 60 minutes at 37°C and afterwards for 5 minutes at 95°C for. Afterwards, cDNA samples were diluted up to 350 µL with RNase-free H2O and frozen in -20°C until further usage.
Table 2. Mastermix for reverse transcription reaction with reagents from Qiagen miScript II RT Kit.

<table>
<thead>
<tr>
<th>Master Mix</th>
<th>Per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× miScript HiSpec Buffer</td>
<td>4 µL</td>
</tr>
<tr>
<td>10× Nucleics Mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>miScript Reverse Transcriptase Mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>subtotal</td>
<td>8 µL</td>
</tr>
</tbody>
</table>

4.7. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

The Qiagen miScript SYBR® Green PCR Kit (cat.no. 218076) was used for qRT-PCR analysis. All primers used were supplied from Qiagen and dissolved in 550 µL of TE buffer (pH 8.0). The procedure for qRT-PCR followed the guidelines described by the supplier (miScript PCR System Handbook).

Briefly, samples were thawed on ice and 3 µL of the cDNA reaction was added to 1.5 µL of RNase-free H2O. A mastermix was prepared according to (Table 3). 4.5 µL of sample and 10.5 µL mastermix were pipetted into 96-well PCR plates (Applied Biosystems®, life technologies™), plates were mixed gently, closed with PCR plate sealing foil (SARSTEDT AG & Co.) and centrifuged at 10000 rpm for 1 minute. Then the plate was placed into a PCR thermal cycler (Applied Biosystems®, life technologies™) and the reaction was carried out using the program (StepOne Software v2.2.2 Applied Biosystems®, life technologies™) with the specification listed below (Table 4) for 40 cycles.
Table 3. Mastermix for qRT-PCR reaction with reagents from Qiagen miScript SYBR® Green PCR Kit.

<table>
<thead>
<tr>
<th>Mastermix</th>
<th>Per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×QuantiTect SYBR Green Master Mix</td>
<td>7.5 µL</td>
</tr>
<tr>
<td>10× miScript Universal Primer</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>10× miScript Primer Assay</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>subtotal</td>
<td>10.5 µL</td>
</tr>
</tbody>
</table>

Table 4. qRT-PCR protocol.

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturing</td>
<td>95°C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Elongation</td>
<td>70°C</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

4.8. Statistics

For behavioral analysis, the difference between time spent freezing before and during the CS presentation was calculated. Differences between two groups were analyzed by two-tailed Student's t tests. For comparisons between three groups a one-way ANOVA followed by a post-hoc Scheffe test was carried out. The level of significance was defined as $p<0.05$ at all instances.

4.9. Bioinformatics

The web platform EIMMo3 from the University of Basel (http://www.mirz.unibas.ch/EIMMo3/) was used to obtain lists for potential miRNA gene targets. Gene targets were searched for the species “mouse”, in the structure “amygdala” and the miRNAs of interest. To further reduce the amount of possible miRNA gene targets, gene ontologies relevant for this thesis were searched within the AmiGO gene ontology database (http://amigo.geneontology.org). Genes related to the ontologies “multicellular response to stress”, “learning or memory”, “regulation of neurotransmitter
levels” and “neurotransmitter transport” were compared to the obtained lists of gene targets for the miRNAs of interest by Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/).
5. Results

5.1. Conditioned safety signals reduce contextual fear

Mice were trained for 3 days in the learned safety and matching learned fear paradigms. 24 hours after the last training session the conditioned stimulus was presented to both groups in the conditioning context and the behavioral fear response as represented by percentage of time spent freezing during the 138 seconds long memory recall test was evaluated. Contextual fear was significantly reduced in animals trained for learned safety during the presentation of the conditioned stimulus. In contrast, in fear conditioned mice contextual fear was significantly increased during the conditioned stimulus display (Figure 6).

![Figure 6](image-url)  
**Figure 6. Display of the CS decreases contextual fear in safety conditioned mice (SC), while contextual fear in mice trained for learned fear (FC) is increased due to the CS presentation.** Fear is evaluated by measuring the percentage of time spent freezing in the conditioning chamber before (preCS) and during presentation of the conditioned stimulus (CS) (n=7-8). Error bars show the standard error from the mean. (**p < 0.01, ***p < 0.001).
5.2. Expression of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 differs in amygdala of learned safety trained animals from fear conditioned mice

To examine the possible function of miRNAs in learned safety, first we analyzed miRNA expression levels in amygdala of safety conditioned mice. Sense and antisense variant of 11 miRNAs, previously associated with stress, depressive disorders and abundant expression in the amygdala, were investigated in this experiment (Table 1).

