# Novel Insights into a microRNA-based Mechanism Underlying Oxytocin-mediated Anxiolysis and Reversal of Social Fear

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*“Of all base passions, fear is most accursed.”*

- William Shakespeare (King Henry IV, First Part)

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# Abstract

Everybody knows these situations, which evoke feelings of discomfort: the lonely walk home by night, a big black spider dangling above the bed or giving a speech in front of strangers. Anxiety and fear are natural responses to real or perceived threats and have been conserved throughout evolution. However, excessive fear and anxiety typically manifests as pathological disease state, such as generalized anxiety disorder, posttraumatic stress disorder or social anxiety disorder. To date, anxiety disorders are a high burden for society and ecology, and the available treatment options are limited and elicit numerous adverse side effects.

In the last decade, the anxiolytic and pro-social neuropeptide oxytocin gained focus amongst researchers as novel treatment option for anxiety disorders. Oxytocin binds to a G protein-coupled receptor, thereby activating intracellular signaling pathways, which have not yet been deciphered in detail. In the early 2000´s, non-coding RNAs, especially microRNAs, have been characterized as potent gene-regulatory molecules. microRNAs form regulatory networks to modulate gene expression on a post-transcriptional level. Due to their high regulatory potential and the availability of simple methods to manipulate microRNAs within the central nervous system, they are suggested to be innovative options for the development of new treatment alternatives.

In this thesis, I focused on the functional involvement of microRNAs in intracellular signaling pathways, which are essential for the anxiolytic and social fear-reversing properties of the neuropeptide oxytocin in rodents. *Via* microRNA expression analysis of the paraventricular nucleus (PVN), I revealed that the transcription of miR-132-3p is induced upon intracerebroventricular oxytocin application in male and female rats, an effect, which is abolished in response to pre-treatment with an oxytocin receptor antagonist. In contrast, chronic activation of the endogenous oxytocin system during lactation did not alter intra-PVN miR-132-3p level, but short-term separation of the mother from its pups increased miR-132-3p transcript levels within the PVN and cerebrospinal fluid of the dams. In a further pilot experiment, I showed that functional inhibition of miR-132-3p *via* a locked nucleic acid (LNA) prevents the anxiolytic properties of oxytocin applied into the PVN, whereas no explicit effect was seen in cued fear conditioning. In summary, oxytocin has been revealed to induce the transcription of miR-132-3p, which is in turn essential for the anxiolytic properties of the neuropeptide.

Moreover, I showed that septal miR-132-3p is involved in the extinction of social fear in male mice: Compared to unconditioned mice, conditioned animals had increased septal miR-132-3p levels after acquisition of social fear. Additionally, functional inhibition of septal miR-132-3p *via* a LNA

impaired extinction, whereas viral overexpression facilitated extinction of social fear. Interestingly, septal LNA-induced miR-132-3p inhibition prevented the oxytocin-induced reversal of social fear and shRNA-mediated downregulation of septal miR-132-3p specifically in oxytocin receptor expressing neurons impaired extinction of social fear. Thereby, miR-132-3p was proven to be essential for the social fear reversing properties of oxytocin. Further analysis of putative septal target messenger RNAs of miR-132-3p *via* microarray analysis revealed several promising candidates, all of which have not been found to be altered after acquisition and extinction of social fear in mice.

In summary, these experimental results expand our understanding of the mechanisms underlying the anxiolytic and social fear-reversing properties of the neuropeptide oxytocin, and reveal that small non-coding ribonucleic acids exert fundamental influence within the central nervous system.

# Zusammenfassung

Jeder kennt diese eigenartigen Momente, die innerliches Unwohlsein auslösen: der nächtliche Nachhauseweg entlang verlassener, dunkler Gassen, die große schwarze Spinne über dem Bett, oder das Halten einer Rede vor größerem Publikum. Aus evolutionsbiologischer Sicht sind ebendiese Angst- und Furchtreaktionen konservierte, natürliche Handlungsweisen als Antwort auf reale oder gefühlte Bedrohungen. Eine exzessive bzw. unangepasste Ausprägung von Angst- oder Furchtzuständen manifestiert sich allerdings für gewöhnlich in pathologischen Krankheitsbildern. Diese beinhalten unter Anderem generalisierte Angststörungen, posttraumatische Belastungsstörungen und soziale Angststörungen. Heutzutage repräsentieren Angsterkrankungen eine Hauptbelastung der modernen Gesellschaft, vor Allem, da die derzeitigen Behandlungsmethoden stark limitiert und von verheerenden Nebenwirkungen geprägt sind.

In den letzten Jahren ist das Neuropeptid Oxytocin aufgrund seiner potenten angstlösenden und pro-sozialen Wirkung in den Fokus der Erforschung neuer pharmakologischer Behandlungsmöglichkeiten für Angsterkrankungen gerückt. Oxytocin bindet an einen G-Protein-gekoppelten Rezeptor und reguliert dadurch komplexe neuronale Signalwege, die bis heute nicht im Detail entschlüsselt sind. In den frühen 2000er Jahren wurden nicht-kodierende Ribonukleinsäuren, im Speziellen sogenannte microRNAs, als potente Regulatoren diverser intrazellulärer Signalwege gefunden. Diese bilden große regulatorische Netzwerke, mit welchen sie die Expression von Genen auf einem post-transkriptionalen Level beeinflussen. Durch ihr hohes regulatorisches Potential und die Tatsache, im zentralen Nervensystem auf einfachem Wege manipulierbar zu sein, stellen microRNAs innovative Angriffspunkte zur Entwicklung neuer Psychopharmaka dar.

Im Zuge meiner Dissertation untersuchte ich die Fragestellung, welche microRNAs in die neuronalen Signalwege von Oxytocin involviert sind und dadurch sowohl Angstverhalten, als auch soziale Furcht in Nagetieren beeinflussen. Mittels Analyse der microRNA Expression konnte ich zeigen, dass die Transkription einer bestimmten microRNA, nämlich miR-132-3p, im *Nucleus paraventricularis* (PVN) der Ratte, durch intracerebroventrikuläre Applikation von Oxytocin geschlechtsunabhängig induziert wird, wohingegen eine vorangehende Infusion eines Oxytocin Rezeptor Antagonisten diesen Effeckt verhinderte. Im Gegensatz dazu, hat die langanhaltende Aktivierung des endogenen Oxytocin-Systems während der Laktation, keine Änderung der miR-132-3p Expression hervorgerufen. Lediglich die kurzfristige Trennung der Jungtiere von der Mutter, führte sowohl im PVN, als auch in der Cerebrospinalflüssigkeit der Muttertiere zu einem Anstieg der miR-132-3p Level. In einem Pilotversuch wies ich nach, dass die lokale Applikation (PVN) sogenannter „Locked Nucleic Acids“ (LNAs), welche

die Funktion der miR-132-3p verhindern, in männlichen Ratten die angstlösende Wirkung von intracerebral appliziertem Oxytocin unterdrücken, wohingegen sich die Effekte auf konditionierte Furcht als nicht eindeutig herausstellten. Zusammenfassend gesagt, konnte ich also aufzeigen, dass die microRNA miR-132-3p durch das Neuropeptid Oxytocin induziert wird und an der angstlösenden Wirkung des Neuropeptids beteiligt ist.

Wie sich herausstellte, ist dieselbe microRNA im *Septum* der Maus in die Auslöschung von konditionierter sozialer Furcht involviert: Nach dem Erlernen der sozialen Furcht zeigten sozial konditionierte Mäuse, im Vergleich zu nicht-konditionierten, eine höhere Expression der miR-132-3p. Ebenso verlangsamte die funktionale Inhibition der miR-132-3p im *Septum*, mittels LNAs, die Auslöschung von sozialer Furcht, wohingegen eine Virus-induzierte Überexpression derselben, diesen Prozess beschleunigte. Zusätzlich verhinderte die funktionale Inhibition mittels LNAs die Oxytocin-vermittelte Auslöschung von sozialer Furcht. Letztlich führte eine Virus-induzierte Repression der miR-132-3p Expression, speziell in Oxytocin Rezeptor exprimierenden Neuronen des *Septums* der Maus, zu einer verschlechterten Auslöschung der sozialen Furcht. Eine weitere Analyse potentieller Ziel-RNAs nach funktionaler Inhibition der miR-132-3p im Maus *Septum* mittels Microarray-Analyse lieferte einige vielversprechende Kandidaten, deren miR-132-vermittelte Regulation der Transkription im Tiermodell der konditionierten sozialen Furcht allerdings nicht bestätigt werden konnte. Kurz gefasst: Ich konnte zeigen, dass die microRNA miR-132-3p essentiell in die Oxytocin-vermittelte Auslöschung von konditionierter sozialer Furcht involviert ist.

Diese experimentellen Ergebnisse erweitern das Verständnis jener Mechanismen, welche der Oxytocin-vermittelten Angstminderung und Auslöschung sozialer Furcht zugrunde liegen, und zeigen, dass auch kleine nicht-kodierende Ribonukleinsäuren im zentralen Nervensystem einen großen Einfluss haben können.

# Introduction

## 1.1 The Social Brain - Implications for Pathologies

Social neuroscience has developed into a major area of current research in behavioral neurobiology and psychiatry. Studies on the neurobiological basis of **social behavior** are of particular interest since numerous psychiatric disorders are symptomized by social deficits (Bludau et al., 2019; Fernández et al., 2018). Social behavior requires perception and integration of socially relevant cues through a complex social cognition process, which involves attention, memory, motivation, and emotion. In general, social situations are essential for the survival and propagation of a species. Hence, brain networks, as well as neurobiological and molecular mechanisms underlying social behavior are evolutionary highly conserved across species. The quantitative variation in the synthesis, release, and/or receptor density of crucial molecules accounts for observed inter- and intra-specific variability of the socio-behavioral response (Fernández et al., 2018). Past, recent, and future studies are shedding light onto how dysregulations of specific neuronal circuits and molecular characteristics could lead to psychopathologies, which include social dysfunctions. The activity of involved brain structures and neural circuits is modulated by several neurotransmitter and neuromodulator systems. Here, **monoaminercic** transmitters as well as neuropeptides, especially the nonapeptide oxytocin (OXT), play a key role.

**Social behavior** is the behavior displayed when individuals of the same species interact with each other. It includes pair bonding, sexual, maternal, aggressive, and defensive behavior as well as social cognition, which is often constituted by a complex combination of olfactory, auditory, visual, and tactile stimuli.

**Monoaminergic** neurotransmitters and neuromodulators include serotonin, dopamine, noradrenaline, and adrenaline.

## 1.2 The Mammalian Oxytocin System

Sir Henry Dale discovered in 1906 that posterior pituitary extracts stimulate uterus contractions in a cat. Hereupon, he named the found substance OXT from the Greek words “ὀξύς τόκος”, meaning quick birth. Accordingly, the most famous peripheral effect of OXT is the promotion of uterine contractions facilitating and accelerating birth (Fuchs and Poblete, 1970). Even today, intravenous OXT infusions are used in preclinical and clinical obstetrics to speed up the birth process

and prevent excessive postpartum hemorrhage *via* myometrial contraction. Beyond that, OXT maintains neuroendocrine signaling during milk ejection (Jurek and Neumann, 2018). OXT signaling within the mammary gland leads to contraction of myoepithelial cells, which promotes milk ejection, an effect, which only recently has been found to be mediated by alterations in calcium oscillations (Stevenson et al., 2019).

The nonapeptide OXT is synthesized in magnocellular and parvocellular neurons of the paraventricular nucleus (PVN), supraoptic nucleus (only magnocellular neurons; SON), and accessory nucleus of the hypothalamus (Althammer and Grinevich, 2017; Meyer-Lindenberg et al., 2011; Rhodes et al., 1981; Swanson and Sawchenko, 1983). Magnocellular neurons of the PVN synthesize the two peptide hormones OXT or arginine vasopressin (AVP), whereas parvocellular neurons of the PVN synthesize OXT, AVP, corticotropin-releasing factor (CRF), and thyreotropin-releasing hormone.

Within magnocellular neurons, OXT along with its carrier proteins called neurophysins is stored and transported in large-dense core vesicles (LDCV). Magnocellular OXT neurons are 20-30 µm in diameter and are densely packed with LDCVs (85% of the total neuronal volume) and thus contain substantial amounts of the neuropeptide (Stoop, 2012; Stoop et al., 2015). The LDCVs are axonally transported from the hypothalamus along the neurohypophysial stalk to the respective axon terminals in the neurohypophysis, which form **neurohemal** contacts, from which OXT is released into the peripheral blood circulation. Within the brain, magnocellular OXT neurons project to forebrain and **limbic** structures, such as the nucleus accumbens (Dölen et al., 2013), lateral septum (LS) (Menon et al., 2018), and central **amygdala** (Knobloch et al., 2012). Local release of OXT from these projections into the respective brain regions has been shown to alter social reward or the fear response. Furthermore these magnocellular OXT projections are suggested to modulate maternal aggression as well as aggressive and dominant behavior in males (Bosch et al., 2005; Calcagnoli et al., 2014). In addition to axonal transport and release of OXT, dendrites have been

**Neurohemal contacts** are defined connections between neurons and the blood stream to release neurosecretory substances into the blood.

The **limbic system** of the brain includes amygdala, hippocampus, thalamus, hypothalamus, basal ganglia, and cingulate cortex.

The **amygdala** is well-characerized as center for memory processing, decisiton-making, and emotional responses. It is constituted of several nuclei: Medial nucleus, central nucleus, basal nucleus, and lateral nucleus. Moreover, clusters of intercalated cells also belong to the amygdala.

found to be a substantial source of neuropeptide release: Electron microscopic profiles demonstrated high peptide immunoreactivity (Armstrong, 1995) and abundant LDCVs have been shown in somatodendritic structures (Pow and Morris, 1989). This dendritic OXT release (central and peripheral) from magnocellular neurons of the SON differs in its temporal dynamics and does not necessarily follow a linear fashion (Ludwig, 1998; Ludwig and Leng, 2006; Neumann et al., 1993a).

In contrast to magnocellular neurons, parvocellular OXTergic neurons (10-20 µm soma diameter) mainly terminate in the spinal cord and brain stem (Swanson and Sawchenko, 1983), where they modulate autonomic functions, such as cardiovascular reactions (Petersson, 2002), breathing (Mack et al., 2002), erection and copulation (Melis et al., 1986), gastric reflexes (Sabatier et al., 2013), and feeding behavior (Atasoy et al., 2012). *Via* PVN-SON interconnections, parvocellular OXT neurons are suggested to control and orchestrate the activity of magnocellular OXT neurons within the SON, thereby facilitating analgesia by repression of nociception (Eliava et al., 2016).

Recently, it has been speculated that the brain OXT system consists of at least four neuronal subpopulations, which are distinguishable by the expression of several genetic markers (Romanov et al., 2017), and axonal projections of parvocellular neurons to the forebrain as well as magnocellular neurons to the midbrain have been reported (Althammer and Grinevich, 2017), which further enhances the necessity of a detailed characterization of the brain´s OXTergic projections.

Upon activation of OXTergic neurons, intracellular $Ca^{2+}$ concentrations are increased, evoking the release of LDCVs (Hökfelt, 1991). The OXT-triggered $Ca^{2+}$ can originate from extracellular sources, such as influx *via* N-type voltage gated $Ca^{2+}$ channels (Fisher and Bourque, 1996) or N-methyl-D-aspartate (NMDA) receptors (Hu and Bourque, 1992), but it is also released from intracellular stores, such as the endoplasmic reticulum (Lambert et al., 1994). The intracellular $Ca^{2+}$ rise leads to rapid and reversible depolymerization of F-actin to G-actin, and the OXT-filled LDCV is transported to the cell membrane. The subsequent binding of

the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complex leads to fusion of the vesicle and plasma membrane, resulting in release of OXT into the extracellular space (Brown, 2016; Ludwig et al., 2016).

Once released, OXT has a half-life time of 20 min in the cerebrospinal fluid (CSF) (Ludwig and Leng, 2006) and approximately 1.5 min in the blood (Higuchi et al., 1986). It is mainly degraded in a process that is performed by aminopeptidases (Stoop, 2012). Due to its hydrophilic peptidergic structure, peripheral OXT can not easily cross the **blood brain barrier** in physiologically-relevant concentrations (Leng and Ludwig, 2016). Only recently, a mechanism by which physiologically-relevant amounts of OXT are transported through the blood brain barrier has been described to involve the vascular receptor for advanced glycation end-products (RAGE) (Yamamoto et al., 2019). This unidirectional transport of OXT from the periphery to the central nervous system (CNS) is suggested to be especially important under conditions of dramatically increased blood OXT concentrations, such as child birth and lactation, which lead to saturation of the peripheral receptor occupancy and thereby promote the transport of the neuropeptide through the blood brain barrier.

Summarized, abovementioned studies reveal that OXT neurons show widespread central projections from the core nuclei of the hypothalamus to distal brain regions, which is essential for the evolution of a fine-tuned interconnected neuro-modulatory network.

The **blood brain barrier** is a highly semipermeable membrane separating the circulating blood from the extracellular fluid of the central nervous system. It allows passive diffusion and selective transport of molecules, which are essential for neuronal function, such as glucose, water, and amino acids.

**GPCRs** (also known as 7-transmembrane domain receptors) constitute a large protein family of receptors. They detect extracellular molecules and hence activate intracellular signal transduction pathways.

### 1.2.1 Brain Oxytocin Receptor Distribution and Intracellular Signaling

The OXT receptor (OXTR) is a G-protein coupled receptor (**GPCR**) including a 7-transmembrane domain. High OXTR expression is found in cortical areas, the olfactory system, the limbic system (especially LS, amygdala, subiculum, thalamus, and hypothalamus) (Gimpl and Fahrenholz, 2001; Jurek and Neumann, 2018). As GPCR, it is coupled to a $G_q$ protein, which activates phospholipase C (PLC). PLC cleaves

inositol-4,5-bis-phosphate ($PIP_2$) resulting in 1,2-diacylglycerol (DAG), which activates protein kinase C, and inositol-1,4,5-triphosphate ($IP_3$) that increases intracellular $Ca^{2+}$ levels (van den Burg and Neumann, 2011; Jurek and Neumann, 2018).

Recently, it was revealed that the $Ca^{2+}$ rise elicited by OXTR activation is mainly mediated by transient receptor potential cation channel subfamily V member 2 (TrpV2) channels in a phosphoinositide 3-kinase-dependent manner (van den Burg et al., 2015). Furthermore, this $Ca^{2+}$ influx from the extracellular space is essential for protein kinase C (PKC), calcium/calmodulin dependent protein kinase I (CaMKI), II, IV, and calcineurin (CaN) cascade activation (Jurek and Neumann, 2018). OXT binding further induces transactivation of the epidermal growth factor receptor (EGFR), subsequent mitogen-activated protein kinase (MAPK) kinase (ERK1/2, ERK5, p38) activation, by which the anxiolytic effect of OXT within the PVN is mediated in male (Blume et al., 2008), as well as female virgin and lactating rats (Jurek et al., 2012). All named signaling cascades converge on the cAMP responsive element binding protein (CREB)-CREB-regulated transcription coactivator (CTRC)/myocyte enhancer factor 2 (**MEF-2**) transcription factor complex (CREB-CRTC/MEF-2), which results in transcriptional activation of target genes. Moreover, *de novo* protein synthesis elicited by OXT is known to be dependent on the eukaryotic elongation factor 2 (eEF2) (Martinetz et al., 2019). However, in-depth information on intracellular signaling cascades and mechanisms is to date still marginal, highlighting that further precise research is required in order to employ OXT as possible treatment option, which is, due to the lack of knowledge, heavily debated.

### 1.2.2 Central Effects of Oxytocin – Focus on Sociability, Fear, and Anxiety

OXT is released within numerous brain regions in response to reproductive, stressful, and social stimuli (Landgraf and Neumann, 2004; Neumann, 2009; Neumann and Slattery, 2016; Neumann et al.,

**MEF2** proteins are a class of transcription factors, which are essential regulators of cellular differentiation and stress response mediation. Four MEF2 isoforms are present in mammals: MEF2A, MEF2B, MEF2C, MEF2D.

1993b; Wotjak et al., 2001; Zoicas et al., 2014). On one hand, synthetic OXT has been found to facilitate sociability and prevent social avoidance in rats and mice (Lukas et al., 2013). On the other hand, optogenetically triggered release of OXT enhances social recognition by modulation of cortical control of early olfactory processing (Oettl et al., 2016), and endogenous OXT is known to regulate pair bonding (Carter et al., 1995; Cho et al., 1999; Insel and Hulihan, 1995; Shapiro and Insel, 1992), and maternal behavior (Bosch et al., 2004, 2005; Neumann et al., 2000; Pedersen and Prange, 1979; Pedersen et al., 2006). In the context of this thesis, the effect of central OXT on social memory formation and maintenance is of especial interest. Infusion of OXT into the rat LS or ventral hippocampus have been revealed to improve juvenile recognition in adult males (Ludwig et al., 2013; Popik et al., 1992). Furthermore, OXT has been found to be crucial for memory formation in a social context, since central OXTR antagonism impairs the maintenance of social memory (Dluzen et al., 2000; Lukas et al., 2013). In a recently established mouse model of social fear, the so called social fear conditioning (SFC), OXT has been found to crucially regulate social fear expression: Infusion of the neuropeptide into the ventricular system or LS is able to reverse social fear (Zoicas et al., 2014). This data further highlights OXTs pro-social effects (for details on OXT in SFC see section 1.5.2 Evaluation of Fear in Rodents).