2 hours after the memory test for safety or fear conditioning, animals were sacrificed. The amygdala was dissected, RNA was extracted and miRNA levels in amygdala tissue were quantified with qRT-PCR as described above. Expressional levels of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 showed a significant reduction in the amygdala of safety conditioned mice compared to fear conditioned mice (Figure 7).
Figure 7. miRNA expression levels are reduced in the amygdala in mice trained for learned safety (SC) compared to fear conditioned (FC) mice. miRNA expression of (A) miR-132, (B) miR-132*, (C) miR-212-5p, (D) miR-15b, (E) miR-92a, (F) miR-100 in the amygdala of safety conditioned and fear conditioned mice. Expression levels were determined by qRT-PCR. All values are normalized to the expression of SNORD61 (n=7-8). Error bars show the standard error from the mean. (*p < 0.05, **p < 0.01).

5.3. Expression of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 is not significantly changed in fear conditioned animals

To determine whether the effect on miRNA expression is specific for learned safety, we compared the expression levels of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 in the amygdala of fear conditioned mice to those of tone alone and shock alone controls. Tone-alone controls received the same number of tone stimuli as the fear conditioned mice, but without being exposed to any shocks. In contrast, shock-alone controls were solely presented with the same count of shocks. Fear conditioning had no significant
effect on expression of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 in the amygdala compared to tone-alone and shock-alone controls (Figure 8).

Figure 8. miRNA expression levels show no significant change between fear conditioned animals and tone alone and shock alone controls. miRNA expression of (A) miR-132, (B) miR-132*, (C) miR-212-5p, (D) miR-15b, (E) miR-92a, (F) miR-100 in the amygdala of fear conditioned mice and tone alone and shock alone control mice. Expression levels were determined via RT-PCR. All values are normalized to the expression of SNORD61 (n=7-8). Error bars show the standard deviation from the mean. n.s. (not significant) p > 0.05.
5.4. miRNAs that show a learned safety specific expression have various potential gene targets related to stress, learning or memory and neurotransmitter regulation

Since miRNAs inhibit translation of target mRNAs, we searched for potential target genes of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100. Accordingly, a bioinformatical scan was conducted, using the EimMo3 database. This database enabled us to find potential target genes for the miRNAs with expression in the amygdala. The same 916 potential target genes were predicted for miR-132 and miR-212, since these miRNAs share the same seed region. In addition, 1497 potential targets for miR-15b, 798 predicted targets for miR-92a and 65 target genes for miR-100 were found. To further characterize target gene predictions, the database AmiGO was searched for gene ontologies that are of interest for our project. Thereby the ontologies “multicellular response to stress”, “learning or memory”, “regulation of neurotransmitter levels” and “neurotransmitter transport” were found and the genes related to these ontologies were compared with the list of the miRNA target candidates. Multiple genes predicted as targets for miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 were associated with stress, learning or memory and neurotransmitter regulation (Figure 9, Table 5-Table 8).
Figure 9. Comparison of target predictions for miR-132, miR-132* and miR-212-5p with genes related to various gene ontologies. Target prediction genes from EIMMo369 (yellow) for miR-132/miR-132*/miR212-5p expressed in the amygdala were compared with genes related to (A) multicellular response to stress, (B) learning or memory, (C) regulation of neurotransmitter levels, (D) neurotransmitter transport. Lists of genes related to the used gene ontologies were obtained from AmiGO71 (blue).

In summary, we found a reduction in the expression level of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 in the amygdala of safety conditioned mice compared to fear conditioned mice. Furthermore, we did not see a change in expression of those six miRNAs in the amygdala of fear conditioned mice compared to tone-alone and shock-alone controls. Therefore we conclude that the reduction of expression in miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 is an effect specific for learned
safety. In addition, we found various potential target genes associated with stress responses, learning or memory and neurotransmitter regulation for miRNAs that showed a reduced expression in learned safety.

Table 5. Potential target genes for miR-132, miR-132*, miR-212-5p, miR-15b and miR-92a related to multicellular response to stress

<table>
<thead>
<tr>
<th>miR-132</th>
<th>miR-15b</th>
<th>miR-92a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pten</td>
<td>Bcl2</td>
<td>Pten</td>
</tr>
<tr>
<td>Mecp2</td>
<td>Bdnf</td>
<td>Grm7</td>
</tr>
<tr>
<td>Htr7</td>
<td>Grm7</td>
<td>Reln</td>
</tr>
<tr>
<td>Grm7</td>
<td>Reln</td>
<td>Cck</td>
</tr>
<tr>
<td></td>
<td>Ret</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drd1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mapk8ip2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vwa1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mecp2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tac1</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Potential target genes for miR-132, miR-132*, miR-212-5p, miR-15b and miR-92a related to learning or memory

<table>
<thead>
<tr>
<th>miR-132, miR132*, miR212-5p</th>
<th>miR-15b</th>
<th>miR-92a</th>
<th>miR-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pten</td>
<td>Btg2</td>
<td>Slc12a5</td>
<td>Adcy1</td>
</tr>
<tr>
<td>Slc6a1</td>
<td>Bdnf</td>
<td>Slc6a1</td>
<td>App</td>
</tr>
<tr>
<td>Mecp2</td>
<td>Grm7</td>
<td></td>
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</tr>
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<td>Foxp2</td>
<td>Reln</td>
<td>Itga8</td>
<td></td>
</tr>
<tr>
<td>Btg2</td>
<td>Foxp2</td>
<td>Pten</td>
<td></td>
</tr>
<tr>
<td>Adnp</td>
<td>Drd1a</td>
<td>Foxp2</td>
<td></td>
</tr>
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<td>App</td>
<td>Jun</td>
<td></td>
</tr>
<tr>
<td>Tmod2</td>
<td>Ric8</td>
<td>Prkar2b</td>
<td></td>
</tr>
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<td>Pja2</td>
<td>Nf1</td>
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</tr>
<tr>
<td>Slc1a4</td>
<td>Prkca</td>
<td>Hif1a</td>
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<td>Rgs14</td>
<td>Ntrk2</td>
<td>Serpinf1</td>
<td></td>
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<tr>
<td>Ppt1</td>
<td>Ptchd1</td>
<td>Prkar1b</td>
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<td>Chl1</td>
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</tr>
<tr>
<td>Adcy1</td>
<td>Tac1</td>
<td>Ephb2</td>
<td>Slc1a2</td>
</tr>
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<td>------</td>
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</tr>
</tbody>
</table>

**Table 7. Potential target genes for miR-132, miR-132*, miR-212-5p, miR-15b and miR-92a related to neurotransmitter transport**

<table>
<thead>
<tr>
<th>miR-132, miR132*</th>
<th>miR-15b</th>
<th>miR-92a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc6a1</td>
<td>Slc6a17</td>
<td>Slc6a1</td>
</tr>
<tr>
<td>Slc6a11</td>
<td>Cplx1</td>
<td>Slc6a11</td>
</tr>
<tr>
<td>Slc6a11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 8. Potential target genes for miR-132, miR-132*, miR-212-5p, miR-15b and miR-92a related to regulation of neurotransmitter levels**

<table>
<thead>
<tr>
<th>miR-132, miR132*</th>
<th>miR-15b</th>
<th>miR-92a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfn2</td>
<td>Wnt7a</td>
<td>Syn2</td>
</tr>
<tr>
<td>Ache</td>
<td>Kcnc4</td>
<td>Slc17a6</td>
</tr>
<tr>
<td>Syn2</td>
<td>Nat8l</td>
<td>Kcnc4</td>
</tr>
<tr>
<td>Ppt1</td>
<td>Nlgn1</td>
<td>Snap91</td>
</tr>
<tr>
<td>Nrxn3</td>
<td>Ppfia3</td>
<td>Cplx1</td>
</tr>
<tr>
<td></td>
<td>Syt4</td>
<td>Vamp2</td>
</tr>
<tr>
<td></td>
<td>Stx1a</td>
<td>Abat</td>
</tr>
<tr>
<td></td>
<td>Nf1</td>
<td>Napa</td>
</tr>
<tr>
<td></td>
<td>Dagla</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dvl1</td>
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</tr>
<tr>
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<td>Htr2c</td>
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</tr>
<tr>
<td></td>
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<tr>
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<tr>
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<td>Napa</td>
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<td></td>
<td>Rab3a</td>
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<tr>
<td></td>
<td>Cadps</td>
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</tr>
</tbody>
</table>
6. Discussion

Results of this thesis demonstrate that the expression of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 are significantly reduced in the amygdala of safety conditioned mice, while the expression of 6 other miRNAs tested, did not show expressional changes after safety learning. Considering that the expression of the 11 miRNAs previously tested in learned safety mice did not differ significantly in amygdala of fear conditioned mice to those of tone and shock alone controls, a finding that parallels observations by Griggs et al.\textsuperscript{56} reporting that the vast majority of miRNAs remain unchanged in their expression in rat amygdala following fear conditioning, it is implied that the decrease in expression of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 in amygdala of safety conditioned mice represents a specific consequence, and/or requirement for learned safety.

miRNAs are known to repress gene expression, hence downregulation of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 is expected to result in an increased expression of target genes. Therefore, the regulation of miRNA expression can be postulated as a mechanism for the modulation of learned safety specific gene expression in the amygdala. To determine whether expression of these miRNAs is required for the behavioral expression and the neuromolecular changes of learned safety, specific miRNA mimics should be expressed in the amygdala and the consequences of the acquisition and expression of learned safety should be tested behaviorally followed by analysis of amygdala gene expression.