In addition to its pro-social properties, central OXT is known to essentially regulate fear- and anxiety-related processes. Intracerebroventricular (icv) application of OXT affects cued fear conditioning (CFC) in rats and mice in a time-dependent manner (Toth et al., 2012a): In rats, administration of OXT prior to acquisition does not alter the fear conditioning response, but decreases fear expression and facilitates fear extinction. Moreover, OXT infusion prior to extinction of cued fear impaires fear extinction in both, rats and mice. This effect of OXT is conserved across species and suggested to prevent the formation of aversive memories during traumatic events. During lactation, the activity of the brain OXT system is enhanced, an effect characterized by elevated hypothalamic OXT synthesis (Knobloch et al.,

2012), suckling-induced peripheral and central OXT release (Neumann et al., 1993b), increased levels of OXTR expression and binding in various brain regions (Insel, 1986; Meddle et al., 2007), and activation of OXTR-coupled signaling cascades (Jurek et al., 2012; Slattery and Neumann, 2008). During lactation, OXT signaling prevents fear-induced **freezing** in an odor fear conditioning paradigm in rats (Rickenbacher et al., 2017) and social fear in the SFC paradigm (Menon et al., 2018). Moreover, studies revealed an anxiolytic phenotype in lactating females (Lonstein, 2005; Neumann, 2001) and in males after mating (Waldherr and Neumann, 2007), which has been suggested to be mediated by OXT. Additionally, local infusions of synthetic OXT into the hypothalamic PVN, central amygdala, or medial prefrontal cortex (PFC) result in acute anxiolysis in male as well as female rodents (Bale et al., 2001; Blume et al., 2008; van den Burg and Neumann, 2011; van den Burg et al., 2015; Jurek et al., 2012; Martinetz et al., 2019; Neumann, 2008; Neumann et al., 2000; Sabihi et al., 2017).

Abovementioned studies clearly illustrate the significance of the neuropeptide OXT in the regulation of sociability and mood, especially fear- and anxiety-related behavior.

## 1.3 Behavioral and Molecular Correlates of Anxiety and Fear

Already in 1919, the direct descendant of Darwin, Walter Cannon, highlighted the emergency adaptive functions of anger and fear in terms of promoting ***fight and flight*** reactions. This made him the primary investigator of emotional, visceral, and autonomic alterations as responses of anxiety and fear. As emotional behaviors essential for survival, fear and anxiety can be both, innate and adaptive, and are expressed in all vertebrates. Nevertheless, among neuroscientists, there are several perspectives on what differentiates anxiety from fear, resulting in numerous different viewpoints, since the terms of fear and anxiety are often used interchangeably. According to established definitions, fear is commonly specified as an emotional response to a real threat or danger, whereas anxiety is an emotional reaction to a

**Freezing** responses are commonly observed in prey animals. They reduce the likelihood to be attacked, because it is more complex for the predator to spot the prey when motionless and it is less likely to be attacked by the predator whenever motionless.

**Fight and flight** reactions are also called hyper-arousal states. They are physiological reactions occurring in response to perceived harmful events, such as attacks or threat to survival.

potential, circumstantial, or anticipated threat or danger (McNaughton and Zangrossi, 2008; Tovote et al., 2015). Conceptually, fear and anxiety relate to brain states that are evoked by external or internal stimuli and elicit a specific combination of measurable behavioral, physiological, hormonal, and autonomic responses (Anderson and Adolphs, 2014; Davis et al., 2010; LeDoux, 2000, 2014). These responses have evolved to enable the organism to survive by adapting to not only beneficial, but also harmful stimuli. Anxiety and fear are highly adaptive and complex responses that are measured through the intensity or persistence of the associated behaviors. Both are coping strategies, which are deployed in dependence of the present situation: (i) Active coping strategies are exerted when escape is feasible. They are primarily mediated by activation of the sympathetic nervous system leading to hypertension and tachycardia (Cannon, 1915; Olds, 1956). (ii) Passive coping strategies are deployed when escape is not possible. They are accompanied by autonomic inhibition, resulting in hypotension and bradycardia (Engel and Schmale, 1972).

### 1.3.1 Anxiety and Fear Responses

The emotional response to anxiety-eliciting stimuli is highly variable and dynamic, which is predicated on the ambiguity of putative threats. Anxiety is an adaptive or innate coping mechanism for dangerous situations and is thereby highly associated with emotional as well as cognitive functions, such as learning and memory. It is apparent that anxiety is adaptive in protecting individuals from danger (Scott, 2013): Anxious avoidance of predators is essential for healthy survival and propagation of species. Anxiety-eliciting stimuli, e.g., open bright spaces, result in species-dependent **approach-avoidance** behaviors in humans and other land-dwelling species, such as rodents and primates. These approach-avoidance behaviors are dependent on the goal and the associated motivation (Kenrick and Shiota, 2014). For example, bright open areas induce avoidance behavior in nocturnal rodents, which naturally prefer protected and dark areas, whereas diurnally

**Approach-avoidance** conflicts originate if a goal has both, positive and negative characteristics or effects. This makes the goal appealing and unappealing simultaneously.

active humans show diverse behaviors in response to the same environment: approach and avoidance behaviors are present in dependence of the individual´s life experience. Approach-avoidance responses are evolutionary highly conserved and beneficial for the organism in that the unknown provides the possibility of both, opportunity and danger. Another conventional strategy to cope with anxiety, are escape behaviors, which allow withdrawal of the organism from the threat and prevention of re-exposure to the same or similar dangerous situations.

Fear can be both, innate and adaptive (Ramachandran, 1994a). Innate fears (e.g., startle responses to unexpected loud noises) have evolutionary been established across centuries as they assist in the organism´s adaptation to the surrounding environment and thereby mitigate harm and ensure survival. Adaptive or learned fears (e.g., aversive events by getting physically attacked) are achieved by direct and indirect (witnessing) experience or by inter-individual assignment. Just as anxiogenic stimuli, fear-eliciting stimuli, such as novel objects or situations, provoke mainly avoidance and escape behaviors, as well as prevention of re-exposure, but also approach behaviors to examine the object or situation. Similar to anxiety, behavioral fear responses have been characterized in numerous species and include avoidance (Blanchard et al., 2003; Edmunds, 1974), flight, freezing, defensive threat, defensive attack, risk assessment, burying the threatening object (Treit et al., 1981), alarm cries (Litvin et al., 2007), and cessation of ongoing behavior (Brady and Hunt, 1951; Estes and Skinner, 1941).

In addition to behavioral alterations, states of fear and anxiety evoke physiological responses. These physiological responses to threat include activation of the autonomic nervous system (Cohen and Randall, 1984; Engel and Schneiderman, 1984), the hypothalamus-pituitary-adrenal (**HPA**) axis (Graeff and Zangrossi Junior, 2010; Korte et al., 1992; Mason et al., 1961), hyperthermia (Adriaan Bouwknecht et al., 2007), pain suppression (Watkins and Mayer, 1982), and a potentiation of somatic reflexes, such as the startle response (Davis et al., 2010; Ray et al., 2009) and eye blink (Weisz and McInerney, 1990) responses.

The **HPA** axis is essential for the body´s stress response and represents the interaction between hypothalamus, pituitary, and adrenal glands. Upon stressful stimulation, the hypothalamus releases corticotropin-releasing hormone, which triggerst the anterior pituitary to release adrenocorticotropic hormone to subsequently stimulate cortisol release from the adrenal glands. As steroid hormone, cortisol acts as negative feedback regulator of the HPA axis at the level of the hypothalamus and pituitary.

The persistence of these behavioral and physiological adaptations in the absence of a real or potential threat leads to detrimental consequences on other pro-survival behaviors, such as self-care and food procurement, but also social interaction and reproduction. Therefore, it is conceivable that inadequate over-activation of mechanisms and circuits involved in anxiety and fear results in debilitating anxiety disorders (see section 1.4 Anxiety Disorders) (Gray and McNaughton, 1996; Hazen et al., 1996).

### 1.3.2 Neurocircuits of Anxiety

Numerous studies suggest that central mechanisms and neuronal substrates of anxiety and fear in rodents and humans are mediated by at least partially overlapping mechanisms (Davis and Whalen, 2001; Davis et al., 2010). However, precise brain circuits that underlie anxiety have not been investigated as much.

Anxiety states are mediated on one hand by local microcircuits and on the other hand by long range projections to distal regions (Tovote et al., 2015). Regions, such as the bed nucleus of the stria terminalis (BNST) or the amygdala, which have major roles in anxiety, mediate anxiogenic as well as anxiolytic behavioral effects. Thereby, the functional consequence within anxiety networks is determined by target-specific and/or cell type-specific connections. For example, two paralleled ventral BNST to ventral tegmental area pathways are capable to mediate anxiogenic as well as anxiolytic behavior (Jennings et al., 2013). Activation of the basolateral amygdala to ventral hippocampus projections is anxiogenic (Felix-Ortiz et al., 2013), whereas activation of the basolateral amygdala to central amygdala circuit is anxiolytic (Tye et al., 2011). Other brain regions that are interconnected with the BNST and/or amygdala and are involved in modulation of anxiety-related behavior, include medial PFC, periaqueductal gray, raphe nucleus, locus coeruleus, LS, and hypothalamus, (Tovote et al., 2015). Importantly, the hypothalamic PVN became apparent as crucial modulator of anxiety responses (Blume et al., 2008; van den Burg et al., 2015; Jurek et al.,

2012; Martinetz et al., 2019). However, distinct components of the anxiety network, such as cell identity and function, within those local and long-range projections remain to be characterized.

### 1.3.3 Anatomy, Neurochemistry, and Function of the Paraventricular Nucleus

The hypothalamus is a small, but essential region of the brain, which is formed by numerous nuclei and nervous fibers. The PVN is a bilateral nucleus of the hypothalamus, which is crucially involved in neuroendocrine and behavioral responses to numerous external and internal stimuli. It is located adjacent to the third ventricle and lies within the periventricular zone. The PVN contains two neurosecretory cell types: magnocellular neurons and parvocellular neurons, which have been described in detail in section 1.2 The Mammalian Oxytocin System.

The PVN receives input from other nuclei of the hypothalamus, such as the suprachiasmatic nucleus (**SCN**) and arcuate nucleus, but also from distal brain regions like periaqueductal gray, parabrachial nucleus, entorhinal cortex, prelimbic cortex, BNST, and amygdala (Hsu et al., 2014). Moreover, it reciprocally interconnects to the contralateral PVN (Jurek and Neumann, 2018). Projections from the PVN reach to the nucleus accumbens, BNST, central and extended amygdala, medial PFC, brainstem, nucleus raphe, and LS (Geerling et al., 2010; Li and Kirouac, 2008; Sofroniew, 1980).

As limbic brain structure, the PVN has been shown to mediate drug relapse (Martin-Fardon and Boutrel, 2012), retrieval of consolidated fear memories (Padilla-Coreano et al., 2012), acute and chronic stress responses (Bhatnagar and Dallman, 1998; Bhatnagar et al., 2002; Hsu et al., 2014), emotional arousal, motivation, and mood, especially anxiety-related behavior (Blume et al., 2008; van den Burg and Neumann, 2011; van den Burg et al., 2015; Jurek et al., 2012; Martinetz et al., 2019; Neumann, 2008; Neumann et al., 2000).

The **SCN** as part of the hypothalamus is seen as the central pacemaker of the circadian rhythm. It is an autonomous nucleus, but also recieves input from the retinothalamic tract to synchronize the day-night-cycle.

### 1.3.4 Neurocircuits of Fear

Most of what we understand about fear originates from studies using **Pavlovian** fear **conditioning**, whereby animals learn to predict aversive events. Hence, the following brief disquisition on neurocircuits involved in acquisition, consolidation, and extinction of fear only includes studies based on conditioned fear.

Within the brain, fear states are mediated by long-range projections between brain regions in conjunction with local microcircuits within essential nuclei of this projection network (Tovote et al., 2015). The major center of the fear circuit is located within the amygdala. Briefly, fear expression is elicited by fearful stimuli that activate thalamic centers and cortical regions, such as primary sensory and association cortices. Input from those regions to several nuclei of the amygdala further mediates fear-related **neuronal plasticity**. Moreover, reciprocal connections between the basal amygdala and the ventral hippocampus as well as the prelimbic cortex modulate this plasticity. The central amygdala projects to hypothalamic, brain stem, and mid brain centers, such as the periaqueductal gray, to modulate neuronal plasticity in order to promote fear behavior and autonomic responses.

Different elements within the same structures mediate extinction of fear: Here the PFC-amygdala pathway is suggested to be most relevant for fear extinction behavior (Muigg et al., 2008, 2009). Bidirectional projections between the infralimbic cortex and basal amygdala or the intercalated cells dampen the fear output from nuclei of the lateral central amygdala to the hypothalamus and periaqueductal grey (Herry et al., 2010; Tovote et al., 2015). Additionally, forebrain to brainstem pathways essentially influence fear extinction, but their identity, connectivity, and specific function remains to be identified. Recently, the group of Valery Grinevich found that magnocellular SON-OXT neurons participate in a fear memory engram, wherein parvocellular OXT neurons from the PVN orchestrate OXT release within distant brain regions, such as the amygdala, in a context-independent manner (Hasan

**Pavlovian conditioning** is a method that causes a reflex response or behavior by training with repetitive or aversive action. It was invented by the Russian physiologist Ivan Petrovich Pavlov, who conditioned dogs to respond in what seemed to be a predictable manner.

**Neuronal plasticity**, also known as brain plasticity or neuroplasticity is the capability of neurons to continuously change throughout life to optimize neural networks. This includes, but is not limited to strengthening and weakening of synapses.

et al., 2019). Moreover, magnocellular OXT projections from the SON to the LS are characterized to mediate social fear extinction in female lactating mice, since silencing of these blocks social investigation in those mice (Menon et al., 2018), revealing the LS as crucial component of the social fear brain circuit.

### 1.3.5 Anatomy, Neurochemistry, and Function of the Septum

As abovementioned, the septal region is highly involved in the expression of fear and its extinction. It is a subcortical forebrain structure located between the lateral ventricles and lies rostrodorsal to the hypothalamus. Anatomically, neurochemically, and functionally the septum is divided into two nuclei: the medial septum (MS) and the LS.

The MS receives ascending input from the hypothalamus, ventral tegmental area, substantia nigra, raphe nucleus, locus coeruleus, and hippocampus (Müller and Remy, 2018; Tsanov, 2017). Within the MS, glutamatergic, γ-aminobutyric acid (GABA)-ergic, and cholinergic neurons are highly interconnected and form a local network to synchronize the septal network (Fuhrmann et al., 2015; Hangya et al., 2009; Huh et al., 2010; Manseau et al., 2005; Müller and Remy, 2018). One major interconnection consists of ascending inputs from the MS as well as the adjacent diagonal band of Broca, which is functionally related to the MS, *via* the fimbria/fornix fiber bundle into the hippocampus (Khakpai et al., 2013). This septo-hippocampal pathway fine-tunes hippocampal physiology and is indispensable for its behavioral functions. Most septo-hippocampal projections are of cholinergic nature (~65%; (Sun et al., 2014)) and are crucially involved in aversive association learning (Lovett-Barron et al., 2014) and formation of spatial memory (Durkin, 1994; Ikonen et al., 2002). Glutamatergic neurons account for ~23% of septo-hippocampal projections (Colom et al., 2005) and are essential for processing of environmental and spatial inputs during initiation of movement episodes, general locomotor state, and running speed (Fuhrmann et al., 2015). GABAergic neurons form the minority of septo-hippocampal

projections and have been implicated in hippocampal neurogenesis (Van der Borght et al., 2005), operant reward learning (Vega-Flores et al., 2014), and, in interaction with the hippocampal cholinergic system, modulation of anxiety-related behavior (Degroot and Treit, 2003; Degroot et al., 2001). Hippocampal GABAergic neurons not only receive GABAergic input from the MS, but also project back to the MS (Alonso and Köhler, 1982; Takács et al., 2008; Tóth et al., 1993), forming a reciprocal long-range circuit, which functionally synchronizes remote areas (Caputi et al., 2013). However, the only neuronal subtype classified to modulate social fear extinction are GABAergic neurons, since overexpression of the OXTR in GABAergic neurons of the LS substantially attenuated SFC-elicited social fear in female mice (Menon et al., 2018).

In comparison to the MS, the LS receives descending glutamatergic input from the hippocampus via the fimbria/fornix bundle (Gallagher et al., 1995). It is mainly composed of GABAergic neurons, which are reciprocally interconnected with the hypothalamus and periaqueductal grey (Sheehan et al., 2004). Moreover, monoaminergic and cholinergic neurons of the amygdala, BNST, medial PFC, locus coeruleus, laterodorsal tegmentum, ventral tegmental area, nucleus accumbens, and entorhinal cortex project to the LS. It is important to note that LS and MS receive reciprocal projections from each other (Risold and Swanson, 1997). Thereby, the LS is a crucial region for integrating cognitive as well as affective functions to directly control appropriate behavioral responses to particular environmental stimuli. The LS is essentially involved in various aspects of social behavior, such as social memory (Engelmann and Landgraf, 1994; Lukas et al., 2013), aggression (Leroy et al., 2018), and social fear (Menon et al., 2018; Zoicas et al., 2014), but also anxiety-related behavior (Sheehan et al., 2004) and avoidance behavior (Troyano-Rodriguez et al., 2019). For example, social instability stress in male rats results in reduced dendritic spines within the LS and is suggested to be the underlying cause of reduced social interaction, impaired social recognition, reduced sexual performance, and increased aggression (Hodges et al., 2019). Further

studies proved that the LS activity negatively correlates with aggressive behavior and consequently, septal lesions are known to induce a "**septal rage**" phenotype (Goodson et al., 2005; Lee and Gammie, 2009; Potegal et al., 1981; Wong et al., 2016). Thus, the LS is crucial for the regulation of aggression, which in principle is a sophisticated component of social behavior. Most importantly, the LS is a key player of social fear, which will be described within this thesis in section 1.5.2 Evaluation of Fear in Rodents. In addition to the regulation of various cognitive social and emotional behaviors, the LS is involved in the physiological stress response by impacting active stress coping and dampening of the HPA axis activity (Herman et al., 1996; Singewald et al., 2011). Further studies implicate sub-populations of neurons, such as CRF receptor 2 expressing neurons, as promoters of stress-induced anxiety (Anthony et al., 2014; Radulovic et al., 1999). In any case, the involvement of the LS as a main regulatory component of the stress response in undeniable.

## 1.4 Anxiety Disorders

In the context of human pathology, inappropriate, exaggerated or prolonged activation of anxiety and fear responses by innocuous stimuli becomes detrimental. Debilitating excessive fear is a significant symptom of many anxiety disorders, such as generalized anxiety disorder (GAD), social anxiety disorder (**SAD**), panic disorder, specific phobia, or obsessive-compulsive disorder (Barton et al., 2014; Craske et al., 2017). Anxiety disorders are associated with immense health care costs and represent a high burden for society and economy. Pursuant to large population-based surveys, they have a constant life-time prevalence of 33.7% throughout the last years (Bandelow and Michaelis, 2015). Additionally, the prevalence in women is approximately twice as high as in men (Angst and Dobler-Mikola, 1985; Bruce et al., 2005; McLean et al., 2011; Regier et al., 1990), which is discussed to be caused by psychological contributors (e.g., childhood trauma), but also genetic, epigenetic, and neurobiological factors. Although prospective studies suggest anxiety disorder as chronic

**Septal rage**, also called sham rage refers to intense dysphoria, hyperexcitability, and anger, which originates from a lesion in the human septum pellucidum.

**SAD** is suggested to be caused by a composition of genetic, epigenetic, and environmental factors. It is symptomized by a combination of emotional, behavioral, and physical symptoms, as well as a general avoidance of social situations.

impairment, prevalence rates decrease throughout age, revealing that an anxiety disorder does not last until old age in most cases (Jacobi et al., 2014). Just as all psychiatric disorders, anxiety disorders show high rates of comorbidity amongst themselves, but also to other psychopathological conditions, such as dysthymia and major depressive disorder (Kessler et al., 2005a). In the following sections, GAD and SAD will be reviewed in detail, as they are the most relevant ones for this thesis.

### 1.4.1 Generalized Anxiety Disorder

In 1980, GAD appeared for the first time as diagnostic category in the third edition of the *Diagnostic and Statistical Manual of Mental Disorders III (DSM-III)*. The distinctive core symptom of GAD is excessive diffuse worry about a number of life circumstances, which is a cognitive aspect of anxiety. According to *DSM-V*, GAD patients show symptoms of restlessness, fatigue, irritability, muscle tension, as well as sleep and concentration deficits. Long-term consequences of worry include the inhibition of emotional processing and perpetuation of anxiogenic conditions (Mathews, 1990). Relief of worry is usually only provided short-term by avoidance of the threatening stimuli (Brown, 1997) or intolerance of uncertainty (Bomyea et al., 2015).