Paralleling our observations on reduction of miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 in learned safety, an animal model for a behavioral intervention in depression, is a finding observed in non-learned helpless (NLH) rats which display a high resilience to stress, a protective factor for the development of several psychopathologies, including depressive disorders\textsuperscript{72}. These rats show a differential miRNA expression pattern in the frontal cortex compared to naïve controls\textsuperscript{73}. Interestingly, in the majority of miRNAs expression is decreased in NLH rats\textsuperscript{73}, which is consistent with the observations made in context of this thesis in learned safety mice. Here, all
miRNAs that were differentially expressed in amygdala of learned safety mice were reduced in their expression. Therefore it would be inviting to test in future experiments whether miRNAs downregulated in amygdala of learned safety mice may show the reverse trend in animal models of depression. First evidence in this direction is provided by results of experiments in rats using the maternal separation model of early adverse life events, known to increase the likelihood for the development of depressive disorders later in life. It was shown that maternal separation in rats induces an increase in expression of various miRNAs in the prefrontal cortex, for example miR-132 and miR-212.\textsuperscript{74} Notably, miR-132 and miR-212 were both downregulated in amygdala of safety conditioned mice, which indicates the importance of those miRNAs in orchestrating the mechanisms of depressive and antidepressive conditions in the brain.

Additionally it would be noteworthy to examine whether the observed changes in miRNA expression are specific to the amygdala or could be also observed in other brain regions forming part of the neural circuitry mediating learned safety, as it has been shown for the regulation of miRNA expression following treatment with pharmacological antidepressants. Baudry \textit{et al.}\textsuperscript{75} demonstrated, that miR-16 expression is increased upon fluoxetine treatment in the raphe nuclei and in contrast reduced in the hippocampus and the locus coeruleus. Complementing this important description of how pharmacological antidepressants act by mediating specific regulation of miRNA expression levels, our findings are, to the best of our knowledge, the first evidence that, in addition to pharmacological antidepressants, a non-pharmacological intervention for depression modulates the expression of a specific set of miRNAs.

miR-132, its miRNA counterpart miR-132* and miR-212, belong to the same miRNA family and are generated by miR-212/132 locus maturation. They are known to play a major role in regulation of various processes in the nervous system including neuronal development, maturation and function\textsuperscript{70} and are highly conserved among vertebrate species\textsuperscript{70}. Since they share an identical seed region, the target binding region of miRNAs, it is likely that miR-132 and
miR-212 have some mRNA targets in common\textsuperscript{70}, including previously validated target genes such as HB-EGF (Heparin binding EGF-like growth factor)\textsuperscript{76,77}, MeCP2 (Methyl-CpG-binding protein)\textsuperscript{78–80} and STAT4 (Signal transducer and activator of transcription 4)\textsuperscript{81}.

Interestingly, the miR-212/132 locus itself has been found to be regulated by the transcription factor CREB\textsuperscript{82}, one of the most prominent molecules implicated in specific forms of learning and memory\textsuperscript{83,84}. Activation of CREB by phosphorylation of CREB\textsuperscript{85} which can be facilitated by various factors such as neuronal activity, neurotrophic factors and cytokines\textsuperscript{82} induces promotion of transcription of its target genes including the miR212/-132 locus. Notably, in mice trained for learned safety electrical activity in the amygdala is decreased\textsuperscript{4}, which could also be paralleled by a reduction in CREB activity, thus reducing the expression of miR-132 and miR-212. Additional evidence for this hypothesis, which remains to be tested in future experiments, arises from the fact that overexpression of miR-132 promotes neurite outgrowth and dendritic morphogenesis\textsuperscript{82}, while in the amygdala of safety conditioned rats, synaptic size is decreased\textsuperscript{25}.

Interestingly, BDNF, which is tightly linked to the pathobiology of depression\textsuperscript{26} is known to promote the expression of miR-132 and miR-212\textsuperscript{65,86}. While BDNF protein expression is known to be enhanced after learned safety in the mouse hippocampus\textsuperscript{6}, its expression in the amygdala has not yet been investigated and region-specific expression of BDNF may be a modulating factor, controlling miR-132 and miR-212 expression related to learned safety mice.

Levels of miR-15b, miR-92a and miR-100 in learned safety were chosen to be analyzed based upon findings describing their upregulation in the amygdala following acute restraint stress (Table 1). In line with these results, we found that learned safety, known for its ability to relief from ongoing stress, reduced the expression of these miRNAs in the amygdala. These observations imply that expression of miR-15b, miR-92a and miR-100 is highly influenced by stress and by mechanisms facilitating relief from stress and further suggest that these miRNAs may also take part in mediating some of the
neurobiological processes associated with the pathophysiology of stress-related diseases, such as PTSD and depression.

miR-15b belongs to the miR-15/107 group of miRNAs, a group in which all members share an AGCAGC sequence starting at the first or second nucleotide from the 5' end. The miR15b/16-2 locus lies within the chromatin/DNA and cell proliferation-related SMC4 genes and miR-15b has been related to basic cellular functions, such as cell division and metabolism\textsuperscript{87}. Additionally, there is evidence for an involvement of miR-15b in the regulation of the stress response, since GC treatment has been shown to regulate the miR-15b/16-2 cluster and overexpression of miR-15b/16-2 \textit{in-vitro} caused an increase in sensitivity to GCs, while reduced expression of miR-15b/16-2 decreased GC sensitivity in cell lines\textsuperscript{88}. Considering the study described above, decreased levels of miR-15b in the amygdala of mice following learned safety, as we found in our experiments may result in a decreased sensitivity to GCs.