The validity of GAD as independent category has been questioned from *DSM-III* to *DSM-V*: On one hand, no clear boundaries between GAD and personality dimensions, other anxiety-spectrum disorders, and non-bipolar depression are the major concerns (Crocq, 2017). On the other hand, epidemiological surveys identified different risk factors for GAD and depressive disorders, revealing a clear separation between the two disorders (Kessler et al., 2008). The lifetime prevalence of 9.0% with an overrepresentation of female patients highlights the need for specific pharmacological treatment options (Kessler et al., 2012). Of all anxiety disorders, GAD has the latest median age onset at approximately 31 years (Bandelow and Michaelis, 2015). Moreover, it is comorbid with other anxiety disorder subtypes and shows particularly high

comorbidity with dysthymia and major depression disorder (Kessler et al., 2005a). In the case of major depression disorder, GAD is discussed as a prodromal, residual, or severity marker of a major depressive episode (Kessler et al., 2008). Interestingly, the comorbidity of GAD with other disorders decreases over the duration of GAD itself (Breslau and Davis, 1985). Long-term medication with benzodiazepines worsens the underlying anxiety (Galanter et al., 2014), an effect, which can be reversed by reduction in the treatment dose (Booth, 1995). However, no treatment options with low side effects and relapse rates are known to date, which highlights the necessity of further research.

### 1.4.2 Social Anxiety Disorder

Although primarily monitoring psychotic patients, the German psychiatrist Emil Kraepelin noted that patients suffering from non-generalized and generalized social phobia experienced "overpowering feelings of aversion […] when they had to establish relations of any kind with other patients", whereas other patients were "unable to urinate or write a letter in the presence of other people" (Kraepelin E., 1904). Today, and in a more therapeutically relevant and epidemiological definition, SAD is characterized by intense fear and avoidance of social situations (Kessler et al., 2005b, 2005a), such as meeting strangers or speaking in public. Amongst anxiety disorders, SAD is the second most common with a lifetime prevalence of 12.1% (Alonso et al., 2011; Kessler et al., 2012). Comparable to the general gender bias of anxiety disorders, 60% of SAD patients are female, although men are overrepresented in the treatment seeking fraction (Xu et al., 2012). According to the *DSM-V*, two subtypes of SAD, generalized SAD and non-generalized SAD, are distinguished. Patients suffering from generalized SAD fear most social situations (Kerns et al., 2013; Vriends et al., 2007), whereas in non-generalized SAD patients fear of only a specific social situation manifests (Bögels et al., 2010). Although generalized SAD is much more debilitating in nature than non-generalized SAD, both lead to significant reduction in the quality of the

patient´s life (Hazen et al., 1996; Stein and Chavira, 1998). In addition, generalized SAD is usually familial, long-lasting, and shows a lower chance of spontaneous recovery, but carries a higher risk of comorbidity. Common comorbidities include major depression (Schneier et al., 1992; Stein and Chavira, 1998), agoraphobia (Magee et al., 1996), or substance abuse (Buckner et al., 2013; Schneier et al., 2010). Here, SAD symptoms usually appear first, suggesting that SAD may be a crucial risk factor for other psychopathologies (Neumann and Slattery, 2016). SAD has an early onset between age 5 to 15, and its symptom of avoidant behavior is mainly considered the biggest hindrance towards extinction or reversal of social anxiety (Stangier et al., 2006). In healthy humans, memory of negative experience is known to decrease over time (Ritchie et al., 2015). Patients symptomized by high levels of social anxiety tend to show an eroding of positive memories, which leads to a perturbation of their fear of social situations and hinders treatment (Glazier and Alden, 2019). To date, no treatment specifically targeting SAD is present, highlighting the need of further detailed understanding of the underlying mechanisms.

### 1.4.3 Treatment of Anxiety Disorders

When symptoms are mild, transient, and without associated impairments in social or occupational function, it is not necessary to treat anxiety disorders. However, most patients show marked distress or suffer from severe complications, such as secondary depression, suicidal ideation, or alcohol abuse, which in conclusion make treatment inevitable. All currently available treatment options are rather unspecific and do not primarily treat all categories of anxiety disorders, but also comorbid psychopathologies such as depression. The state-of-the-art therapy consists of a combination of behavioral/psychological and pharmacological treatment to achieve improved remission rates (Fedoroff and Taylor, 2001). Behavioral/psychological therapy includes patient-specific cognitive-behavioral therapy (**CBT**) (Choy et al., 2007; Singewald et al., 2015; Stangier, 2016). Conjunctive pharmacotherapy

**CBT** involves individual coping strategies and exposure-based therapies including the controlled exposure to anxiogenic stimuli for systemic desensitization.

comprises selective serotonin reuptake inhibitors (**SSRIs**; Escitalopram, Fluoxetine, Paroxetine), serotonin-noradrenalin reuptake inhibitors (**SNRIs**; e.g., Duloxetine, Venlafaxine), and tricyclic antidepressants (e.g., Clomipramine), but also calcium modulators (e.g., Pregabalin), serotonin receptos 1A agonists (e.g., Buspirone), and reversible monoamine oxidase A inhibitors (e.g., Moclobemide) (Bandelow et al., 2017). All named drugs treat different combinations of indicated symptoms, have variable pre-post effect sizes, and elicit numerous side effects, including sedation, constipation, sexual dysfunction, and a high risk of a toxic overdose (Ravindran and Stein, 2010). Although, plenty treatment options exist, many patients fail to respond, achieve only partial remission of symptoms, or show a high relapse rate after treatment discontinuation (Blanco et al., 2002). The numerous risks, side effects, and low treatment response or high relapse rates of the established anxiolytic drugs, give a strong impetus for future research and the development of new therapeutic strategies for the treatment of anxiety disorders. Numerous of those novel strategies involve a potential use of endogenous or exogenous modulators of glutamate and neuropeptide signaling. Particularly, anxiolytic activities of antagonists of the CRF receptor, glutamate receptor, as well as anxiolysis by neuropeptides such as OXT, AVP, neuropeptide S (NPS), neuropeptide Y, substance P, orexin, galanin, and cholecystokinin are under investigation (Mathew et al., 2008). Moreover, no disease subtype-specific pharmacological treatment option is available. This endeavor requires a more detailed understanding of neuronal and molecular alterations underlying anxiety disorder subtypes. Thus, effective research on anxiety and fear using appropriate animal models is essential.

**SSRIs** are a group of antidepressant and anxiolytic drugs, which inhibit the uptake of serotonin within the brain.

**SNRIs** selectively inhibit the uptake of serotonin and norepinephrine and are used as antidepressant and anxiolytic drugs.

## 1.5 Modelling Anxiety and Fear in Rodents

From an evolutionary point of view, neuronal and hormonal systems controlling anxiety and fear behavior contain mechanisms and components, which are highly conserved among species (McNaughton

and Zangrossi, 2008). In this regard, a behavioral animal model resembling a human pathology has to fulfill three basic criteria:

I. *Face validity*: The behavior of the animal appears analogous to the respective human behavior.
II. *Predictive validity*: The animal model is capable to predict the outcome of a specific manipulation.
III. *Construct validity*: The animal model is capable to recruit the same neurobiological substrate as its respective human disorder.

Based on abovementioned criteria, it is – even if all three criteria are met – almost impossible to develop an animal model, which entirely mimics a human psychopathology, such as anxiety disorder. Usually, only subtype-specific animal models exist, which at least partly resemble occurring human symptoms.

### 1.5.1 Evaluation of Anxiety-related Behavior in Rodents

Numerous animal models resembling symptoms of pathologies exist. For example, different knockout models (Scherma et al., 2019) and rodents selectively bred for behavioral extremes, e.g. high vs low anxiety-related behavior, are commonly used (Wegener et al., 2012). In addition to animal models, behavioral tests of anxiety-related behavior in rodents, such as the elevated plus-maze (EPM) (Lister, 1987), light dark-box (LDB) (Bourin and Hascoët, 2003), open field test (OFT) (Stanford, 2007), and novel object investigation test (NOI) (Toth et al., 2012b) are widely used. They are based on exploratory behavior, conflict behavior, and defensive behavior (Rotzinger et al., 2010). All three tests allow the assessment of innate and adaptive anxiety-related behavior in rats and mice, and make use of the conflictive drive of rodents to explore novel areas versus avoiding elevated and brightly lit spaces. Moreover, locomotive effects of putative anxiogenic or anxiolytic treatments can be evaluated. In the present thesis, all four

tests were used to measure levels of anxiety-related behavior in mice and rats after pharmacological, functional, or genetic manipulation.

### 1.5.2 Evaluation of Fear in Rodents

Pavlovian fear conditioning is based on associative learning mechanisms and generates an adaptive response to environmental stimuli. In this paradigm, an initially neutral stimulus (the conditioned stimulus (CS), e.g., a tone) is associated with an aversive event (the unconditioned stimulus (US), e.g., a foot shock), thereby evoking a conditioned response (e.g., freezing) even when confronted with the CS alone (Fendt and Fanselow, 1999; Maren, 2001). A further repeated presentation of the CS without the US – called fear extinction - leads to a gradual decline of the conditioned response until the rodent is no longer fearful (Myers and Davis, 2007). Though Pavlovian fear conditioning is an adequate model to study anxiety disorders in a general context, it does not resemble SAD since the presence of a social component is missing. Social contact is a heavily rewarding stimulus for rodents and humans. In contrast, SAD patients, who feel punished by the social environment, avoid social contact whenever possible. This legitimate conflict of acceptance versus avoidance in social situations needs to be significantly incorporated when developing an animal model to resemble SAD.

In 2012 a mouse model for SAD, so called SFC (explained in detail in section 2.6.1.1 Social Fear Conditioning) was developed in male (Toth et al., 2012b, 2013) and later also in female (Menon et al., 2018) mice. The SFC paradigm is based on operant fear conditioning principles: a social stimulus is associated with a mild foot shock (punishment) as consequence of social contact, ultimately leading to acquisition of social fear (avoidance of social stimulus) in mice. During extinction of social fear, mice are presented in their home cage with unknown conspecifics, thereby choosing to approach or avoid the respective social stimulus. Usually, social fear-conditioned mice show avoidance behavior (decreased social investigation) towards the social stimulus at first.

Perceiving the absence of a foot shock during social investigation results in a gradual, but complete extinction of social fear after multiple exposures to social stimuli. SFC generates social anxiety-like symptoms in mice without other confounding symptoms, such as depressive-like behavior or general anxiety-related behavior. Unlike other models of social avoidance, such as exposure to acute social defeat (Lukas et al., 2011; Toth and Neumann, 2013), SFC leads to generalized social fear, which persists up to at least two weeks, without affecting general anxiety-related behavior, depressive behavior or locomotor activity (Toth et al., 2012b). These essential features make SFC a unique paradigm to study the neurobiological mechanisms underlying SAD. Using the SFC paradigm, the pro-social and anxiolytic neuropeptide OXT has been shown to completely reverse SFC-induced social fear when applied into the mouse LS prior to extinction of social fear (Zoicas et al., 2014). Moreover, OXT release in the LS is attenuated, whereas OXTR binding within the LS and other regions is increased in socially fear-conditioned mice in comparison to unconditioned mice. Interestingly, lactating mice, which have a highly activated brain OXT system (Insel, 1986; Jurek et al., 2012; Knobloch et al., 2012; Meddle et al., 2007; Neumann et al., 1993b; Slattery and Neumann, 2008), do not express social fear (Menon et al., 2018). Additionally, antagonizing septal OXTRs in lactating mice reinstates social fear, whereas silencing of OXTergic LS projections blocks social investigation in these mice. Taken together, the septal OXT system is an essential modulator of social fear in male and female mice, however, the precise molecular alterations resulting in the observed behavioral phenotypes have not been identified yet.

## 1.6 Molecular Changes Underlying Anxiety Disorders and Conditioned Fear

Generally, the understanding of molecular mechanisms underlying complex emotional behavior is parsimonious; however, dysregulation of several systems is implicated in the pathophysiology of anxiety disorders and has been extensively studied. However, describing all of

them within this thesis exceeds its limit. Thus, only a brief summary of the involved intracellular pathways and several target genes will be given below.

Numerous candidate genes involved in anxiety have been identified (Sokolowska and Hovatta, 2013). Amongst those, the regulator of G-protein signaling 2 is to be mentioned, since it mediates the anxiolytic effect of OXT within the amygdala of female mice (Okimoto et al., 2012). Several monoamine neurotransmitter systems, including glutamate, GABA, serotonin, dopamine, and norepinephrine (Heninger and Charney, 1988), as well as peptidergic neurotransmitter systems like OXT, AVP (Neumann and Landgraf, 2012), NPS (Grund and Neumann, 2018; Grund et al., 2017; Ionescu et al., 2012), substance P (Singewald et al., 2011), and CRF (Krysiak et al., 2000) are involved in anxiety. For example, local infusions of OXT into the hypothalamic PVN, central amygdala, or medial prefrontal cortex (PFC) result in acute anxiolysis in male as well as female rodents (Bale et al., 2001; Blume et al., 2008; van den Burg and Neumann, 2011; van den Burg et al., 2015; Jurek et al., 2012; Martinetz et al., 2019; Neumann, 2008; Neumann et al., 2000; Sabihi et al., 2017).

Numerous candidate genes involved in fear acquisition, extinction, and recall have been identified over the last decades. The majority of mechanistic factors of fear learning and memory affect synaptic plasticity. Intracellular signaling cascades are altered by transcriptional and epigenetic mechanisms, which ultimately modulate behavioral responses, such as fear (Stoppel et al., 2006). Transgenic mouse models, as well as gene expression analysis strongly associate glutamate receptors and ion channels with acquisition and extinction of conditioned fear. For example, the shift of the NMDA receptor subunit 2B positively correlates with the strength of acquisition and extinction of fear memory. This effect is suggested to be mediated by driving the insertion of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors into the postsynaptic membrane, leading to activation of “silent synapses” (Rumpel et al., 2005). Additional glutamatergic effects are mediated by metabotropic glutamate

receptors (mGluRs), which affect fear conditioning directly or indirectly *via* tyrosine kinase-mediated modulation of ionotropic receptors (Kojima et al., 2005; Miyakawa et al., 1994). Intracellularly, signaling and transcription factors such as CaMK II and CaMK IV are indispensable for fear conditioning (Chen et al., 1994; Mayford et al., 1996; Wei et al., 2002). Additionally, protein kinase A as well as the MAPK pathway, and its executing transcription factors, such as CREB, are activated and mediate persistent storage of conditioned fear (Dhaka et al., 2003), whereas blockade of pathways, especially the MAPK cascade, decreases fear memory performance (Lin et al., 2001). Generally, modulation of NMDA receptor function, AMPA receptor trafficking, and spinogenesis, thus also fear memory formation, all rely on actin filament dynamics (Fischer et al., 2004). In fact, altered mRNA expression of actin and α-actinin as well as the extracellular matrix molecule tenascin and cell adhesion molecule neuroligin are induced by fear conditioning (Ressler et al., 2002; Stork et al., 2001). Interestingly, the cell recognition factor neural cell adhesion molecule, which mediates neuromodulatory and hormonal effects on conditioned fear is induced in the amygdala and hippocampus of fear conditioned rodents (Merino et al., 2000; Sandi, 2004; Stork et al., 1999, 2000). Obviously, components of the HPA axis, especially CRF, modulate fear. For example, inhibition of CRF receptor 2 expression in the LS attenuates fear conditioning (Ho et al., 2001). Last but not least, monoamine transmitter, such as norepinephrine, dopamine, and serotonin, and their respective systems impact conditioned fear (Stoppel et al., 2006), but will not be discussed in detail here.

However, the precise molecular underpinnings of anxiety as well as acquisition, consolidation, and extinction of fear, especially social fear, remain elusive. A novel class of RNAs, so called non-coding RNAs, has attracted the neuroscientific community within the last decade, since they were found to significantly influence emotional responses.

## 1.7 MicroRNAs – Biogenesis, Function, and Regulation

With the striking identification of microRNAs (miRNAs) in *Caenorhabditis elegans* in 1993 by ***Lee** et al.* (Lee et al., 1993) followed by almost 10 years of numerous discoveries in understanding small RNA function (Lagos-Quintana et al., 2002; Lau et al., 2001; Lee et al., 2001), the longstanding central dogma of minutious gene regulation has been significantly redefined. Ever since, miRNAs are widely accepted as potent post-transcriptional regulators of gene expression. Over the past decades, numerous studies have in detail deciphered miRNA biogenesis and function, which are tightly controlled at various levels (Treiber et al., 2018). To date, aberrant miRNA expression has been associated with a broad range of somatic and affective disorders (Jiang et al., 2009), highlighting the tremendous gene regulatory power of these small RNAs.

### 1.7.1 Mechanisms of microRNA Biogenesis

miRNAs are a class of evolutionary highly conserved small (~19-24 nt in length) **non-coding RNA**s that are endogenously expressed in almost all eukaryotes, with the exception of *Saccharomyces cerevisiae*. In animals, miRNAs can be biosynthesized *via* two different pathways: the canonical biosynthesis and the non-canonical biosynthesis.

*Canonical biosynthesis:* miRNAs, which are synthesized according to the canonical biogenesis pathway (figure 1), are encoded in the genome as individual genes, clusters that contain a few to several hundreds of miRNAs, or in introns of host genes. If clustered, they are transcribed together as polycistronic transcript and further processed to singular mature miRNAs. Although many miRNAs derive from introns of protein-coding genes (Bartel, 2009; Carthew and Sontheimer, 2009; Ha and Kim, 2014), functional interactions between host-gene and miRNA are rare. During the canonical biogenesis, miRNAs are transcribed by RNA polymerase II as polyadenylated and capped primary miRNAs (pri-miRNAs) (Lee et al., 2004) that form a hairpin loop with the mature miRNA sequence in its stem. Within the nucleus, the pri-miRNA is

In 1993, ***Lee** et al.* found that the lin-4 gene does not encode for a protein, but rather for a small 21 nt long RNA product that regulates translation of the protein lin-14, a crucial factor for the nematode´s development, *via* antisense RNA-RNA interaction. Thereby, lin-4 was the first miRNA to be discovered.

**Non-coding RNA**s are classified according to their length: Small non-coding RNAs are up to 200 nts in length, whereas long non-coding RNAs are longer than 200 nts.

processed by a protein complex called microprocessor, resulting in a 70 nt-long single hair pin called precursor microRNA (pre-miRNA) (Lee et al., 2003). The microprocessor protein complex is composed of the RNase III Drosha, a RNA binding protein (RBP) DiGeorge critical region 8 (DGCR8) dimer, and several less-understood factors (e.g. DEAD Box RNA helicases p68 (DDX5) and p72 (DDX17)) (Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Lee et al., 2004). By direct interaction with the export receptor exportin 5 (Exp5), pre-miRNAs are exported into the cytoplasm in a Ran-GTP dependent manner (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Subsequently, the RNase III Dicer processes a double-stranded RNA of 20-25 nts in length from the stem of the pre-miRNA (Grishok et al., 2001; Ketting et al., 2001). Human Dicer functions in conjunction with the *trans*-activation-responsive RNA binding protein (TRBP). Across species, Dicer interacts with different double-stranded RBPs, revealing general mechanistic features that are highly conserved across evolution. In the final process called RNA-induced silencing complex (**RISC**) loading, the double stranded miRNA is transferred to a member of the argonaute (**AGO**) protein family, which selects one strand as guide strand that becomes the mature miRNA, whereas the other strand (passenger strand) is discarded (Kobayashi and Tomari, 2016). miRNA strand selection depends on the last nucleotide of the 5´ end and the thermodynamic stability of the miRNA (Frank et al., 2010; Gregory et al., 2004; Khvorova et al., 2003; Schwarz et al., 2003; Suzuki et al., 2015): The guide strand is preferentially selected based on an uracil or adenosine at its 5´ end, whereas the passenger strand shows a guanine/cytosine-bias. Moreover, the strand with weaker complementary binding at its 5´end is preferred as guide strand and loaded into RISC. The strand selection ratio for several miRNAs varies and is dependent on the cell cycle, developmental stage, and disease state, suggesting the importance of a tight control of the strand selection (Meijer et al., 2014). miRNA-loaded AGO proteins dissociate and form RISC, wherein they exhibit their function.

The multiprotein complex **RISC** is a ribonucleoprotein, which incorporates a single stranded (e.g., miRNA) or double stranded (e.g., small interfering RNA) RNA fragment. Within RISC, the RNA single strand acts as template to recognize complementary mRNA transcripts.

In mammals, the **AGO** protein family comprises eight members, subclassified in 2 subfamilies: PIWI and AGO proteins. PIWI proteins interact with PIWI-interacting RNAs (piRNAs), whereas AGO proteins interact with e.g. miRNAs. Four **AGO** proteins are currently known. Although AGO 1-4 are capable to load miRNAs, exclusively AGO2 possesses endonuclease activity and can possibly lead to target mRNA cleavage.