Stress, a predisposing factor for depression, induces the production of GCs by the adrenal cortex\textsuperscript{89}. GCs in return regulate brain structures related to emotional processing and cognition, such as the amygdala and the hippocampus by binding to GC receptors\textsuperscript{90}. Stress and even a single dose of GC administration have been reported to cause dendritic spine hypertrophy in the basolateral amygdala\textsuperscript{91}. Interestingly, spine synapses are decreased in size in the lateral amygdala of learned safety trained rats\textsuperscript{25}. It remains to be investigated in further experiments, if a reduced sensitivity to GC in the amygdala, caused by a decrease in miR-15b levels accounts for this decrease in synapse size in the lateral amygdala.

miR-92a is transcribed from the miR-17~92 cluster and its regulation has been extensively linked to various types of cancer\textsuperscript{92}. Although the precise mechanisms of action of miR-92a remain to be elucidated, its angiogenic capacities have been robustly demonstrated\textsuperscript{93}. Given the angiogenic-neurogenic link it would be tempting to speculate, that miR-92a could also be involved in the regulation of adult neurogenesis, a process related to depression and response to antidepressant treatment\textsuperscript{26,94}. Reduced
expression of miR-92a could then be mediating the enhanced survival of newly generated cells in the hippocampal dentate gyrus following safety learning\textsuperscript{6}, possibly through PTEN, one of its target genes\textsuperscript{95}. Additional support for a role of miR-92a in depression can be derived from reports describing a stimulation of the miR-17~92 cluster by interleukin-6 (IL-6)\textsuperscript{96}, a cytokine prominently linked to the pathophysiology of depression\textsuperscript{97,98}. However, cytokine expression in learned safety and its potential effect on the expression of miR-92a in the amygdala remain to be investigated.

miR-100, a member of the miR-99 family, has been demonstrated to target mammalian target of rapamycin (mTOR), a protein involved in the TrkB/PI3K/Akt signaling pathway\textsuperscript{99,100}. The TrkB/PI3K/Akt pathway is activated by binding of BDNF to its receptor TrkB, a neurotrophic factor highly involved in learned safety and moreover in the pathophysiology of depression. Activation of the TrkB/PI3K/Akt pathway promotes translation of mTOR\textsuperscript{101}. Considering the fact, that miR-100 expression is reduced in the amygdala following learned safety, one could speculate that the downregulation of miR-100 causes increased levels of its target mTOR. Interestingly inhibition of mTOR has been shown to disrupt consolidation and reconsolidation of amygdala dependent auditory fear memory\textsuperscript{102}. In an additional study, it was demonstrated that predictable chronic mild stress in adolescence had an antidepressant effect later in life and that this effect is mediated by increased mTOR signalling\textsuperscript{103}. The unpredictable chronic mild stress paradigm, an animal model for depression, caused a decrease in activated mTOR in the amygdala\textsuperscript{104}. Taking together the observations described above, the idea that miRNA-100 dependent mTOR regulation is involved in the mechanisms mediating the antidepressive effects of learned safety remains promising and has to be tested in further experiments.

Following up on miRNA expression studies, a bioinformatical scan was performed, to search for potential targets for miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 using several established algorithms. The platform EIMMo3 predicts targets based on complementarity level between miRNA seed regions and mRNA sequences and on evolutionary conservation.
of target sites\textsuperscript{105}. In total, 2476 target genes were found for miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100, which were further classified by gene ontologies “multicellular response to stress”, “learning or memory”, “regulation of neurotransmitter levels” and “neurotransmitter transport” using the gene ontology database AmiGO\textsuperscript{71}. These gene ontology groups have been chosen based upon their presumed relevance for learned safety and related neurobiological mechanisms. Various target genes for miR-132, miR-132*, miR-212-5p, miR-15b, miR-92a and miR-100 have been found to be associated with one or more of these gene ontologies (Table 9). Nevertheless, \textit{in-silico} predicted target genes need to be confirmed \textit{in-vitro} and/or \textit{ex-vivo} in further experiments, since computational targetscan predictions bear the potential for providing both false-positive and false-negative hits\textsuperscript{106}. 
Table 9. Target genes predicted by the EIMMo3 target scan for 2 or more microRNAs and/or related to two or more gene ontologies (GO). 132=miR-132, miR-132*, miR-212-5p; 15b=miR-15b; 92a=miR-92a; 100=miR-100. ST=multicellular response to stress; LM= learning or memory; REG= regulation of neurotransmitter levels; NT=neurotransmitter transport.