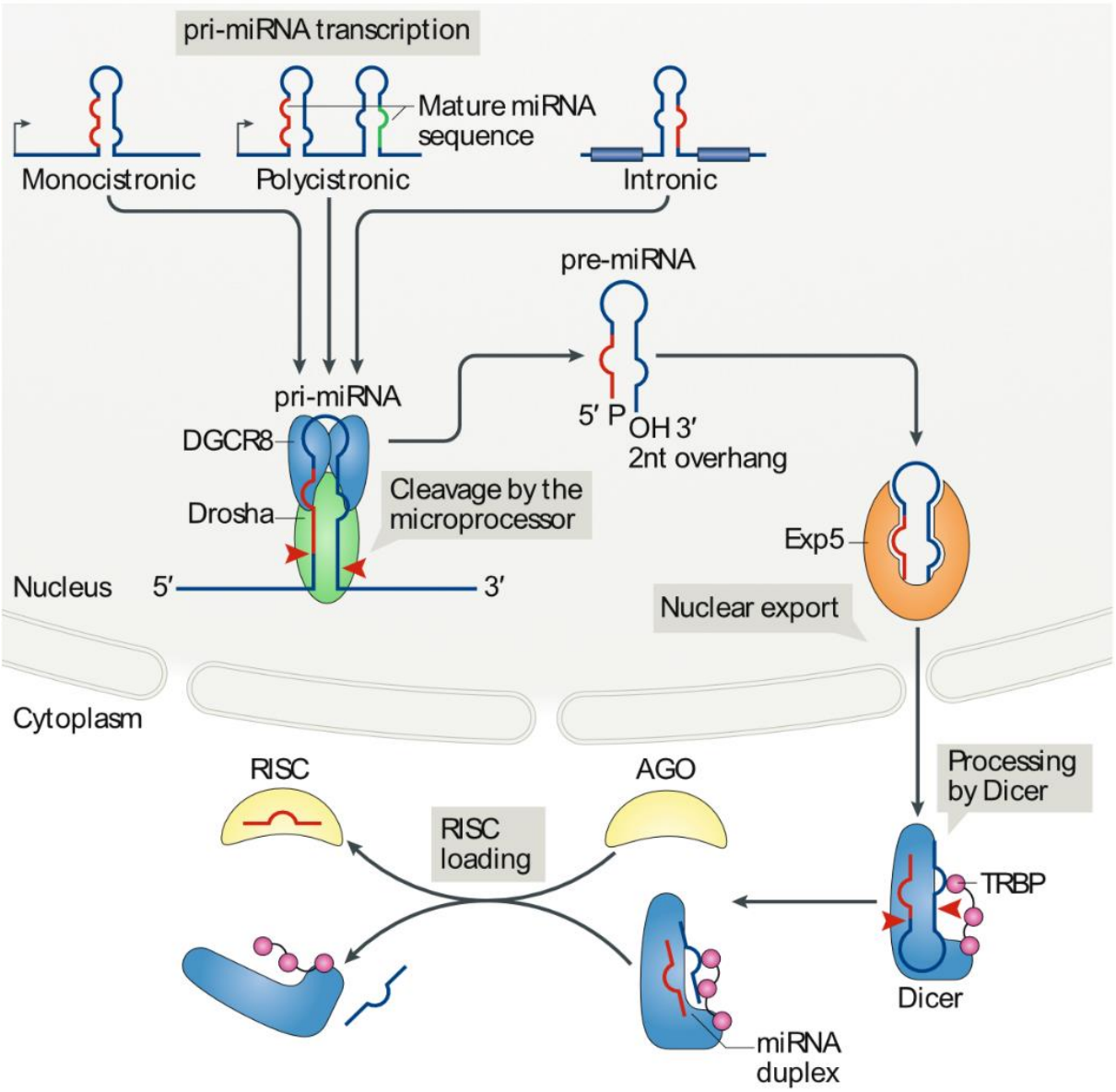


**Figure 1 – Canonical miRNA biogenesis.** In animals, miRNAs are encoded in a monocistronic (individual gene), polycicstronic (gene clusters) or intronic (in introns of host genes) manner. The microprocessor complex, consisting of dimeric DiGeorge critical region 8 (DGCR8) and Drosha, processes primary miRNA transcripts (pri-miRNA), which contain hairpins as well as 5´and 3´flanking sequences. Drosha cleaves at the stem of the pri-miRNA hairpin (red arrowheads) leading to the liberation of a precursor miRNA (pre-miRNA). The pre-miRNA is characterized by a 3´hydroxyl group (OH), 2 nt overhangs, and a 5´phosphate (P). Exportin 5 (Exp5) binds pre-miRNAs to facilitate their export into the cytoplasm. Within the cytoplasm, Dicer cleaves pre-miRNAs within their stem close to the terminal loop (red arrowheads), resulting in a miRNA duplex intermediate. Dicer in conjunction with *trans*-activation-responsive RNA-binding protein (TRBP) and an Argonaute (AGO) protein assembles as the RNA-induced silencing complex (RISC) loading complex. One strand of the miRNA duplex is transferred to the AGO protein, leading to the formation of a functionally active RISC (adapted from (Treiber et al., 2018)).

*Non-canonical biosynthesis:* Recently, novel miRNA biogenesis pathways have been deciphered (Kim et al., 2016). Individual depletion of Drosha and Dicer does not lead to abolished miRNA expression, revealing a non-canonical pathway for the biosynthesis of miRNAs. Equally, Exp5 depletion influences miRNA biogenesis only modestly, implicating alternative export mechanisms. So far, several Drosha-independent or Dicer-independent classes of miRNAs have been identified (Treiber et al., 2018): (i) A major class of non-canonical miRNAs is derived from introns of protein encoding genes. After intron splicing, the so called 'mirtrons' function as pre-miRNA, thereby bypass cleavage by the microprocessor complex, and are immediately exported into the cytoplasm. Mirtrons are present in *Arabidopsis thaliana*, *C. elegans*, mice, and humans (Berezikov et al., 2007; Meng and Shao, 2012; Okamura et al., 2007; Ruby et al., 2007). (ii) Another class of non-canonical miRNAs is derived from other non-coding RNAs, such as small nucleolar RNAs (**snoRNAs**) or tRNAs. This important knowledge was extricated from studies in embryonic stem cells or postmitotic neurons of DGCR8 knockout and Dicer knockout mice, which still express various miRNAs that are independent of the microprocessor, but do rely on Dicer processing (Babiarz et al., 2008, 2011). (iii) Non-canonical miRNAs can be synthesized from nascent, capped hairpin transcripts in microprocessor-independent steps (Xie et al., 2013). It is important to mention that due to the 5´ 7-methyl-guanosine-triphosphate (**m$^7$G**) cap, pri-miRNAs generated by this pathway are exported from the nucleus by exportin1, and RISC-loading of these miRNAs is restricted to the 3p arm. (iv) A rather rare non-canonical miRNA biogenesis is microprocessor-dependent and Dicer-independent. The only known miRNA, miR-451, is directly cleaved by the RNase H-like endonuclease AGO2 and is, after further trimming, loaded into RISC (Cifuentes et al., 2010).

In the last years, the biochemical basis for miRNA processing, especially sequence and structural features of pri-miRNA transcripts, particular microprocessor and Dicer characteristics, binding partners, and RISC loading has been in the focus of research (Treiber et al., 2012, 2019),

**snoRNAs** are a class of small RNA molecules, which primarily guide post-transcriptional modifications of other RNAs (mainly ribosomal RNAs, transfer RNAs, and small nuclear RNAs).

The **m$^7$G** structure at the 5´ end of mRNAs promotes their translation and protects them from degradation.

and adds even more complexity to the multilayered mechanisms of miRNA-mediated gene regulation.

### 1.7.2 Regulation of microRNA Biogenesis

Similar to most intracellular pathways, protein components of the miRNA biogenesis can be regulated on a co-transcriptional, post-transcriptional or post-translational level, which results in altered miRNA transcription profiles.

*Co- and post-transcriptional regulation of biogenesis factors:* Extensive co- and post-transcriptional regulation of miRNA transcripts is implemented by specific RBPs or long non-coding RNAs (**lncRNAs**), which facilitate the cross-talk between different RNA pathways or directly affect miRNA processing and RISC-loading.

RBPs positively or negatively regulate the biogenesis of numerous miRNAs at the level of Dicer or Drosha and interestingly, many RBPs with functions, for example in mRNA processing, also interact with the miRNA biogenesis intermediates, pri-miRNAs and pre-miRNAs. *In vitro* pulldown experiments followed by mass spectrometry identified 180 potential RBPs for more than 70 pri-miRNAs (Treiber et al., 2017). Moreover, a recent genome wide crosslinking and immunoprecipitation study demonstrated 116 RBPs that bind to miRNA loci (Nussbacher and Yeo, 2018).

One of the most famous examples of RBPs regulating miRNA biogenesis is the pluripotency factor LIN28. The LIN28 protein family consists of two homologs, LIN28A and LIN28B, both of which negatively regulate let-7 expression, but by different mechanisms (Ha and Kim, 2014). In order to eliminate let-7 expression in stem cells, cytoplasmic LIN28A binds pre-let-7 transcripts and recruits the terminal uridyl transferase 4 (TUT4) or 7 (TUT7) (Heo et al., 2008, 2009; Rybak et al., 2008; Viswanathan et al., 2008). TUT4 or TUT7 add a short oligo(U) stretch to the 3´end of pre-let-7, hence inhibiting Dicer processing, leading to subsequent degradation by the 3´-5´exonuclease DIS3-like exonuclease 2 (Chang et al., 2013; Faehnle et al., 2014; Ustianenko et al., 2013).

**lncRNAs** are non-coding RNAs of >200nt length, which function as gene transcription regulators, post-transcriptional regulators, and as epigenetic factors during imprinting and chromosome inactivation.

Unlike LIN28A, LIN28B is located in the nucleus of somatic cells, recognizes pri-let-7, and sequesters it away from the microprocessor complex (Piskounova et al., 2011).

Additionally, long non-coding RNAs (lncRNAs), such as NEAT1, Uc.283+A, and RNC4, regulate miRNA biogenesis at the level of Drosha by (i) scaffolding RBPs and the microprocessor to enhance pri-miRNA transcripts, (ii) preventing pri-miRNA cleavage, or (iii) stimulating pri-miRNA processing (Jiang et al., 2017; Krol et al., 2015; Liz et al., 2014).

*Post-translational regulation of biogenesis factors:* Adequate adaptation of the transcriptome following external stimuli requires alterations of miRNA processing and activity. Post-translational modifications (PTMs) of mechanistically involved proteins possess high regulatory impact on the affected down-stream mechanisms. In the case of miRNA biogenesis, various phosphorylation, ubiquitination, and sumoylation states on synthesizing proteins are established.

On the level of the microprocessor complex, MAPK and ABL tyrosine kinase generate several phosphorylations on DGCR8 that increase miRNA transcript levels (Paroo et al., 2009). DGCR8 can further be sumoylated at specific lysines, which prevents its ubiquitination-mediated degradation, thereby increases its stability and elevates miRNA transcripts (Zhu et al., 2015). In addition, the ribonuclease III Drosha can be post-translationally modified. On one site, Drosha is known to be phosphorylated by MAPK p38 under stress conditions, resulting in reduced interaction with DGCR8, nuclear export and degradation of Drosha, thereby leading to increased cell death (Yang et al., 2015). On the other side, MAPK-activated protein kinase 2, which is activated by p38, promotes processing of distinct pri-miRNAs by phosphorylation of the microprocessor co-factor p68 (Hong et al., 2013). Moreover, glycogen synthase kinase 3β phosphorylates Drosha, which is required for its nuclear localization (Tang et al., 2010) and thereby increases its activity (Fletcher et al., 2016).

Furthermore, activation of the MAPK pathway leads to phosphorylation of TRBP, thus stabilizes the Dicer-TRBP complex, resulting in increased

miRNA biogenesis (Paroo et al., 2009). TRBP is also known to possess a sumoylation site. When sumoylated, AGO-binding and RISC-loading is increased (Chen et al., 2015).

Activation of the EGFR, which is well known in hypoxic conditions, leads to phosphorylation of AGO at Threonine 393, resulting in reduced AGO-Dicer association, which decreases a subset of miRNAs (Shen et al., 2013).

### 1.7.3 Mechanisms and Regulation of microRNA Function

After loading of the mature miRNA into RISC, the miRNA guides RISC to complementary sequences that are mainly located in the 3´untranslated region (**3´UTR**) of mRNAs. Dependent on the sequence complementarity, miRNAs exhibit specific functions (figure 2): Perfect or nearly perfect complementarity leads to RNA interference (**RNAi**)-like cleavage of the mRNA, which is the most common miRNA function in plants (Meister and Tuschl, 2004). Since full complementarity is rather rare in animals, mammalian miRNAs predominantly inhibit mRNA translation and initiate mRNA decay. Here, the miRNA target sites are only complementary to nucleotides 2 – 8, which are referred to as **seed sequence** and required for target site recognition (Bartel, 2009), whereas the rest only exhibits partial complementarity (Chen and Rajewsky, 2007), but is nevertheless considered to supplement and even enhance target affinity (Sheu-Gruttadauria et al., 2019). Briefly, at early stages of miRNA-mediated gene silencing, the target mRNA is still stable, but its translation is inhibited at the initiation step. At later stages, mRNA poly(A) tails are shortened, which renders mRNA degradation (Béthune et al., 2012; Djuranovic et al., 2012). In detail, upon formation of a miRNA-mRNA interaction, AGO recruits a member of the GW protein family (in mammals: trinucleotide repeat-containing gene 6 proteins). GW proteins are widely unstructured, contain multiple glycine-tryptophan (GW) repeats and possess an AGO-binding domain, as well as a silencing domain (Pfaff et al., 2013; Schirle and MacRae, 2012). The silencing domain interacts with the deadenylase complexes

The **3´UTR** controls many aspects of mRNA metabolism, such as transport, localization, translation efficiency, and mRNA stability. It can extend up to several kilobases and generally contains numerous binding sites for regulatory proteins and miRNAs, thereby allowing dynamic and combinatorial gene regulation.

**RNAi** refers to the biological mechanism by which RNA molecules inhibit gene expression or translation by targeted neutralization of mRNAs.

Nucleotide positions 2-8 from the 5´ end of the miRNA are called **seed sequence**. They generally perfectly base-pair with the target mRNA and are essential for defining the target repertoire of a miRNA.

poly(A)-specific ribonuclease 2 (PARN2)-PARN3 or carbon catabolite repressor 4 (CCR4)-negative regulator of transcription (NOT), leading to poly(A) tail shortening of the target mRNA and 3´-5´mRNA decay (Behm-Ansmant et al., 2006; Eulalio et al., 2008a, 2008b). Additionally, the GW protein silencing domain interacts with poly(A)-binding proteins residing on the mRNA poly(A) tail, which also leads to a stimulation of **deadenylation** and mRNA decay (Chen et al., 2009). Since a short or absent poly(A) tail is a well-known signal for mRNA decapping, the mRNA-decapping enzyme 1 (DCP1)-DCP2 complex, in conjunction with several stimulatory proteins (e.g. DEAD-box helicase 6; DDX6), is recruited to the 5´ mRNA end, where it removes the $m^7G$ cap (Jonas and Izaurralde, 2015). Because of the unprotected 5´end, the mRNA is degraded by the 5´-3´exoribonuclease 1. DDX6 is the pivotal factor harmonizing translational repression and mRNA decay: It represses translation and stimulates decapping, both of which are indispensably required for mRNA decay. *Via* interaction with the CCR4-NOT complex, DDX6 further couples the process of decapping and deadenylation (Chen et al., 2014; Mathys et al., 2014; Ozgur et al., 2015). Generally, one miRNA can repress numerous genes and, *vice versa*, one gene can be regulated by various miRNAs, revealing a complex gene regulatory network (Bartel, 2009).

Recently, competing endogenous RNAs (**ceRNAs**), i.e., lncRNAs and circular RNAs (circRNAs), have been found to regulate miRNA function by competing for binding to the respective target mRNA or by sponging of miRNAs. For example, the lncRNAs MIAT and TUG1 act as ceRNAs of miR-132. Both bind to miR-132 *via* complementary base pairing, thereby sterically blocking putative seed sequences necessary for target RNA binding, which ultimately upregulates the downstream mRNA targets, and affects cellular pathways (Li et al., 2018, 2017a; Liu et al., 2018). circRNAs are single-stranded non-coding RNAs, which form a covalently closed loop. They are dynamically expressed, function as miRNA sponges, and hence interfere with RISC-loading, thereby de-repressing respective miRNA targets (Barrett and Salzman, 2016; Patop et al., 2019). By containing several consecutive miRNA binding sites, it

**Deadenylation** refers to the removal of the poly(A) tail from the mRNA 3´ end. It´s the first step in mRNA decay, which is followed by removal of the $m^7G$ cap and exonucleolytic 5´ to 3´ degradation of the mRNA.

**ceRNAs** regulate various mRNA transcripts by competing for the pool of shared miRNAs.

is suggested that, especially in neurons, where spatially separated zones of mRNA translation exist, local miRNA-mRNA-interactions are mediated by circRNAs, thereby reducing target mRNA suppression (O'Carroll and Schaefer, 2013). The invention of these interacting non-coding RNAs further bedevils the miRNA regulatory-network.

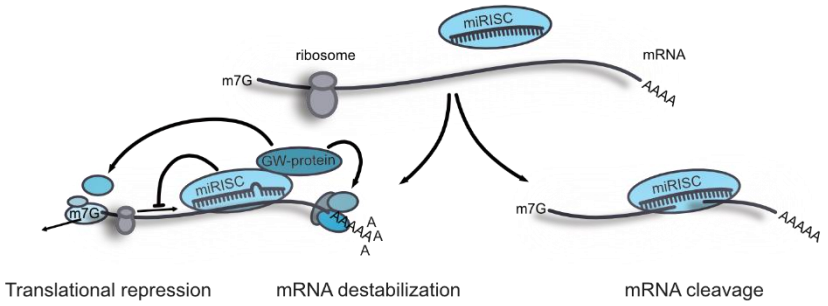


**Figure 2 – Mechanism of miRNA function.** miRNAs are incorporated into the RNA-induced silencing complex (miRISC). Partial complementarity of the miRNA to its target mRNA leads to translational repression or mRNA decay *via* GW-protein-mediated shortening of the poly-A tail and decapping of the 5´end ($m^7G$) (mRNA destabilization), whereas full complementarity results in cleavage of the target mRNA.

## 1.8 microRNAs in the Central Nervous System

Within the mammalian brain, almost 50% of all identified miRNAs are expressed (Landgraf et al., 2007) in a region-, neuron type-, and intraneuronal compartment-specific manner (O'Carroll and Schaefer, 2013). miRNAs are uniquely positioned within the cell to fundamentally contribute to structural and physiological alterations of neuronal activity: miRNAs are transported into the dendritic compartment, whereas specific miRNAs are enriched at the synapse (Edbauer et al., 2010; Schratt et al., 2006). They undergo rapid turnover, which allows dynamic adaptation to neuronal input (Krol et al., 2015). Interestingly, miRNA turnover in neurons is by far faster than in non-neuronal cells, with an average half-life of 30 minutes to one hour, compared to 6 to 24 hours in non-neuronal cells (Gatfield et al., 2009). The neuronal transcript level of certain miRNAs is significantly higher than that of others. Some miRNAs, such as let-7, miR-132, miR-124, and miR-128 are highly expressed in neurons, whereas other miRNAs are only present in

very few copies per neuron (O'Carroll and Schaefer, 2013). This diversity is suggested to be at least partly due to the **multiplicity** of **genes** encoding for brain-enriched miRNAs (Griffiths-Jones et al., 2008), which further supports the interneuronal diversity of miRNA transcript levels (O'Carroll and Schaefer, 2013). This combination of neuronal expression, intraneuronal localization, and rapid turnover strengthens their vital function in the regulation of local protein synthesis involved in neuronal development, maturation, as well as plasticity in response to neuronal activity (Kosik, 2006; Saba and Schratt, 2010; Schratt, 2009; Siegel et al., 2011; Vo et al., 2010).

Numerous miRNAs have been found to impact physiology and morphology of neuronal dendrites and synapses. miR-132, the main focus of this thesis and expounded below, was one of the earliest miRNAs identified to influence neuronal plasticity (Vo et al., 2005). Within the group of plasticity-related miRNAs, miR-132 is unique in the sense that it promotes dendrite and spine outgrowth (Impey et al., 2010; Wayman et al., 2008). Contrastingly, miR-134, -125b, and -138, amongst others, all decrease spine volume and impede neuronal facilitation (Olde Loohuis et al., 2012). One exception among plasticity-regulating miRNAs is miR-124, which, on one hand, mediates spine shrinkage, but, on the other hand, stimulates neurite branching and increases the number of primary neurites.

Manipulation of different miRNAs throughout various behavioral paradigms further pinpoints towards miRNA-mediated gene expression alterations as crucial regulators of adequate behavioral responses. As studied in relevant animal models, miRNAs have been causally linked to anxiety-related behavior (Aten et al., 2019; Haramati et al., 2011; Murphy and Singewald, 2018, 2019), depressive-like behavior (Fonken et al., 2016; Smalheiser et al., 2011), learning and memory (Gao et al., 2010; Zovoilis et al., 2011), social behavior (Cheng et al., 2018; Gascon et al., 2014), as well as fear conditioning-elicited fear (Dias et al., 2014; Murphy et al., 2017; Wang et al., 2013; Xu et al., 2019). Moreover, human miRNA dysregulation has been associated with a multitude of psychiatric and neurological disorders, such as frontotemporal

**Gene multiplicity** is a common mechanism to back-up an adequate expression level of important genes, such as several miRNAs, in the case of mutation of one gene.

dementia (Arrant and Roberson, 2014; Gascon et al., 2014), Alzheimer's disease (Banzhaf-Strathmann et al., 2014; Swarbrick et al., 2019; Zovoilis et al., 2011), fragile X syndrome (Im and Kenny, 2012), schizophrenia (Beveridge et al., 2010; Perkins et al., 2007), major depressive disorder (Li et al., 2013; Lopez et al., 2014; Serafini et al., 2014), post-traumatic stress disorder (Giridharan et al., 2016), autism spectrum disorder (**ASD**) (Abu-Elneel et al., 2008; Issler and Chen, 2015), and general anxiety disorders (Malan-Müller et al., 2013; Muiños-Gimeno et al., 2011), among others. All these studies highlight the profound significance of fine-tuned miRNA transcript levels and their crucial function in the healthy cellular state, making miRNAs potent biomarkers for CNS diseases (Rao et al., 2013). The detailed contribution of miR-132, miR-124, and miR-134 to neuronal function, animal behavior and human psychopathologies is expounded in section 1.8.2 Selected miRNAs Relevant for Anxiety and Fear.