<table>
<thead>
<tr>
<th>Target of 2 or more miRs related to 2 or more GOs</th>
<th>Targeted by 2 or more miRs and related to 2 or more GOs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adcy1 (132,100) Bdnf (ST, LM)</td>
<td>Grm7 (132,15b,92a)(LM,ST)</td>
</tr>
<tr>
<td>App (100,15b) Cplx1 (NT, REG)</td>
<td>Mecp2 (132,15b) (ST,LM)</td>
</tr>
<tr>
<td>Btg2 (132,15b,92a) Drd1a (ST,LM)</td>
<td>Pten (132,92a) (ST,LM)</td>
</tr>
<tr>
<td>Cnr1 (132,15b,92a) Htr7 (ST,LM)</td>
<td>Reln (15b,92a) (ST,LM)</td>
</tr>
<tr>
<td>Foxp2 (132,15b,92a) Mapk8ip2 (ST,LM)</td>
<td>Slc6a1 (132,15b,92a)(LM,NT)</td>
</tr>
<tr>
<td>Itga8 (132,92a)</td>
<td></td>
</tr>
<tr>
<td>Kcnc4 (15b,92a)</td>
<td></td>
</tr>
<tr>
<td>Napa (15b,92a)</td>
<td></td>
</tr>
<tr>
<td>Slc1a4 (132,15b)</td>
<td></td>
</tr>
<tr>
<td>Slc6a11 (15,92)</td>
<td></td>
</tr>
<tr>
<td>Syn2 (132,92)</td>
<td></td>
</tr>
</tbody>
</table>

Some of those predicted target genes appear as prime candidates for future corroboration in molecular experiments due to their proposed role in emotional (dys)regulation and potential target genes predicted for two or more miRNAs that are related to two or more gene ontologies are further discussed below.

Metabotropic Glutamate Receptor 7 (Grm7)

L-glutamate is the main excitatory neurotransmitter in the central nervous system, exerting its action by binding to ionotropic and metabotropic glutamate receptors. The metabotropic glutamate receptor 7 (mGluR7) which is highly expressed in the amygdala and the hippocampus, two brain regions critically involved in learned safety, is located presynaptically and acts as autoreceptor by inhibiting L-glutamate release, thereby creating a negative feedback loop. Interestingly, mGluR7 knockout mice display impairments in...
amygdala-dependent learning, including fear extinction, which, such as learned safety is fear-restraining mechanism fear\textsuperscript{109}. mGluR7 has also been linked to the pathophysiology of depression\textsuperscript{108}. In experimental animals, activation of mGluR7 has been shown to reduce behavioral despair in the FST\textsuperscript{110} and treatment of primary neuronal cultures with the mood stabilizers lithium and VPA lowered miR-34a levels herby increasing protein levels of its target mGluR7\textsuperscript{55}. The results of this thesis show that expression of miR-34a is unchanged in the amygdala of safety and fear conditioned mice. Nevertheless, decreased expression of microRNAs potentially targeting the mGluR7 in the amygdala, such as miR-132, miR-132*, miR-212-5p, miR15b and miR-92a observed herein may cause an increase of mGluR7 with potential relevance for the behavioral and cellular effects of learned safety.

**Phosphatase and tensin homolog (Pten)**

PTEN, a prominent tumor suppressor, acts as a lipid and protein phosphatase. Aside from its tumor suppressive function, PTEN regulates neuronal migration\textsuperscript{111,112} and controls neuronal size\textsuperscript{113} in the brain.

PTEN has been demonstrated to inhibit CREB\textsuperscript{114}, a transcription factor involved in regulation of a multitude of genes, ranging from synaptic plasticity genes to genes involved in the pathophysiology of depression\textsuperscript{115,116}. Furthermore, CREB has been shown to induce the expression of miR-132 and miR-212\textsuperscript{82}. PTEN, an already validated target of miR-92a\textsuperscript{95} and a potential target for miR-132, miR-132* and miR-212, all miRNAs that show a decrease in expression in the amygdala following learned safety, may therefore be increased in the amygdala following safety learning. An increase in PTEN could further cause a decrease in CREB activation, thereby regulating expression of miR-132 and miR-212 in a negative feedback loop.

Conditional inactivation of PTEN in mice demonstrated that these mice exhibit social deficits and an impairment of social learning. In addition, these mice display an increased initial startle response and enhanced anxious behavior in the light-dark box and the open field test, but not in the elevated plus maze\textsuperscript{117}. It needs to be determined, if elevated PTEN levels elicited by decreased levels of miR-92a, miR-132, miR-132* and miR-212-5p could contribute to the fear-inhibitory properties of learned safety.
**Methyl CpG binding protein 2 (MeCP2)**

MeCP2 is a protein that binds to methylated cytosins in the DNA, and further recruits histon deacetylases. Deacetylation of histones causes the formation of heterochromatin, and thereby transcriptional inhibition\(^\text{118}\). In contrast, MeCP2 has recently also been reported as transcriptional activator by interacting with CREB at promoters of active genes\(^\text{119}\). MeCP2 is a validated target of miR-132\(^\text{64}\) and miR-212\(^\text{80}\).