### 1.8.1 Experimental Manipulation of microRNAs *in vivo*

Numerous miRNA sequencing studies reveal different alterations of miRNAs in various behavioral paradigms, pathological states, as well as after pharmacological treatment conditions. Nevertheless, experimental *in vivo* confirmations of causal relationships between miRNA-mediated target gene regulation and behavioral or pathological alterations are rare.

Since miRNAs are – just like all other intracellular effectors – genomically encoded, transgenic knockout of singular or clustered miRNAs is feasible, and to date hundreds of miRNA knockout mouse lines are available (International Mouse Strain Resource, http://www.findmice.org). Single nucleotide polymorphisms (**SNP**s) or mutations in the miRNA coding sequence (Gong et al., 2012) have global effects, since the miRNA-target binding affinity and selectivity is severely altered. Surely, selective breeding of rodents (e.g., breeding for extremes in emotional responses) can also result in genomic miRNA

**ASD** is a neurodevelopmental disorder characterized by impaired social interaction and communication, as well as by restricted and repetitive behavioral patterns. ASD is suggested to be caused by a combination of genetic, epigenetic, and environmental factors.

A **SNP** is a substitution of a single nucleotide at a specific position in the genome, where each variation is present, to some degree, within a population.

sequence alterations. However, no such case has been reported and investigated in the context of social or emotional behavior so far.

To reveal the functional involvement of a distinct miRNA or miRNA cluster in the CNS, overexpression and knockdown studies are commonly performed (figure 3). The gain-of-function, overexpression of miRNAs, is achieved by selective lentivirus (Zöllner et al., 2014) or adeno-associated virus (**AAV**) (Xie et al., 2015) infection, as well as transfection of mimic sequences (Wang, 2011). By overexpression of a particular miRNA, its numerous targets are extensively repressed, thereby leading to dysregulations of intracellular components and finally behavioral maladaptation. The loss-of-function approach is mainly obtained by short hairpin RNA (shRNA)-mediated knock down, which can be achieved by viral infection (Xie et al., 2015) or functional blockage by locked nucleic acids (LNAs; so called AntimiRs) complementary to the miRNA sequence (Veedu and Wengel, 2009), all of which lead to a de-repression of mRNA targets of the respective miRNA.

An **AAV** is a small virus particle, which is able to infect human and some other primate species. Due to its lack of pathogenity, it is currently not known to cause disease. AAVs are attractive vector delivery tools for scientific investigations. In many cases, they integrate into the genome of dividing and quiescent cells, but can also persist in an extrachromosomal state.

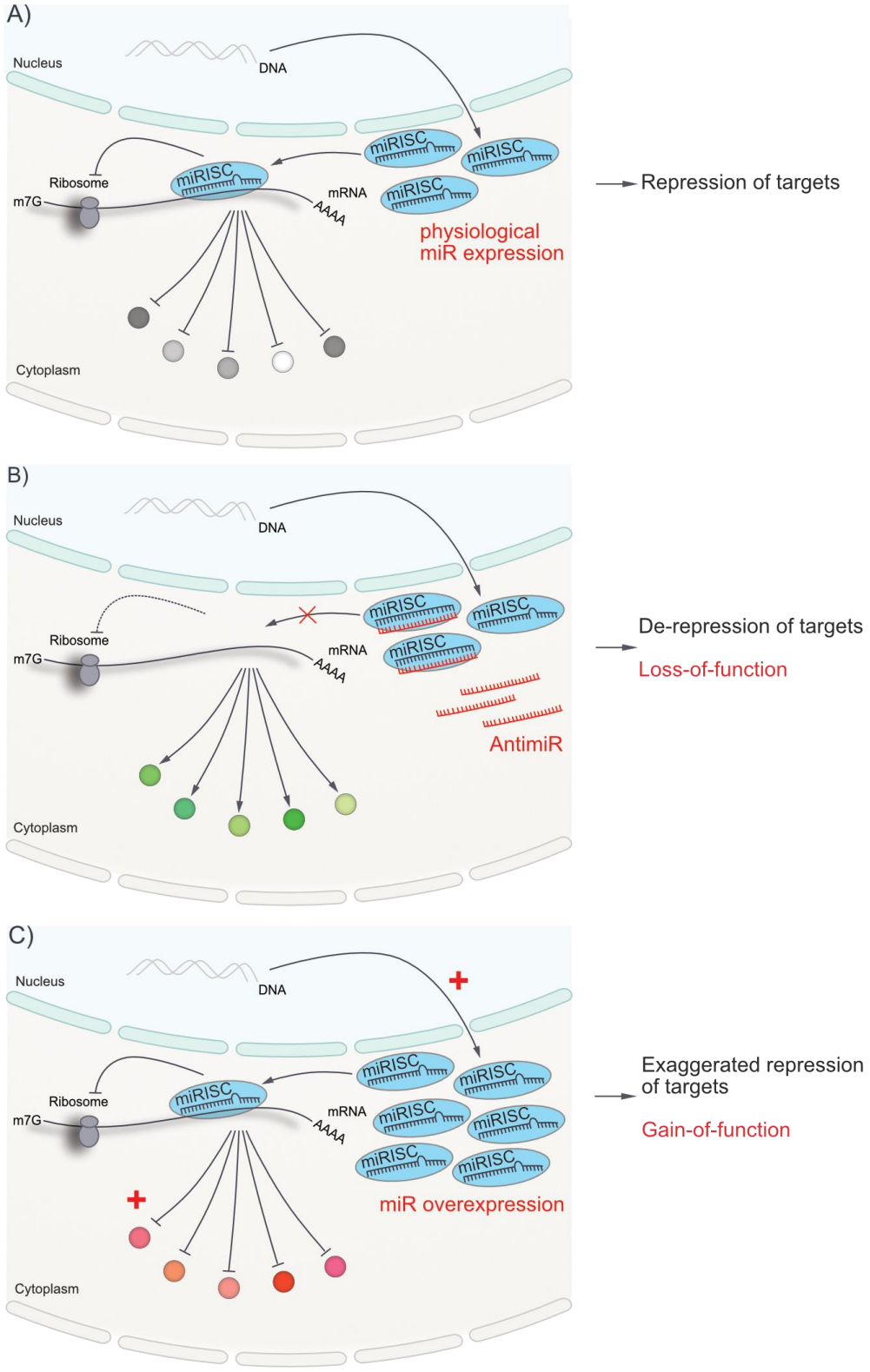


**Figure 3 – Effect of physiological and manipulated miRNA levels on target mRNA expression.** A) Under physiological conditions, miRNAs adequately repress their target mRNAs. B) Transfection with an AntimiR (complementary sequence to target miRNA) leads to functional repression of the miRNA and thereby de-repression of the respective target mRNAs. C) Overexpression of miRNAs leads to excessive RNA-induced silencing complex (RISC) loading and thereby to exaggerated repression of targets (m7G: 5´-mRNA cap; miRISC: miRNA loaded into RNA-induced silencing complex; AAAA: poly-A tail of target mRNA).

For the first time, LNAs have independently been synthesized by Takeshi Imanishi *et al.* (Obika et al., 1997) and Jesper Wengel *et al.* (Koshkin et al., 1998a). They are nucleic acid analogues, which present unprecedented binding affinity and excellent specificity towards complementary DNA and RNA oligonucleotides. As indicated by their name, LNAs are composed of nucleic acids, which are locked by an O2´-C4´-methylene linkage at their ribose ring (figure 4). This imposes conformational restriction to adopt an *N*-type sugar puckering (Koshkin et al., 1998b, 1998a; Obika et al., 1998; Singh et al., 1998).

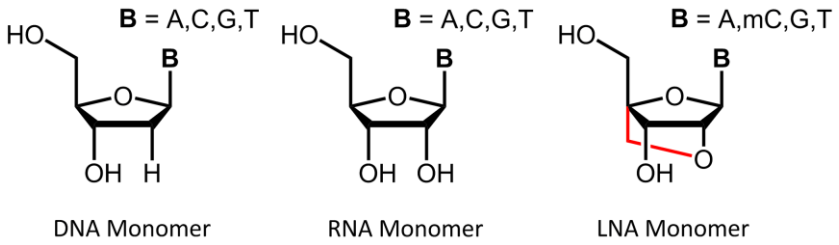


**Figure 4 – Representation of a DNA, RNA, and locked nucleic acid (LNA) monomer.** The structure of a DNA (left), RNA (middle), and LNA (right) monomer is schematically represented. LNAs consist of an additional O2´-C4´-methylene linker depicted in red (B: base, A: adenine; C: cytosine; mC: methyl-cytosine; G: guanine; T: thymine; adapted from biomers.net).

LNAs offer crucial properties, which are essential for successful therapeutic exploitation: (i) unprecedented RNA (and DNA) binding affinity, (ii) excellent base pairing specificity (up to 1 nt), (iii) high bio-stability and resistance to nucleolytic degradation, (iv) low toxicity in animals (for the majority of LNAs), and (v) eligible chemistry for proper manufacturing and structural modification (Veedu and Wengel, 2009). To date, various sugar-phosphate backbone modifications, cholesterol substitution or addition of fluorescent dyes are synthesizable and offer manifold application possibilities. The substitution of miRNA targeting LNAs with fluorophores is typically conducted at the 5´ end of the LNA, since labeling of the 3´end carries the risk of poor complementary base pair interaction to the corresponding miRNA by steric interference, which renders them functionally not effective.

### 1.8.2 Selected microRNAs Relevant for Anxiety and Fear

In all described miRNA studies of this thesis, transcript levels of numerous miRNAs have been evaluated. However, I want to focus on only three of them: miR-132-3p, miR-134-5p, and miR-124-3p. All three of them have been selected based on the analysis of a previously performed miRNA Deep Sequencing and literature-based research. All three miRNAs are brain-enriched miRNAs and have been shown to mediate anxiety, fear, and/or socio-behavioral responses (for details see section 1.8.2 Selected miRNAs Relevant for Anxiety and Fear). However, their specific involvement in OXT-induced anxiolysis and OXT-mediated reversal of social fear has not been investigated so far.

#### 1.8.2.1 Neuronal miR-132/212

In 2002 miR-132 was identified as one of the first brain-specific miRNAs (Lagos-Quintana et al., 2002). The highly conserved locus of miR-132/212 lies in an intergenic region of the long arm of chromosome 11 (qB5) in mice and in humans on the short arm of chromosome 17 (p13.3) with Mir-212 being 200 bp upstream of Mir-132 (Haeussler et al., 2019). It is located on the plus strand between the two overlapping genes of the serine-hydrolase Ovca2 and Dph1, which are located ~2 kb downstream on the minus strand, and the transcription factor Hic1 that is ~3 kb upstream on the minus strand. Nevertheless, the pri-miR-132/212 transcript is independent from the genes flanking its locus. Within the promoter of Mir-132/212, three CREB responsive elements (Impey et al., 2010; Vo et al., 2005), one MEF-2A, and one repressing REST binding site is present (TRANSFAC® Professional). miR-132 and miR-212 are **polycistronic transcripts** that are transcribed into two isoforms. Isoform 1 is 5.1 kb long and is expressed in brain and testes, whereas isoform 2 is only 2.3 bp in length and expressed in brain, testes, heart, and mammary gland among others (Ucar et al., 2010). As polycistronic transcript, both isoforms are processed into separate pre-miR-132 and pre-miR-212 transcripts that undergo all further steps of biosynthesis. Mature miR-132 and miR-212 possess the same seed

**Polycistronic transcripts** are genes that function as hereditary unit and derive from multiple regions.

**Exemplified nomenclature of miRNAs:** The numbering of miRNA genes is simply sequential.

Example:
mmu-miR-132-3p
Mmu:
prefix stating species
MiR-132:
gene encoding for miRNA
mir-132:
pre-miRNA transcript
miR-132:
mature miRNA transcript
-3p:
derived from the 3´arm
-5p:
derived from the 5´arm

sequence, which classifies them as components of the same miRNA-family. Both are expressed at high levels in the brain and testis, and at lower levels in other tissues and cell types, such as macrophages, tumor endothelial cells, ovarian granulose cells, gonadotrope cells, mammary stromal cells, and sperm (Anand et al., 2010; Benito et al., 2018; Fiedler et al., 2008; Luers et al., 2010; Shaked et al., 2009; Ucar et al., 2010; Yuen et al., 2009). Interestingly, miR-132 exhibits a rostral to caudal pattern within the mouse brain with the highest expression in the forebrain and the lowest in cerebellum (Olsen et al., 2009). Moreover, its expression increases post-natally, reaching its maximum on day 28, which is equal to its expression level within the adult brain (Impey et al., 2010; Nudelman et al., 2010). Interestingly, neurons transfer miR-132 into the peripheral blood *via* secreting exosomes (Xu et al., 2017), which illustrates a novel avenue for neurovascular communication. This trafficking seems to be functionally essential for the maintenance of the brain´s vascular integrity, where neurons transfer miR-132 to endothelial cells, resulting in adequate regulation of the adherens junction protein endothelial cadherin by directly targeting the eEF2 kinase.

miR-132 is the first identified activity-dependent miRNA. Since the discovery of miR-132/212, a wide variety of stimuli has been shown to induce their expression. Amongst those stimuli, the brain derived neurotrophic factor (BDNF) was the first of numerous trophic factors and extracellular signals found to increase miR-132 expression (Vo et al., 2005). Additionally, miR-132 transcription is enhanced by neuronal depolarization elicited in cultured hippocampal neurons *via* e.g., potassium chloride and treatment with forskolin (activation of adenylyl cyclases) or the $GABA_A$ antagonist bicuculline, all of which signal *via* CREB (Klein et al., 2007; Nudelman et al., 2010; Wayman et al., 2008). This increase of miR-132 is present within the soma as well as the dendrites of cultured hippocampal neurons. Here, induction of the pre-miR-132 transcript is much more robust than induction of the mature miR-132. This suggests that processing of miR-132 from the precursor to the mature form is regulated, however, the factors mediating this

regulation remain elusive (Nudelman et al., 2010; Wibrand et al., 2010). *In vivo*, miR-132 is induced in specific brain regions after seizures (Huang et al., 2014) and several behavioral paradigms that involve neuronal activation. For example, contextual fear conditioning (neuronal activation in the hippocampus), odor exposure (neuronal activation in the olfactory bulb), exposure to predator-scent or multimodal stress (neuronal activation in the hippocampus and amygdala), learned safety (neuronal activation of the basolateral amygdala), non-learned helplessness (neuronal activation of the frontal cortex), and cocaine injection (neuronal activation in the striatum) all cause miR-132 to increase in the respective brain regions (Aten et al., 2019; Nudelman et al., 2010; Ronovsky et al., 2019; Shaltiel et al., 2013; Smalheiser et al., 2011). Similarly, it was found that social isolation of female mice leads to increased miR-132 expression in the cerebral cortex, concomitant with decreased miR-134 levels (Kumari et al., 2016), suggesting a miRNA mediated regulation of synaptic plasticity. Moreover, exposing mice to light leads to increased miR-132 within the SCN, a crucial brain region for the regulation of the circadian clock (Cheng et al., 2007). Within the SCN miR-132/212 is also known to modulate entrainment to seasonal photoperiods by altering dendritic spine density and morphology *via* methyl-CpG binding protein 2 (MeCP2) and its downstream factors BDNF and the kinase mTOR (Mendoza-Viveros et al., 2017).

Electrophysiological studies depicted the significant contribution of miR-132 and/or miR-212 in synaptic transmission and function by regulation of long-term potentiation (LTP). miR-132 overexpression has been shown to enhance synaptic transmission (Edbauer et al., 2010; Lambert et al., 2010), whereas viral miR-132 inhibition impairs synaptic transmission (Luikart et al., 2011; Remenyi et al., 2013). The effect on LTP is suggested to be, on one side, based on a presynaptic influence of miR-132 either on the probability of neurotransmitter release or the number of synapses, and, on the other side, a miRNA-mediated regulation of postsynaptic AMPA receptors. The beneficial influence of miR-132/212 on LTP has been further evidenced *in vivo* (Benito et al., 2018). Environmental enrichment is known to increase hippocampal

LTP and results in increased miR-132/212 expression in the hippocampus of male mice. Interestingly, increased miRNA transcript levels have also been found in the sperm of male mice exposed to environmental enrichment, wherein they are involved in intergenerational, but not transgenerational, inheritance of the enhanced LTP phenotype. Further *in vivo* studies revealed that induction of LTP by high-frequency stimulation of the perforant path in rats increased miR-132 and miR-212 expression in the dentate gyrus in a mGluR-dependent and NMDA receptor insensitive manner (Wibrand et al., 2010). The significant capacity of miR-132/212 in regulation of LTP underscores the crucial involvement of both miRNAs in synaptic plasticity, but also suggest them as highly potent candidates for learning and memory processes.

To date, several studies provide causal evidence for miR-132/212 being a pivotal factor for cognitive capacity and emotional behavior. Transgenic mice overexpressing miR-132 in forebrain neurons display significant deficits in novel object recognition (Hansen et al., 2010). Similarly, extreme supra-physiological levels of miR-132 (>3-fold) inhibit learning, whereas low hippocampal miR-132 overexpression enhances cognitive capacity in the Barnes maze (Hansen et al., 2013). Constitutive or conditional double-knockout of miR-132/212, or inhibition of only miR-132, in mice results in significant cognitive deficits in formation and retention of spatial, temporal, and recognition memory, as well as in tests of novel object recognition (Hansen et al., 2016; Hernandez-Rapp et al., 2015; Wang et al., 2013). This contradictive effect showing that both, supra- and infra-physiological levels of miR-132/212, reveal the same effect on cognitive capacity in mice, is explained by its effect on neuronal plasticity. Overexpression of miR-132/212 is known to lead to elevated dendritic spines, which is suggested to be the cause of abovementioned cognitive deficits (Hansen et al., 2013). Generally, awareness and appropriate response to imminent threats are essential for preventing chronic stress and anxiety, as well as for the wellbeing and preservation of a species. Learned safety is an animal model utilizing imminent aversive stimuli, and includes a fear inhibitory

mechanism that regulates fear responses, promotes episodes of safety, and generates positive affective states (Kong et al., 2014). Here, the miR-132/212 family members are identified as safety-related miRNAs within the basolateral amygdala (Ronovsky et al., 2019). Overexpression of miR-132 impairs the behavioral recall of learned safety, whereas miR-132/212 knockout enhanced learned safety deciphering another essential role for miR-132/212 in fear avoidance learning. In any case, those deficits caused by manipulation of miR-132/212 further emphasize the crucial role of maintaining a limited range of miR-132/212 expression within the brain to ensure normal learning and memory, as well as cognitive tasks.

Although controversially found, miR-132/212 is suggested to be a regulator of anxiety-related behavior. Constitutive miR-132/212 knockout does not alter anxiety-related behavior measured on the EPM (Hernandez-Rapp et al., 2015), whereas a recent study showed that miR-132 overexpression, as well as conditional miR-132/212 knockout, significantly increases anxiety-related behavior (Aten et al., 2019). Moreover, social isolation-induced anxiety is suggested to be partly mediated by increased miR-132 in the cerebral cortex or PFC of female mice (Kumari et al., 2016). Similarly, maternal separation has not only been associated with increased anxiety-related behavior, but also with elevated miR-132 transcripts in the PFC in rats (Uchida et al., 2010). Despite controversial or without a causal relation, abovementioned studies clearly pinpoint towards a regulatory function of miR-132 in anxiety-related behavior and therefore suggest miR-132 to be possibly involved in anxiety disorders (Malan-Müller et al., 2013).

So far, alterations of miR-132 and miR-212 expression were observed in numerous diseases ranging from vascular/immune/neoplastic to metabolic and neurological disorders. Analysis of miR-132/212 transcript levels across various tissues, blood, and tumor cells reveals dysregulated miR-132/212 transcript levels in numerous forms of cancer (Haller et al., 2010; Hatakeyama et al., 2010; Incoronato et al., 2010; Park et al., 2011; Wang et al., 2018), rheumatoid arthritis (Pauley

et al., 2008), type two diabetes (Klöting et al., 2009), non-alcoholic and fatty liver disease (Estep et al., 2010).

Given the crucial role of miR-132 in neuronal development and function, it is reasonable that alterations of its transcript levels are associated with certain neurological disorders. **Rett syndrome**, an ASD-associated syndrome affecting young girls, is caused by mutations in MeCP2, which is a known target of miR-132 (Klein et al., 2007). miR-132 reduces MeCP2 expression, which decreases BDNF levels, subsequently leading to reduced miR-132, which in turn allows MeCP2 levels to increase. Via this self-regulatory pathway, miR-132 is suggested to contribute to regulation of Rett- and autism-related genes (Lyu et al., 2016). Further evidence for the involvement of miR-132 in ASD-related behavioral dysfunctions is provided by prenatal exposure to the anticonvulsant and mood-stabilizing **valproic acid** in rats, which is a well-established animal model of ASD (Markram et al., 2008; Schneider and Przewłocki, 2005). Here, prenatal valproic acid treatment leads to increased miR-132 transcript levels within the embryonic brain, which is suggested to underlie the ASD-like behavioral malfunctions (Hara et al., 2017). Since valproic acid is a histone deacetylase inhibitor (Phiel et al., 2001), the observed behavioral alterations are likely mediated by histone PTMs that alter the expression pattern of disease-related genes, such as miR-132. Likewise, miR-132 is downregulated in *post mortem* PFC tissue of schizophrenia patients (Kim et al., 2010; Miller et al., 2012). Subsequently, target genes are abnormally expressed which contributes to the neurodevelopmental and neuromorphological pathologies present in schizophrenia. This is further evidenced by a mouse model of schizophrenia in which miR-132 levels are reduced within the PFC and hippocampus (Stark et al., 2008). Moreover, the miR-132 level in peripheral blood mononuclear cells is proposed as superior biomarker to discriminate first-onset schizophrenia patients from healthy controls (Yu et al., 2015). Prior to treatment, miR-132 was downregulated in the blood of psychotic patients, whereas after antipsychotic treatment miR-132 was markedly increased compared to pre-treatment levels. Further confirmation in a dizocilpine-induced rat

**Rett syndrome** is symptomized by problems with language and coordination, as well as by repetitive movements. So far, there is no cure for Rett syndrome; treatment is directed to improve symptoms.

Children prenatally exposed to **valproic acid** have an increased absolute risk (cumulative incidence) of being diagnosed with ASD.

model of schizophrenia revealed down regulated miR-132 in peripheral blood mononuclear cells, as well as whole-brain samples. Moreover, plasma miR-132 levels are increased in untreated major depressive disorder patients compared to healthy controls, as well as citalopram-treated depressive patients, suggesting mir-132 as indicator for the current depression status (Fang et al., 2018). This increased plasma miR-132 expression is associated with visual memory deficit, a core feature of depression that further correlates with comorbid anxiety symptoms (Liu et al., 2016). Abovementioned studies clearly highlight the involvement of miR-132 in the pathogenesis of neuropsychiatric disorders. However, the distinct contribution of miR-132 on disease-related genes needs further elucidation.

Given its crucial contribution in maintaining synaptic homeostasis, it is not surprising that miR-132 has been associated with neurodegenerative diseases, such as Alzheimer´s disease, Parkinson´s disease, Huntington´s disease, amyotrophic lateral sclerosis, and frontotemporal dementia in addition to Rett syndrome and ASD (Salta and De Strooper, 2017). Although partially contradictive, abovementioned review reveals a strong impact of miR-132 dysregulation in neurodegenerative diseases and suggests potential therapeutic treatment strategies.

#### 1.8.2.2 Neuronal miR-124

In mice, miR-124 is encoded on the plus strands of chromosome 2, 3, and 14, whereas in humans the gene is located on the minus and plus strand at different regions of chromosome 8 and on the plus strand of chromosome 20. The present gene multiplicity illustrates the functional importance of miR-124, which is further emphasized by the fact that it is the most abundant miRNA in the mouse brain as it accounts for 25-48% of all brain-expressed miRNAs (Lagos-Quintana et al., 2002).

miR-124 increases the number of primary neurites in cortical neurons, promotes differentiation of neuronal stem cells, and stimulates neurite branching (Silber et al., 2008). In differentiating mouse P19 cells and in

primary cortical neurons, miR-124 reduces F-actin density, increases tubulin acetylation, and possibly regulates cytoskeletal reorganization, which overall stimulates neuronal outgrowth (Yu et al., 2008). Within the mouse subventricular zone, miR-124 reduces SRY-box containing gene 9 and thereby regulates adult neurogenesis (Cheng et al., 2009).

The miR-124 dosage within the mouse PFC is suggested to modulate adequate PFC function. Heterozygous knockout of miR-124 in mice results in impaired **prepulse inhibition**, metamphetamine-induced hyperactivity, and social behavior deficits (Kozuka et al., 2019). The sensorimotor gating disruptions revealed by prepulse inhibition are found to be mediated by miR-124-dependent dopamine receptor D2 de-repression.

Interestingly, cortical miR-124 is also downregulated in a frontotemporal dementia mouse model and in human patients, suggesting it as crucial contributor for the development of present socio-behavioral dysfunctions (Arrant and Roberson, 2014; Gascon et al., 2014). The critical miR-124 target mediating reduced sociability is the AMPA receptor subunit GluA2. GluA2 containing AMPA receptors are calcium impermeable, whereas those lacking GluA2 are calcium permeable. Viral overexpression of miR-124 or GluA2 knockdown in the medial PFC corrects sociability deficits in the frontotemporal dementia mouse model, confirming the miRNA-mediated pathogenic effects of altered AMPA receptor subunit composition on social investigation. Moreover, it negatively constrains serotonin-induced long-term facilitation and converts short-term into long-term synaptic facilitation in *Aplysia californica*, revealing a memory-regulatory capacity of miR-124 (Rajasethupathy et al., 2009).

**Prepulse inhibition** is a neurobiological phenomenon in which a weaker pre-stimulus (prepulse) inhibits the organism´s reaction to a subsequent strong startling stimulus (pulse). Used stimuli are commonly acoustic, tactile and visual.

Within the hippocampus, expression of the glucocorticoid receptor (GR) is regulated by cAMP and miR-124, indicating miR-124 as essential component for proper feedback regulation of the stress response within the brain and along the HPA axis (Aesoy et al., 2018). Acute restraint stress in mice leads to a partly sex-dependent decrease in amygdalar and hippocampal miR-124 transcript levels, and, thereby, de-repression

of the mineralocorticoid receptor (MR), which is an important effector of the early stress response (Mannironi et al., 2013). Moreover, hippocampal miR-124 is downregulated in mice exposed to chronic ultra-mild stress and viral overexpression of neuronal miR-124 leads to resilience, whereas miR-124 inhibition results in susceptibility to this mild stress paradigm (Higuchi et al., 2016). Abovementioned studies highlight a possible involvement of miR-124 in depression, and some studies already suggest miR-124 as potential disease biomarker. Voluntary exercise, which is related to higher resilience to psychological stress, decreases hippocampal miR-124 transcripts, thereby increasing GR expression, in single-housed mice (Pan-Vazquez et al., 2015).

In recent years, miR-124 has been repeatedly emphasized as crucial factor of innate and adaptive immune responses, as well as of peripheral, tumor, and CNS immune-related diseases. In peripheral immune-diseases, such as inflammatory bowel disease, rheumatoid arthritis, and ankylosing spondylitis, alterations of miR-124 are suggested to severely affect the disease etiology and progression, making miR-124 a possible candidate for future treatment (Qin et al., 2016). Furthermore, numerous studies support the potential application of miR-124 as immunotherapeutic agent for tumors and as novel antiviral treatment.

With respect to CNS-affecting disorders, miR-124 has been suggested to be a potential factor for brain recovery after stroke. In a middle cerebral artery occlusion mouse model of stroke, application of synthetic miR-124 reduced brain injury and functional impairment as well as enhanced angiogenesis and neurovascular remodeling (Doeppner et al., 2013). Moreover, plasma miR-124 is suggested to be a reliable biomarker for stroke identification (Sun et al., 2015). Autoimmune encephalomyelitis (Ponomarev et al., 2011), spinal cord injury (Louw et al., 2016), and chronic pain (Willemen et al., 2012) all lead to activation of microglia, which can be alleviated or even prevented by miR-124 application. High miR-124 levels decrease microglia activation and infiltration, as well as activation of macrophages, thereby reducing the inflammatory state. However, in

epilepsy miR-124 does not act as repressor, but activator of microglia (Brennan et al., 2016). In epileptic states, miR-124 is a dual regulator, which on one hand attenuates epileptogenesis, but on the other hand promotes epilepsy by exaggerating inflammation. Furthermore, miR-124 is proposed as novel inhibitor of glioblastoma and neuroblastoma cell differentiation and invasion (Fowler et al., 2011; Huang et al., 2011), making miR-124 a potential therapeutic target for brain tumor treatment.

So far, no direct evidence for miR-124 as important gene regulator involved in mood disorders, such as anxiety and depression is existing. Nevertheless, substantial evidence pinpoints towards miR-124-mediated alterations, which could impact the susceptibility to develop mood and anxiety disorders in adulthood (Malan-Müller et al., 2013). Additionally, plasma miR-124 is increased with depression and antidepressant treatment, making it a possible biomarker for diagnosis and treatment response (Fang et al., 2018).

Just as miR-132, dysregulation of miR-124 has been implicated in neurodegenerative diseases, such as Alzheimer´s disease (Smith et al., 2011), Parkinson´s disease (Li et al., 2017b), Huntington´s disease (Johnson and Buckley, 2009), and frontotemporal dementia (Arrant and Roberson, 2014; Gascon et al., 2014). Although vast progress has been made over the last years, further detailed research is needed to understand miR-124-mediated regulation of disease-related genes for specific future diagnosis and possible treatment of abovementioned disorders.

#### 1.8.2.3 Neuronal miR-134

A **cluster** of genes refers to a group of two or more genes found within the organism´s genome, which encode for similar polypeptides or proteins that are often located in close proximity (a few thousand base pairs) and share a generalized function.

The intergenic miR-134 is encoded on the plus strand of chromosome 12 of mice and in humans on the plus strand of chromosome 14. miR-134 is part of the largest known mammalian miRNA **cluster**, the so called miR-379-410 cluster, which is maternally imprinted and encompasses 38 miRNAs in mice and 40 miRNAs in humans (Jimenez-Mateos et al., 2012; Seitz et al., 2004). The miR-379-410 cluster is brain-

specific and its functions seems to be only conserved in mammals, where it is discussed in terms of epigenetic control and gene regulation during development (Seitz et al., 2004).

During embryonic development, miR-134 is broadly expressed, but its postnatal expression is largely restricted to the brain (Landgraf et al., 2007; Schratt et al., 2006). Within the brain, miR-134 expression is cell-type specific: It is highly expressed in pyramidal neurons of the hippocampus (Jimenez-Mateos et al., 2012; Schratt et al., 2006), but not in cells of glial origin (Landgraf et al., 2007). miR-134 regulates cortical neuron migration (Gaughwin et al., 2011), activity-dependent dendritogenesis (Fiore et al., 2009), dendritic spine development (Schratt et al., 2006), LTP (Gao et al., 2010), and homeostatic synaptic downscaling (Fiore et al., 2014). Of course, other members of the miR-379-410 cluster, such as miR-381, miR-329, and miR-485, have also been found to control neuronal plasticity (Lackinger et al., 2019), but their specific contribution in this regard is out of the scope of this thesis.

Several studies investigated behavioral alterations in different miR-379-410 knockout mice, which all suggest miR-379-410 in emotional processing. The lack of this miRNA cluster is found to be associated with abnormal emotional responses, since knockout mice display increased anxiety-related behavior in unfamiliar environments, like EPM and OFT, without changes in spontaneous exploration, general locomotion, depressive-like behavior, and sociability (Marty et al., 2016). In contrast to the unchanged sociability found in abovementioned study, recently the group of Gerhard Schratt revealed that juvenile (23 days old) miR-379-410 knockout mice display a hyper-social and anxiogenic behavior illustrated by increased social interaction and ultrasonic vocalizations (Lackinger et al., 2019). The pro-social effect was accompanied by increased excitatory synaptic transmission and exaggerated expression of hippocampal ionotropic glutamate receptor complexes. Moreover, the hypersocial effect in miR-379-410 cluster knockout mice was abolished in adolescent male, but not female individuals. Within both studies, miR-134 is suggested to be the most important miRNA of the

miR-379-410 cluster, which results in the observed behavioral alterations.

Additionally, miR-134-mediated dysregulation of neuronal plasticity within the ventromedial PFC relates to depressive-like behavior in chronically stressed rats (Fan et al., 2018). Exposure to chronic unpredictable mild stress (CUMS) upregulates miR-134 transcripts within the ventromedial PFC, leading to repression of synapse-associated proteins. Sponging and thereby blocking miR-134 function within the ventromedial PFC of CUMS-exposed rats *via* an AAV significantly ameliorates neuronal plasticity alterations and depressive-like behavior. To date, it is well known that environmental enrichment ameliorates cognitive impairments and depressive-like behavior elicited by CUMS exposure. Only recently, improvement of these cognitive and emotional dysregulations has been shown to be dependent on miR-134-mediated regulation of sirtuin 1 (SIRT1) within the hippocampus (Shen et al., 2019). SIRT1 is characterized by a histone deacetylase activity and is involved in synaptic plasticity by regulating miR-134 expression (Bicker and Schratt, 2010). Furthermore, loss-of-function of SIRT1 impairs memory, which is illustrated by decreased freezing 24 hrs after contextual fear conditioning, a poor discrimination index in the novel object discrimination test, and increased escape latency in the Morris water-maze test (Gao et al., 2010). These profound memory impairments were suggested to be mediated by dysregulated miR-134 expression in response to manipulation of SIRT1 expression levels. Under physiological conditions, SIRT1 together with several co-factors restricts miR-134 transcription, leading to high CREB levels, which enable functional synaptic plasticity (Gao et al., 2010). Loss-of-function of SIRT1 causes high miR-134 transcript levels, which lead to translational repression of CREB proteins resulting in impaired synaptic plasticity.

Increasing evidence indicates miR-134 as essential factor of human carcinoma and tumor cell proliferation, apoptosis, invasion, and metastasis, as well as cancer diagnosis, treatments and prognosis (Pan et al., 2017). miR-134 functions as both, tumor repressor but also cancer

promoter of numerous cancer types, such as lung cancer, breast cancer, colorectal cancer, colon cancer, prostate cancer, gastric cancer, glioma, and glioblastoma.

As crucial regulator of neuronal plasticity, cognitive functions, and memory, it seems not surprising that also miR-134 is associated with various psychiatric and neurologic diseases. In peripheral blood mononuclear cells of bipolar patients, miR-134 expression is decreased (Rong et al., 2011). The same alteration is observed in the blood of schizophrenic patients (Gardiner et al., 2012), whereas increased miR-134 transcript levels are found *post mortem* within the dorsolateral PFC (Santarelli et al., 2011). Furthermore, miR-134 expression is increased in peripheral blood of autistic children, highlighting the potential use of miRNAs as biomarkers and therapeutic targets in neurologic disorders (Hirsch et al., 2018). In neurodegenerative disorders, especially Alzheimer's disease and mild cognitive impairment, a set of miRNAs, including miR-134, is increased within the plasma (Sheinerman et al., 2012). Abovementioned studies pinpoint miR-134 as a potential regulator of neurological, psychiatric, and neurodegenerational disorders. Nevertheless, the specific contribution of miR-134 within those diseases remains elusive.

## 1.9 Aims and Outline of the Thesis

Both GAD and SAD have a surpassing lifetime prevalence and hence represent an enormous burden for society and economy. Experiencing them decreases life quality, may induce social isolation, and substance abuse, all of which in turn promote anxiety. In order to escape this vicious cycle, specialized and specific treatment is particularly required. The neuropeptide OXT represents a potential treatment option due to its anxiolytic and pro social activity described in preclinical studies. Moreover, the manipulation of miRNAs *via* inhibition or overexpression is a promising option: In 1999, the first antisense-oligonucleotide therapeutic Fomivirsen (CIBA Vision Europe) was launched in Germany for the treatment of cytomegalo-virus retinitis. Numerous preclinical and clinical studies are currently conducted and in 2018, the first ever RNAi based therapeutic, called Onpattro (patisiran) (Alnylam Pharmaceuticals), was launched in Germany for the treatment of Hereditary Transthyretin-Mediated Amyloidosis in adults. Although the knowledge about OXT´s central actions and miRNA-based gene expression modulation increases continuously, many aspects of the mechanisms causing or contributing to pathologies remain elusive at a molecular, cellular, and tissue-wide level. So far, not a single study deciphers the involvement of miRNAs in OXT´s modulatory effect on socio-emotional behavior. To shed further light onto this uncharted field, I focused on two major research questions in this thesis.

1. ***"Is miR-132 within the PVN Involved in Oxytocin-induced Anxiolysis?"***

A previously performed miRNA Deep Sequencing of rat PVN tissue and rat embryonic hypothalamic cells revealed numerous miRNAs that are altered in their expression profile 3 hours after either icv OXT infusion or stimulation with synthetic OXT, respectively. Based on analysis of the Deep Sequencing data and literature research of putative miRNA targets, I aimed to validate the expression of selected miRNAs, especially miR-132/212 family members, after icv OXT infusion in male and female rats in a time-dependent manner. I hypothesized that especially miR-132 underlies the local signaling of OXT within the PVN. I aimed to prove this by pharmacological studies utilizing icv OXT infusions or a combinatorial treatment of an OXTR antagonist (OXTR-A) with subsequent OXT infusions in rats. Here, I found that miR-132-3p is induced by activation of the OXTRs within the PVN of male and female rats. Based on the first hypothesis and the well-established anxiolytic effect of OXT during lactation, I further hypothesized that intra-PVN miR-132 underlies this anxiolytic effect of endogenous OXT. Therefore, I intended to measure miRNA expression levels within the PVN and CSF in mid-lactation (lactation day 5, LD5), as well as after icv OXTR-A application. Unfortunately, lactating rats did not show increased intra-PVN miR-132-3p level. Only

short-term removal of the pups resulted in increased miR-132-3p. Additionally icv application of an OXTR-A decreased miR-132-3p within the PVN of lactating rats. Moreover, I aimed to assess mRNA expression levels of the OXTR to validate equal activation properties between lactating and virgin females. Since miR-132 is associated with anxiety disorders (Malan-Müller et al., 2013), I hypothesized that local PVN miR-132 inhibition increases anxiety- and fear related behavior in rats. Hence, I aimed to inhibit miR-132 within the PVN in combination with local OXT infusion prior to anxiety and fear assessment. Inhibition of intra-PVN miR-132-3p resulted in a prevention of OXT-mediated aniolysis, whereas the effect on cued fear was not explicit.

Taken together, I showed that miR-132 underlies intracellular OXTR signaling and might thereby be involved in OXT-mediated anxiolysis.

### *2. "Does miR-132 Mediate the Oxytocin-induced Reversal of Social Fear?"*

Based on abovementioned studies in rats (see first research aim), I hypothesized that miR-132 within the LS is involved in extinction of social fear. In order to assess this research question, I aimed to analyze septal miRNA transcript levels in male mice at various time points (30 min, 90 min, 3 hrs, and 24 hrs) after acquisition and extinction of social fear. Here, I found miR-132 as crucially regulated miRNA, which might be involved in social fear behavior. Based on the first hypothesis, I assumed that manipulation of septal miR-132 significantly alters social fear extinction behavior. Hence, I intended to perform SFC after septal inhibition or overexpression of miR-132 within the LS. Here, inhibition impaired, whereas overexpression facilitated social fear extinction in male mice. Based on the results found, I hypothesized that septal miR-132-3p underlies the OXT-mediated reversal of social fear found in male and female mice (Menon et al., 2018; Zoicas et al., 2014). Therefore, I aimed to inhibit septal miR-132-3p prior to local OXT infusion and social fear extinction assessment. Septal miR-132-3p inhibition prevented the OXT-mediated reversal of social fear in male mice. Since all methodological approaches (inhibition, overexpression of miR-132) affect all neuronal and glial subtypes, I further hypothesized that miR-132 within OXTR positive neurons of the LS is the mediator of the observed fear reversing effect of OXT. Therefore, I intended to deplete miR-132 only within OXTR expressing neurons using a SICO-shRNA based AAV-approach, which ultimately confirmed my hypothesis: shRNA-mediated knockdown of septal miR-132-3p resulted in impairments of social fear extinction. Last, but not least, I hypothesized, that septal miR-132 is repressing several target mRNAs, which ultimately result in the observed behavioral adaptations. Therefore, in a biochemical approach, I intended to identify miR-132 target mRNAs within the LS

*via* AGO-co-immunoprecipitation (AGO-IP) and Microarray, revealing numerous promising mRNA candidates, which I aimed to validate further.

The obtained results provide the first evidence for miR-132 within OXTR expressing neurons to not only impair extinction, but also prevent OXT-mediated reversal of social fear.

# Material and Methods

## 2.1 Animals and Animal Husbandry

Male and female Wistar rats (8-9 weeks at start of experiment; Charles River Laboratories, Germany), male CD1 wildtype mice (8 weeks at start of experiment; Charles River Laboratories, Germany), and male OXTRCre mice with CD1 background (8 weeks at start of experiment; University of Regensburg, Germany) for conditional Cre-mediated knockdown of miRNAs in OXTR expressing neurons (Li et al., 2016; Nakajima et al., 2014) were housed in groups of 4 under standard laboratory conditions (12/12 hrs light/dark cycle; lights on at 07:00 a.m., 21-23°C, 55-60% humidity) with food and water *ad libitum*. Female rats were mated with sexually experienced males and single housed on pregnancy day (PD) 18. Icv cannulas were stereotaxically implanted on PD 18. The day of birth was set as LD 1. Behavioral assessment as well as euthanasia was performed in mid lactation (LD 5). All experiments were performed between 08.00 a.m. and 12.00 a.m. in accordance with the Guide for the Care and Use of Laboratory Animals by the NIH, and were approved by the government of the Oberpfalz.