MeCP2 binds to the BDNF gene and represses its transcription, however upon neuronal activation, MeCP2 is phosphorylated and dissociates from the BDNF gene, thereby contributing to the mechanisms behind activity-dependent expression of BDNF\(^\text{120}\). It has been shown that learned safety causes a reduction of neuronal activity in the amygdala\(^\text{4}\). When these observations are further translated, one may speculate that the BDNF gene remains repressed by MeCP2 in the amygdala following learned safety. In addition, reduced miR-132, miR-132* miR-212-5p and miR-92a levels in the amygdala following learned safety may lead to an increase of MeCP2.

Enhanced levels of MeCP2 following learned safety is in agreement with previous reports on increased levels of anxiety in mice with a specific deletion of MeCP2 in the basolateral amygdala. In addition, these mice exhibited deficiencies in auditory fear conditioning further highlighting the importance of MeCP2 for amygdala dependent associative learning processes\(^\text{121}\). To obtain conclusive insights into the involvement of MeCP2 in the molecular mechanism of learned safety, further experiments need to be conducted addressing the expression of MeCP2 following learned safety and examining its requirement for safety learning *in-vivo*. 
**Reelin (Reln)**

In the brain, the glycoprotein reelin has multiple functions ranging from regulation of neuronal cell migration and proper brain lamination in early development to an involvement in neurogenesis and synaptic plasticity during adulthood\(^{122}\).

A multitude of postmortem studies have been establishing a link between the *Reln* gene and various neuropsychiatric disorders including schizophrenia, bipolar disorder, autism, major depression, lissencephaly and Alzheimer’s disease\(^{123}\), demonstrating lower levels of reelin in patient brains\(^{124}\). Moreover, in experimental animals, reelin overexpression had an antidepressant effect in the FST\(^{125}\) and the antidepressant citalopram, which acts as a selective serotonin reuptake inhibitor (SSRI) has been shown to increase reelin levels in the brain\(^{126}\). Interestingly, in mice trained for learned safety behavioral despair is reduced in the FST in the presence of the conditioned stimulus\(^6\), as it is seen in response to overexpression of reelin. We found that miR-15b and miR-92a are decreased in expression in the amygdala following safety learning. If reelin will be validated as target of miR-15b and miR-92a in a future experiment, one could further speculate that reelin is a crucial factor in mediating the mechanisms of learned safety and contributes to the antidepressant effects of learned safety.

In a further study, Lin et al.\(^{127}\) found that miR-128b is increased due to fear extinction, which, similar to learned safety, acts as a fear inhibitory mechanism. It has also been shown there that miR-128b targets the reelin mRNA *in-vitro*. However, reelin was increased following fear extinction\(^{127}\). Therefore, it has to be further evaluated if reelin might be a target of miR-92a and miR-15b and is increased when the levels of those two miRNAs are downregulated, which may be a fundamental mechanism of facilitating inhibition of fear.
GABA Transporter 1 (Slc6a1)

GABA transporters, including GABA transporter 1 (GAT1) remove GABA, the main inhibitory neurotransmitter in the mammalian brain, from the synaptic cleft back into the intracellular space of neurons and glia hereby terminating its action on postsynaptic receptors\textsuperscript{128}. GAT1 is abundantly expressed on presynaptic nerve terminals of the limbic system including the amygdala and the hippocampus\textsuperscript{129}. It was demonstrated that GAT1 knockout mice exhibit antidepressive and anxiolytic behavior\textsuperscript{130} and that acute and chronic administration of a selective GAT1 inhibitor induces an antidepressive and anxiolytic effect\textsuperscript{131}.

Fear conditioning requires neuronal activation of the amygdala by sensory processing regions in thalamus and cortex\textsuperscript{18}. This activation is under control of GABAergic neurons, which are thought to prevent generalization of fear, pointing out the importance of GABA transmission in the fear conditioning circuit\textsuperscript{132}. In an additional study, it was demonstrated that fear extinction reduces the mRNA level of GAT1 in the amygdala\textsuperscript{133}.

Our results show that learned safety reduces amygdalar expression of miR-132*, miR-132, miR-212-5p, miR-92a and miR-15b and all of these miRNAs potentially target GAT1 mRNA. If GAT1 can be validated as target for these miRNAs, decreased miRNA levels may result in an increase of GAT1 and postsynaptic inhibition by GABA may be reduced in the amygdala. An increase in GAT1 levels following learned safety would therefore contrast the observations described above- namely the antidepressant and anxiolytic property of GAT1 inhibition, the property of GABA to inhibit fear generalization and the decrease of GAT1 following fear extinction. Therefore the potential role of GAT1 in learned safety still has to be determined by further studies.
7. Conclusion and perspectives for the future