## 2.2 Surgical Procedures

All animals were allowed at least one week of habituation prior to surgical procedures. Animals received a subcutaneous injection of the analgesic Buprenovet (Bayer, Germany, 0.05 mg/kg Buprenorhine) and the antibiotic Baytril (Baxter, USA, 10 mg/kg Enrofloxacin) 30 min prior to surgery. All stereotaxic surgeries (guide cannula implantation and microinfusion of LNAs or AAVs; see table 1; Kopf stereotactic frame 922, Kopf Instruments, Canada) were performed under isoflurane (Orion Pharma, Germany) inhalation anesthesia and in semi-sterile conditions. The following coordinates for targeting the selected brain areas were established based on the rat (Paxinos and Watson, 2014) or mouse (Franklin and Paxinos, 2013) brain atlas:

| Stereotaxic surgery | AP | ML | DV | Angle | Volume |
|---|---|---|---|---|---|
| **Rat - Guide cannula implantation** | | | | | |
| Icv (12 mm; 21 G) | -1.0 | -1.6 | -2.0 | 0° | - |
| Above PVN (12 mm; 23 G) | -1.4 | +1.8 / -2.1 | -6.3 | 10° | - |
| **Rat - Microinfusion** | | | | | |
| Intra-PVN | -1.8 | ±0.3 | -8.0 | 0° | 280 nl |
| **Mouse – Guide cannula implantation** | | | | | |
| Icv (8 mm; 21 G) | +0.2 | +1.0 | -1.4 | 0° | - |
| Above dorsal LS (8 mm; 21 G) | +0.3 | ±0.5 | -1.6 | 0° | - |
| **Mouse - Microinfusions** | | | | | |
| Intra-LS | +0.3 | ±0.5 | -3.8 | 0° | 70 nl |
| | | | -3.4 | | 70 nl |
| | | | -3.1 | | 70 nl |
| | | | -2.8 | | 70 nl |

**Table 1 – Stereotaxic coordinates (in mm) used for guide cannula implantation or microinfusions of locked nucleic acids (LNAs) and adeno-associated viruses (AAVs) accordant to bregma** (AP: anterior-posterior axis; ML: medio-lateral axis; DV: dorso-ventral axis; PVN: paraventricular nucleus; LS: lateral septum).

### 2.2.1 Implantation of Guide Cannulas

For icv infusions, a guide cannula was stereotaxically implanted 2 mm above the right lateral ventricle, whereas for local infusions, guide cannulas were implanted bilaterally 2 mm above the PVN or LS. Cannulas were fixed with dental cement and the help of two stainless steel screws. Animals were single-housed after surgery, allowed to recover for at least 5 days, and handled daily to habituate them to the infusion procedures to minimize non-specific stress responses during experiments. Guide cannulas were further closed with a dummy cannula (stylet), which was cleaned daily with 70% ethanol during the handling procedure.

### 2.2.2 Intracerebral Microinfusion of Locked Nucleic Acids and Adeno-associated Viruses

To manipulate miR-132-3p transcript levels *in vivo*, either LNAs or AAVs (Table 2) were bilaterally microinfused using a calibrated 5-µl glass micropipette (VWR, USA; inner diameter: 0.3 mm), which was pulled to create a long narrow tip. The micropipette shaft was labelled with a 1-mm scale,

which corresponds to a volume of approximately 70 nl. After stereotaxically targeting of the desired region by the corresponding xyz coordinates (see table 1), LNAs (Exicon, The Netherlands) or AAVs (Vector Biolabs, USA) were slowly infused into the PVN or LS by manual pressure infusion (rat PVN: 1 x 140 nl / hemisphere; mouse LS: 4 x 70 nl / hemisphere). After every infusion, the micropipette was kept in place for 2 min to assure adequate diffusion of LNAs and AAVs. The skull was closed using bone wax (Ethicon, USA), and the skin was sutured using a sterile nylon fiber (Ethicon, USA). After the surgical procedure, animals were carefully observed until completely awake and single-housed for 48 hrs (LNA infusion) or 3 weeks (AAV infusion) to recover from anesthesia and allow the LNAs and AAVs to attain their full function. The used LNAs were substituted with a 6-carboxyfluorescein (FAM) at their 5´end. The infused AAVs lead to the expression of eukaryotic green fluorescent protein (eGFP) in wildtype mice or a combination of constitutive mCherry expression with GFP expression only upon shRNA-mediated knockdown in OXTRCre mice.

| Microinfusion | nmol | GCs |
|---|---|---|
| **LNAs** | | |
| scrambled control (Scr) | 0.5 | - |
| miR-132-3p inhibitor (Inh) | 0.5 | - |
| | | |
| **AAVs** | | |
| $scAAV_1$-EF1a-mmu-miR-132-eGFP (Mimic) | - | $4.9 \times 10^3$ |
| $scAAV_1$-EF1a-ctrl-miR-eGFP (Ctrl) | - | $4.9 \times 10^3$ |
| $AAV_9$-EF1-mCherry-SICO-GFP-mmu-miR-132-3p-shRNA (shRNA) | - | $9.4 \times 10^6$ |
| $AAV_9$-EF1-mCherry-SICO-GFP-scrmb-shRNA (Scr) | - | $9.4 \times 10^6$ |

**Table 2 – Concentration of locked nucleic acids (LNAs) and genome copies (GCs) of adeno-associated viruses (AAVs) microinfused bilaterally into the septum of male mice or paraventricular nucleus of rats (only LNAs).** All AAVs expressed the encoded construct under the constitutive elongation factor 1 (EF1) promoter (sc: self-complementary; SICO: system for Cre-inducible shRNA expression; scrmb: scrambled; shRNA: small hairpin RNA).

## 2.3 Drug Infusion in Conscious Animals

Substances for icv or local infusion were diluted in sterile Ringer´s solution (B. Braun Melsungen AG, Germany) on the day of experiment to avoid repeated freezing-thawing cycles. For icv or local infusions (PVN, LS), the dummy cannula was replaced by an infusion cannula. After manual pressure-infusion of the appropriate volume (see table 3) the infusion cannula was kept in place for approximately 10 sec to allow local substance diffusion. Sterile Ringer´s solution was infused as

control (vehicle; Veh). None of the infused animals showed signs of tremor or convulsion, when placed back into their home cages.

| Substance | Concentration infused | Infusion cannula |
|---|---|---|
| **Rat** | | |
| Icv OXT | 0.1 µg / 5 µl | 25 G, 14.7 mm |
| Icv OXTR-A | 0.75 µg / 5 µl | 25 G, 14.7 mm |
| Intra-PVN OXT | 1 µg / 2 µl / hemisphere | 27 G, 14 mm |
| | | |
| **Mouse** | | |
| Icv OXT | 0.1 µg / 2 µl | 27 G, 10mm |
| Intra-LS OXT | 5 ng / 0.2 µl / hemisphere | 27 G; 10 mm |

**Table 3 – Substances and concentrations infused *via* intracerebroventricular (icv) or local guide cannulas (PVN: paraventricular nucleus; LS: lateral septum) in rats and mice using the corresponding infusion cannulas.** The oxytocin receptor antagonist (OXTR-A; desGly-NH2, d(CH2)5[Tyr(Me)2,Thr4]OVT) was a kind gift from Prof. Dr. Maurice Manning (Manning et al., 2008).

## 2.4 Verification of Cannula and Probe Placement

After behavioral experiments, animals were deeply anesthetized with Ketamine (10%, 1 ml/kg, WDT) and Xylazin (2%, 0.5 ml/kg, Bernburg) and perfused transcardially with 0.01 M PBS (pH 7.4) and 4% paraformaldehyde (in PBS, pH 7.4). Brains were removed, post-fixed overnight in a 4% paraformaldehyde solution, cryo-protected in 30% sucrose for 3 days, and snap-frozen in pre-chilled N-methylbutane on dry ice. Cannula placement and/or localization and efficiency of LNA transfection or AAV transduction was verified in 40-µm thick coronal cryosections according to the brain atlas. Further immunohistochemical labeling of the LNA or AAV tag (FAM, GFP, and mCherry) of brain sections was not necessary, since immunofluorescence was strong enough for visualization of the corresponding infusion sites. Only animals with correctly localized cannulas and/or probes were included in the statistical analysis. Animals used for evaluation of RNA expression were not perfused transcardially. The verification of the cannula placement was carried out in histological 40-µm thick coronal cryosections.

## 2.5 Extraction of Cerebrospinal Fluid from the *Cisterna Cerebromedullaris* of Rats

To isolate and analyze exosomal miRNAs released into the CSF, virgin and lactating female rats were anesthetized with 25% urethane (1.2 g/kg). After dissection of the neck skin and muscles,

approximately 100 µl CSF was drawn with the help of a infusion cannula (25 G, 20 mm), which was inserted 2 mm into the *cisterna cerebromedullaris* and connected to a 100 µl Hamilton syringe. Drawn CSF was snap-frozen on dry ice and exosomal miRNAs were isolated.

## 2.6 Behavioral Tests and Paradigms

Combinations of the following behavioral tests and paradigms for anxiety-related behavior and fear were performed. A detailed specification of the assessed behavior per experiment can be found in section 2.9 Experimental Design. The transition from rats to mice was experimentally necessary, since the expected anxiolytic and social fear reversing effects of central OXT application are established only in rats and mice, respectively. The following sections, which describe the behavioral methods in detail, are specifically explained for the species (rat or mouse), in which they were conducted during this doctoral thesis.

### 2.6.1 Fear Behavior

#### 2.6.1.1 Social Fear Conditioning

In order to assess the effect of septal miR-132-3p manipulation on social fear, SFC was performed in male mice on three consecutive days as described previously (figure 5) (Toth et al., 2012b, 2013; Zoicas et al., 2014).

*Social fear acquisition (day 1, figure 5 A):* During social fear acquisition, mice were transferred from their home cage into the conditioning chamber (45 x 23 x 36 cm; transparent Perspex box with a stainless-steel grid floor, 300 lux). After a 30-s habituation period, mice were presented an empty wire mesh cage (7 x 7 x 6 cm) for 3 min as non-social stimulus. This stimulus was subsequently replaced with an identical small cage containing an unfamiliar, but sex and age-matched, conspecific. Unconditioned ($SFC^-$) control mice were allowed to freely approach and investigate the social stimulus in the conditioning chamber for 3 min without receiving any aversive foot shock. Contrastingly, social fear conditioned ($SFC^+$) mice were given an approximately 1-sec foot shock (0.7 mA) each time they investigate the social stimulus. If the animal did not re-contact the stimulus already after a single foot shock, it was observed for additional 6 min for social contact. Here, lack of social contact with the conspecific was interpreted as acquired social fear, and the animal was returned to its home cage. However, if the experimental mouse investigated the conspecific again within these 6 min, it was interpreted as lack of social fear learning and the mouse received another foot shock. The mouse was returned to its home cage, when no further contact was made for

further 2 min. On average $SFC^+$ mice received 2-3 foot shocks until successfully avoiding the conspecific.

*Social fear extinction training (day 2, figure 5 B):* The subsequent day after acquisition of social fear, each $SFC^-$ and $SFC^+$ mouse was exposed to three non-social stimuli (empty cages) within their home cage to assess their general non-social investigation as parameter of non-social fear and general anxiety-related behavior. Subsequently, mice were exposed to 6 different unknown social stimuli - 6 different unfamiliar age and sex-matched conspecifics - each in a different small wire-mesh cage to assess social investigation as parameter of social fear. Each stimulus was presented for 3 min followed by a 3-min inter-stimulus interval. A gradual increase in social investigation (percentage of time spent in contact with the social stimulus) of $SFC^+$ mice was interpreted as measure for successful social fear extinction.

*Social fear extinction recall (day 3, figure 5 C):* One day after extinction training, mice were again exposed to 6 different unfamiliar, sex and age-matched social stimuli within their home cage for three minutes with a 3-min inter-stimulus interval (analogous to social fear extinction training).

All experiments for the evaluation miRNA transcript levels in response to acquisition and extinction of social fear were further complemented by two control groups: mice randomly receiving two shocks during acquisition (shock control), and mice exposed to the conditioning chamber without shock application (context control).

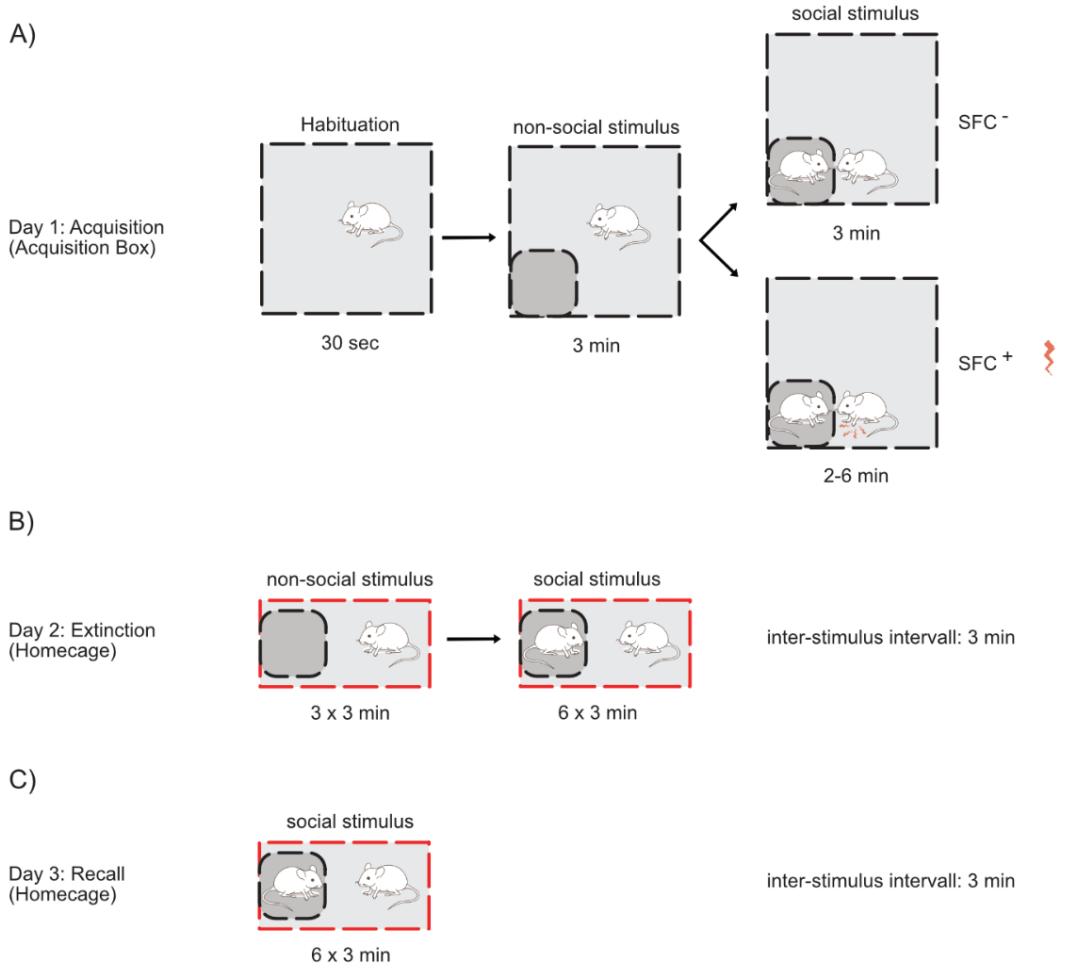


**Figure 5 – Scheme depicting the three days of the social fear conditioning (SFC) paradigm.** A) On day 1 during acquisition of social fear mice are habituated to the chamber. Subsequently a non-social and a social stimulus is presented for 3 min each. Unconditioned ($SFC^-$) mice are allowed to explore the social stimulus freely, whereas conditioned ($SFC^+$) mice receive an aversive foot shock when investigating the social stimulus. B) On day 2 during social fear extinction, mice are exposed to 3 non-social and 6 social stimuli leading to a gradual loss of social fear. C) During social fear recall on day 3 mice are again exposed to 6 social stimuli to evaluate whether extinction of social fear was successful (adapted from (Menon et al., 2018)).

#### 2.6.1.2 Cued Fear Conditioning

In order to reveal whether intra-PVN miR-132-3p inhibition affects the cued fear-impairing effect of OXT, CFC of rats was performed as described previously (Toth et al., 2012a).

*Cued fear acquisition (day 1):* During cued fear acquisition, rats were transferred from their home cage into the conditioning chamber (45 x 45 x 40 cm; transparent Perspex box with a stainless-steel grid floor: context A, 300 lux) and allowed to habituate for 2 min. Afterwards, 5 repetitions of a 30 sec tome (80 dB) co-terminated with a two second foot shock (0.7 mA) and a 2-min inter-CS-US pairing interval were applied to all animals. Acquisition of cued fear was finalized by a 2-min

acclimatization period before removing animals from the chamber. During cued fear acquisition, the percentage of time spent freezing during presentation of the tone (CS) was assessed as fear.

*Cued fear extinction (day 2):* On the following day, rats were transferred to the conditioning chamber (45 x 45 x 40 cm; black Perspex box with a smooth floor: context B, 0 lux). After a 2-min adaptation period, animals were exposed to 30 repetitions of a 30 sec tone (80 dB) interval with an inter-interval pause of 5 sec. Cued fear extinction was terminated by a final 2-min acclimatization phase. The gradual loss of the percentage of freezing during tone presentations was interpreted as extinction of cued fear. Animals that showed high percentage of freezing during the initial habituation phase of day 2 were removed from analysis due to possible effects of contextual fear.

*Cued fear retention (day 3):* On day three, rats were again transferred to the conditioning chamber with context B (see day 2). After a 2-min habituation period, 5 repetitions of a 30-sec tone and 5-sec pause combination were presented. Here, the percentage of time spent freezing was interpreted as fear. Animals that showed high percentage of freezing during the initial habituation phase of day 3 were removed from analysis due to possible effects of long-lasting contextual fear.

### 2.6.3 Anxiety-related Behavior

Following the respective treatment, general anxiety-related behavior was assessed using the EPM, LDB, OFT, and NOI. In general, these tests are based on the natural conflict between the explorative drive and the innate fear of open, lit, and exposed spaces. The more anxious an animal is, the less time it spends in the potentially insecure bright compartment and *vice versa*.

#### 2.6.3.1 Elevated Plus-Maze

To assess non-social anxiety-related behavior, the EPM was performed in mice (Lister, 1987) and rats (Pellow et al., 1985) as described previously (table 4). The apparatus was elevated 35 cm above the floor and consisted of two open and two closed arms radiating from a central platform. Animals were placed on the central platform facing a closed arm and allowed to explore the maze for 5 min. The percentage of time spent on the open arms indicated general anxiety-related behavior, and the number of closed arm entries was used as indicator for locomotor activity.

| | Size mouse (cm) | Light mouse (lux) | Size rat (cm) | Light rat (lux) |
|---|---|---|---|---|
| Central platform | 6 x 6 | - | 10 x 10 | - |
| Open arms | 6 x 30 x 0.2 | 100 | 50 x 10 x | 80 |
| Closed arms | 6 x 30 x 16 | 30 | 50 x 10 x 30 | 10 |
| Elevation | 35 | - | 70 | - |

**Table 4 – Technical parameters of the elevated plus-maze (EPM).** Size, elevation, as well as lux of the compartments of the EPM, which was used to assess non-social anxiety in mice and rats.

#### 2.6.3.2 Light Dark-Box

Another test to evaluate non-social anxiety-related behavior in mice (Crawley and Goodwin, 1980) and rats is the LDB (table 5). It consisted of two chambers; one brightly lit box (light box) and one dark box, which were connected by a small opening enabling the transition between compartments. Animals were placed in the light box facing the wall opposite to the transition opening and were allowed to explore the LDB for 10 min. The percentage of time spent in the light box was evaluated as anxiety-related behavior, whereas the distance travelled was taken as indicator of locomotor activity.

| | Size mouse (cm) | Light mouse (lux) | Size rat (cm) | Light rat (lux) |
|---|---|---|---|---|
| Light box | 27 x 27 x 27 | 350 | 40 x 50 x 40 | 100 |
| Dark box | 18 x 27 x 27 | 0 | 40 x 30 x 40 | 0 |
| Opening | 6 x 6 | - | 7.5 x 7.5 | - |

**Table 5 – Technical parameters of the light dark-box (LDB).** Size and lux of the light and dark compartment and opening between the two compartments, which were used to assess non-social anxiety-related behavior in mice and rats.

#### 2.6.3.3 Open Field Test and Novel Object Investigation

The third test used to measure non-social anxiety-related behavior and locomotor activity in mice was the OFT in combination with a NOI test (table 6). Mice were placed in the center of the open-field box and allowed to explore it for 5 min. During a break of 5 min, animals were returned to their home cages, the chamber was cleaned, and a novel object (metal cylinder, 2 cm diameter, 1 cm height) was placed in the middle of the center zone. Mice were re-exposed to the open field including the novel object for 5 min. The time spent in the center zone as well as the time spent investigating the novel object were taken as measurements for anxiety-related behavior. Moreover, the distance travelled was evaluated as locomotor activity.

| | Size mouse (cm) | Light mouse (lux) |
|---|---|---|
| Box | 40 x 40 x 20 | - |
| Center Zone | 20 x 20 | 150 |

**Table 6 – Technical parameters of the open field test (OFT) and novel object investigation test (NOI).** Size and lux of the OFT/NOI, which was used to assess non-social anxiety and locomotor activity in mice.