In context of this thesis, it was found that expression of the 6 miRNAs miR-132, miR-132*, miR-212-5p, miR15b, miR-92a and miR-100 is selectively modulated in the amygdala following learned safety. The molecular and behavioral consequences of this learned safety specific expressional modulation remain to be investigated in further experiments. Considering the function of miRNAs as post-transcriptional regulators of target genes, it is likely that miRNAs showing learned safety-specific expression regulate various genes thereby forming the molecular basis for the neural mechanisms underlying learned safety. Various potential target genes for miR-132, miR-132*, miR-212-5p, miR15b, miR-92a and miR-100 have been identified in-silico in context of this thesis. Further validation of these target genes in-vitro and in-vivo will increase our knowledge about the function of miRNA modulation in learned safety and the mechanisms by which learned safety elicits its molecular and behavioral effects.
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10. List of abbreviations

B6. C57Bl/6J
BDNF. brain derived neurotrophic factor
CS. conditioned stimulus
dIPFC. dorsolateral prefrontal cortex
fMRI. functional magnetic resonance imaging
FST. forced swim test
GAT1. GABA transporter 1
GC. glucocorticoids
IL-6. interleukin-6
MDD. major depressive disorder
MeCP2. Methyl CpG binding protein 2
mGluR7. metabotropic glutamate receptor 7
miRNA. microRNA
mTOR. mammalian target of rapamycin
PTEN. phosphatase and tensin homolog
PTSD. post-traumatic stress disorder
qRT-PCR. quantitative real-time polymerase chain reaction
RT. room temperature
Si. sensory insula
Sli. Svmj
SSRI. selective serotonin reuptake inhibitor
SPT. sucrose preference test
US. Unconditioned Stimulus
VPA. valproate
11. Appendix

11.1. Zusammenfassung


**Methoden:** In einem ersten Experiment wurden die Expressionslevels von elf microRNAs in Gewebe der Amygdala von gelernter Sicherheit und gelernter Angst trainierten Mäusen durch qRT-PCR analysiert. In einem zweiten Experiment wurde die Expression der selben elf microRNAs in für gelernte Angst trainierten Mäusen mit Ton-allein und Schock-allein Mäusen verglichen. Schlussendlich wurde eine bioinformatische Untersuchung durchgeführt um nach potentiell durch die microRNAs regulierten Genen zu suchen.

**Ergebnisse:** Es wurde gefunden, dass sich die Expression von fünf microRNAs in der Amygdala von für gelernte Sicherheit und gelernte Angst trainierten Mäusen signifikant unterscheidet. Keine Unterschiede in der Expression dieser microRNAs wurde in der Amygdala von für gelernte Angst trainierten im Vergleich zu Ton-allein und Schock-allein Mäusen gefunden. Durch die bioinformatische Untersuchung wurden mehrere Gene entdeckt, die potentiell durch microRNAs mit für gelernte Sicherheit spezifischer Expression reguliert sein könnten.

**Diskussion:** Die selektive Modulierung der Expression von fünf verschiedenen microRNAs in der Amygdala aufgrund von gelernter Sicherheit legt nahe, dass diese microRNAs für die Regulation von unterschiedlichen Genen verantwortlich sind, die die molekulare Basis der gelernten Sicherheit zugrunde liegenden neuronalen Mechanismen formen.
11.2. Abstract

**Background:** To detect and interpret safety signals and moreover to react appropriately to those signals is crucial for mental health. Learned safety involves learning about signals indicating protection from danger, thus modulating fear responses. The amygdala is a brain region where specific gene expression changes resulting from safety learning have been observed in mice. The aim of the present study is to try to understand how gene expression in the amygdala during learned safety is regulated focusing on the role of microRNAs.

**Methods:** In a first experiment, expressional levels of 11 miRNAs were analyzed in amygdala tissue of learned safety and learned fear trained control mice by qRT-PCR. In a second experiment, expression of the same 11 miRNAs was analyzed in mice trained in a learned fear paradigm and compared to tone alone and shock alone controls. Finally, a bioinformatical scan was performed, to search for potential microRNA target genes.

**Results:** Amygdala expression of 5 miRNAs has been found to differ significantly between learned safety and learned fear trained animals. No differences in the expression of these miRNAs were found between learned fear and tone-alone and shock-alone control groups. A bioinformatical scan revealed various potential target genes for the miRNAs with learned safety specific expression related to stress and depression.

**Discussion:** The selective modulation of expression of 5 specific miRNAs in the amygdala following learned safety suggests that these miRNAs may account for the regulation of various target genes forming the molecular basis for the neural mechanisms underlying learned safety.
11.3. Danksagung


Weiters möchte ich mich bei allen Mitgliedern dieser Arbeitsgruppe bedanken, die mir die Zeit hier so lustig und lehrreich gemacht haben, besonders bei Giorgia Savalli, mit der es dank ihrer Hilfe und Geduld eine große Freude war zusammen zu arbeiten.

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