### 2.6.4 Scoring of Behavior

The animal´s behavior displayed in the SFC, and EPM was scored manually by an observer blind to treatment using JWatcher (V1.0, Macquarie University and UCLA, USA), cued fear expression during CFC was scored computerized using the TSE fear conditioning system (TSE System GmbH, Germany), and the expressed behavior during LDB and OF was scored computerized using Etho Vision® XT (Noldus, the Netherlands).

## 2.7 Molecular Methods

### 2.7.1 RNA Isolation from Tissue

After anesthesia and rapid decapitation, rat brains were removed, and the PFC, PVN, and amygdala were dissected from fresh tissue. Brains of the experimental mice were removed after anesthesia and rapid decapitation, snap-frozen in ice-cold 2-methylbutane, and LS, PVN, amygdala, and dorsal as well as ventral hippocampus were punched (1mm diameter) from 100-µm cryo-cut coronal sections. In both species, the dissected tissue is only enriched of the desired region. Nevertheless, it will be referred to as PFC, PVN, amygdala, LS, and hippocampus in this thesis. Total RNA was isolated according to the manufacturer´s protocol using the Nucleo Spin miRNA kit (Macherey und Nagel, Germany). Quantity of total RNA and/or miRNAs was measured using Qbit (RNA extended range or miRNA kit, Thermo Fisher Scientific, USA) according to the manufacturer´s protocol. Quality of total RNA was analyzed at 260/280 nm using the NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific). 1000 ng total RNA was reverse transcribed to complementary DNA (cDNA) using poly-A tailing (Hurteau et al., 2006) (universal RT primer 5´- AAC GAG ACG ACG ACA GAC TTT TTT TTT TTT TTT V -3´; V = variable base) in combination with the Super Script IV first-strand synthesis system (Invitrogen) for relative miRNA assessment and only the Super Script IV first-strand synthesis system for relative mRNA quantification.

### 2.7.2 miRNA Isolation from Cerebrospinal Fluid

To isolate and analyze exosomal miRNA released into the CSF, the miRCURY™ RNA Isolation Kit for biofluids (Exiqon, The Netherlands) including the optional DNase digestion was utilized according to the manufacturer's protocol. A total volume of 100 µl of CSF extracted from virgin and lactating rats was used. The cDNA synthesis was performed as described for tissue samples (see section 2.7.1 RNA Isolation from Tissue).

### 2.7.3 Quantitative Real-Time PCR

Relative miRNA and mRNA quantities were analyzed *via* SYBR Green-based quantitative real-time PCR (qPCR) using the $\Delta\Delta C_t$ method and the Quantstudio 5 (Applied Biosystems). SYBR Green (PowerUp™SYBR™Green Master Mix, Applied Biosystems, USA) master mix component concentrations was as proposed by the manufacturer. miRNAs were normalized to 18S ribosomal RNA expression, whereas mRNAs were normalized to glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*). The following primers (Metabion, Germany) were used (table 7):

| Gene | Accession ID | 5´-3´ sequence | Amplicon size (bp) |
|---|---|---|---|
| Universal PCR primer | | AAC GAG ACG ACG ACA GAC TTT | |
| 18S-RNA | NR_003278.3 | CGT AGG TGA ACC TGC GGA A | 60 |
| miR-124-3p | MIMAT0000134 | TAA GGC ACG CGG TGA ATG CC | 53 |
| miR-132-3p | MIMAT0000144 | TAA CAG TCT ACA GCC ATG GTC G | 55 |
| miR-212-5p | MIMAT0017053 | TAA CAG TCT CCA GTC ACG GCC A | 55 |
| **Mouse** | | | |
| Peg3 | NM_008817.2 | TTC CCA GCA AGG GGA GAT CA<br>GGT GTC GTC TTC GTG ATG GT | 132 |
| Pparg | NM_001127330.2 | GAG GGA CGC GGA AGA AGA G<br>CCG CTT CTT TCA AAT CTT GTC TGT | 236 |
| Htr2C | NM_008312.4 | CGT TTG GGC AAT ATC AAT AGG AGT<br>CAG TTC CTC CTC GGT GTG AC | 241 |
| Dvl3 | NM_007889.3 | TCT CTA TGG TCC CCC GAT GC<br>AGA GGG GCC CTG ATC ACA TC | 185 |
| Ncoa1 | NM_010881.2 | CCA GGA CAA GAA GAC AGA AGA GTT<br>CTG AAC CTG CTG CAC CTG TAG A | 77 |
| Robo4 | NM_001309390.2 | AAG CCC AGG TCC AAA CTC TG<br>GTT GCG GTG AAG TTG TGG TC | 88 |
| Gapdh | NM_008084 | AAG GGC TCA TGA CCA CAG<br>CAG GGA TGA TGT TCT GGG | 93 |

| | | | |
|---|---|---|---|
| **Rat** | | | |
| Oxtr | NM_012871.3 | CTG GAG TGT CGA GTT GGA CC<br>AGC CAG GAA CAG AAT GAG GC | 136 |
| Gapdh | NM_017008.4 | TGA TGA CAT CAA GAA GGT GG<br>CAT TGT CAT ACC AGG AAA TGA G | 164 |

**Table 7 – Accession ID, sequence, and amplicon size of primers used for quantitative real-time PCR analysis of miRNAs and target mRNAs.** All miRNA primers used are species-independent, since the sequence is highly conserved across mammals.

### 2.7.4 Argonaute RNA-Immunoprecipitation-Microarray Analysis

In concordance with the behavioral alterations *in vivo*, 48 hrs after septal infusion of a miR-132 inhibitor or scrambled control LNA, the septum of male mice was dissected and AGO2 with the incorporated miRNA as well as the adjacent mRNAs was pulled by means of AGO2-IP (6F4 hybridoma; mAGO2 antibody) using protein G beads (performed by Daniela Zeitler, Regensburg, Germany). Further sample processing was performed at the Affymetrix Service Provider and Core Facility "KFB – Center of Excellence for Fluorescent Bioanalytics" (http://www.kfb-regensburg.de/; Regensburg, Germany) using the Affymetrix Clariome S mouse array, and bioinformatic analysis was conducted by Uwe Schwartz (Regensburg, Germany). Briefly, the analysis is based on a Bioconductor tutorial (Klaus and Reisenauer, 2016) and data was further processed using the Bioconductor oligo package (Carvalho and Irizarry, 2010). Probe intensities were further normalized using the RMA function. Low intensity probes were removed and only probes, which exhibited intensities greater than 5 in at least two IP samples, were kept for further analysis. To identify significant changes in RNA composition of the pulled down AGO complex a linear model was fit to samples. After normalization for multiple testing 0 hits were detected, passing the significance threshold of 0.1. All genes were sorted by the non-adjusted p-value.

## 2.8 Statistical Analysis

For statistical analysis GraphPad Prism version 6.0 for windows (GraphPad Software, San Diego California, USA) was used. Normal distribution of data was evaluated using the Kolmogorov-Smirnov test with Dallal-Wilkinson-Liliefor P value. If half or more groups revealed a normal distribution, a parametric statistical analysis was performed, whereas if less than half of the analyzed groups showed normal distribution, a non-parametric test was performed. Data were analyzed by student´s t-test, Mann Whitney U-test, one way or two way analysis of variance (ANOVA) followed by a Bonferroni´s *post-hoc* analysis, or two way ANOVA for repeated measures

followed by a Bonferroni´s *post-hoc* analysis whenever appropriate (exception: shRNA-mediated knockdown of pre-miR-132 in the septum of OXTRCre mice: Least significant difference (LSD) post hoc). Statistical significance was set at $p \leq 0.05$. Overall statistics and post-hoc tests have been stated for each experiment separately in the results section (3.1 and 3.2).

## 2.9 Experimental Design

The following section contains a graphical illustration of the experimental designs of the separate experiments performed throughout this thesis. The headlines refer to the two main aims, respective results and discussion paragraphs of the thesis.

### 2.9.1 Part I: "Is miR-132 within the PVN Involved in Oxytocin-induced Anxiolysis?"

To examine, whether miRNA expression levels are altered within the PVN in response to OXT application, male and female rats were implanted with an icv guide cannula. After 5-7 days of habituation, rats were infused with Veh or OXT 90 min or 3 hrs prior to euthanasia and tissue collection (figure 6 A). To further analyze, whether the miRNA expression-altering effect of OXT is mediated via OXTRs, male rats were infused with Veh or an OXTR-A combined with subsequent Veh or OXT application (figure 6 B) 3 hrs prior to euthanasia and tissue collection. To further reveal, if chronic activation of the brain OXT system as seen during lactation is associated with altered intra-PVN miRNA levels, brain tissue was extracted on LD5 (figure 6 C). Hence, rats were mated with sexually experienced males and the day of birth was set as LD1. As control groups, pups were removed from lactating dams either 24 hrs or 4 hrs prior to euthanasia. Maternal behavior, especially milk ejection reflexes, were observed for 4 hrs as indirect indicator for adequate central OXT release. As further proof of possible miRNA alterations within the PVN, an OXTR-A or Veh was infused icv in lactating rats (LD5) 3 hrs prior to euthanasia (figure 6 D). To analyze putative functional effects of miRNA-132 inhibition on OXT-mediated anxiolysis and CFC, male rats were infused with a LNA scrambled of miR-132-3p inhibitor 48 hrs prior to evaluation of the anxiolytic properties of intra-PVN OXT treatment on anxiety-related behavior and locomotor activity (figure 6 E). Furthermore, these animals underwent CFC to reveal possible effects of miR-132-3p inhibition on fear behavior.

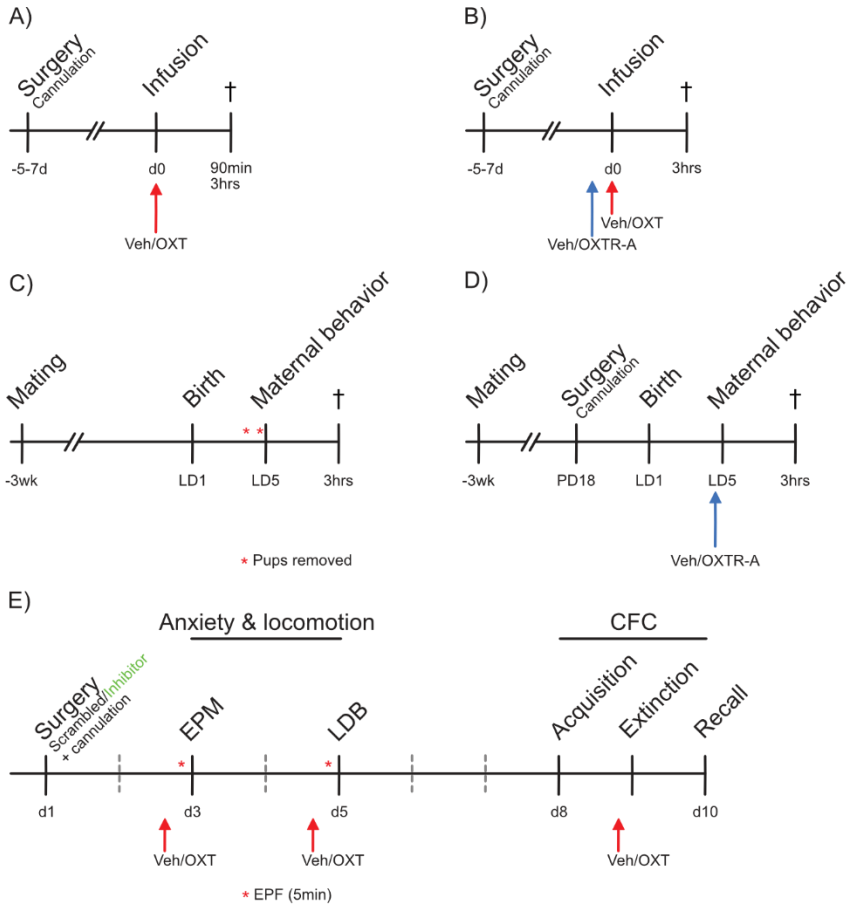


**Figure 6 – Experimental design employed to evaluate the possible involvement of miRNAs in the anxiolytic and fear attenuating effect of oxytocin in rats.** A) Timeline of miRNA expression analysis after icv oxytocin (OXT; 0.1 µg / 5 µl) or vehicle (Veh) infusion. 90 min or 3 hrs after icv infusion, rats were euthanized, and miRNAs were analysed from fresh tissue punches. B) Timeline of miRNA expression analysis after icv OXT receptor antagonist (OXTR-A; 0.75 µg / 5 µl) infusion and subsequent OXT infusion. Veh or an OXTR-A was infused 10 min prior to local Veh or OXT infusion. Animals were euthanized 3 hrs post 2$^{nd}$-infusion. C) Timeline of miRNA expression analysis in lactating rats. A) Maternal behavior was observed on lactation day (LD) 5 and dams euthanized 4 hrs after the start of the behavioral observation. As further control, pups were removed either 4 hrs or 24 hrs prior to observation. miRNA expression was normalized to virgin rats. D) On LD 5, lactating and virgin rats were infused with Veh or OXTR-A and euthanized 3 hrs post-infusion. E) Timeline of the functional significance of miR-132 inhibition within the PVN on OXT-mediated anxiolysis and fear. 48 hrs prior to anxiety and locomotor activity testing, rats were infused with a miR-132 inhibitor (0.5 nmol) or scrambled control (0.5 nmol) locked nucleic acid. Animals were locally infused with Veh or OXT (1 µg / 2 µl / hemisphere) 10 min prior to anxiety and fear testing. Moreover, they were exposed to 5-min elevated platform (EPF) stress before being placed on the elevated plus-maze (EPM) on day 3 or light dark-box (LDB) on day 5. On day 8-10, rats underwent the cued fear conditioning (CFC) procedure (d: day; wk: week; PD: pregnancy day; OXTR-A: desGly-NH2, d(CH2)5[Tyr(Me)2,Thr4]OVT; † euthanasia).

### 2.9.2 Part II: "Does miR-132 Mediate the Oxytocin-induced Reversal of Social Fear?"

To evaluate miRNA transcript levels within the LS at various time points after acquisition and extinction of social fear, male CD1 mice were euthanized 30 min, 90 min, 3 hrs or 24 hrs after behavioral testing (figure 7 A-B). Just as in the studies in rats mentioned in part I, LS miRNA expression was analyzed 90 min and 3 hrs after icv OXT versus Veh infusion (figure 7 C). To reveal whether repeated social contact in unconditioned and conditioned mice alters miRNA expression within the LS, mice were exposed to either 3 non-social and subsequent 6 social stimuli or only 9 non-social stimuli (figure 7 D-E). For a functional analysis of miR-132-3p within the LS, miR-132-3p was inhibited 48 hrs prior to acquisition of social fear (figure 7 F) or overexpressed 3 weeks prior to acquisition (figure 7 I). Animals were further tested for general anxiety-related behavior and locomotor activity (figure 7 F, I). Additionally, the effect of miR-132-3p inhibition on OXT-mediated reversal of social fear (figure 7 G), as well as the influence of shRNA-mediated knockdown of pre-miR-132-3p only in $OXTR^{+}$ neurons on acquisition and extinction of social fear (figure 7 H) was evaluated.

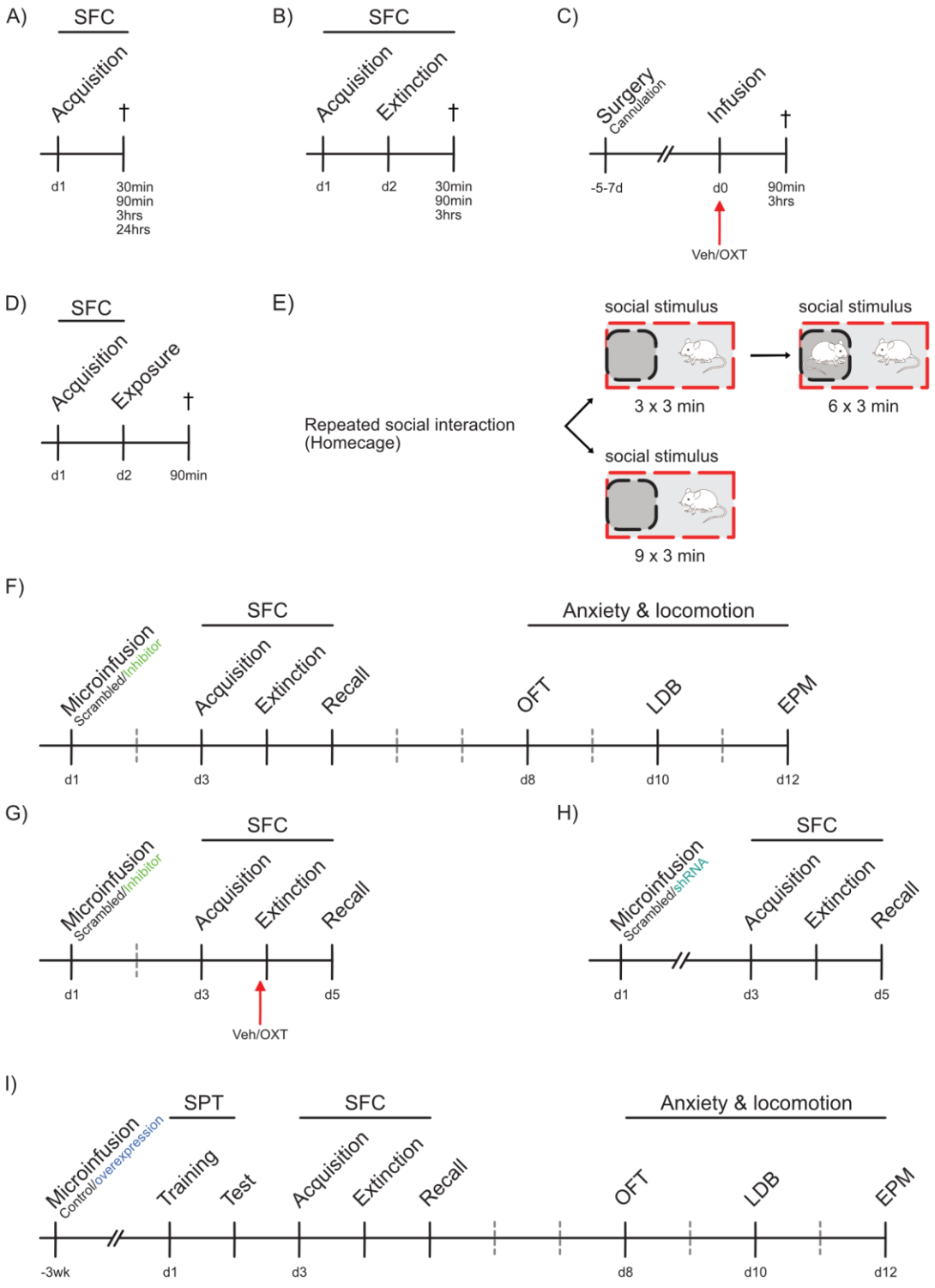


**Figure 7 – Experimental design employed to evaluate expression alterations and the functional significance of miRNAs in the mouse lateral septum throughout the social fear conditioning (SFC) paradigm.** A) 30 min, 90 min, 3 hrs or 24 hrs after acquisition of social fear, animals were euthanized, their brains were cryo-cut, and miRNA expression was analyzed via real-time quantitative PCR. B) 30 min, 90 min or 3 hrs after extinction of social fear, animals were euthanized, the brain was cryo cut, and miRNA expression was analyzed via real-time quantitative PCR. C) 90 min or 3 hrs after icv infusion of vehicle (Veh) or oxytocin (OXT; 0.1 µg / 2 µl), mice were euthanized, and miRNAs were analysed from cryo cut brains. D) Mice were euthanized 90 min after exposure to 3 non-social followed by 6 social stimuli or 9 non-social stimuli (continued on next page).

E) To assess effects of repeated social contact, mice were exposed to either 3 non-social and subsequent 6 social stimuli or 9 non-social stimuli in their home cage one day after acquisition of social fear. Stimulus exposure was 3 min with a 3-min inter-stimulus interval. F) Scrambled control (0.5 nmol) or miR-132 inhibitor (0.5 nmol) locked nucleic acids (LNAs) were infused 48 hrs prior to acquisition of social fear in the SFC paradigm. On the subsequent days, extinction and recall of social fear was performed and after two days anxiety-related behavior and locomotion were tested in the open field test/novel object investigation (OFT), light dark-box (LDB), and elevated plus-maze (EPM). G) Scrambled control or miR-132 inhibitor LNAs were infused 48 hrs prior to acquisition of social fear in the SFC paradigm. On the subsequent day and 10 min prior to extinction of social fear, Veh or OXT (5 ng / 0.2 µl / hemisphere) was locally infused into the LS. On the next day, recall of social fear was performed. H) A scrambled shRNA ($9.4 \times 10^6$) or shRNA knocking down pre-miR-132 expressing AAV ($9.4 \times 10^6$) was infused 3 weeks prior to acquisition of social fear. I) A miRNA control ($4.9 \times 10^3$) or miR-132 overexpression ($4.9 \times 10^3$) AAV was infused 3 weeks prior acquisition, extinction, and recall of social fear. On day 8, 10, and 12, animals were tested for anxiety-related behavior and locomotor activity in the OFT, LDB, and EMP (d: day; wk: week; † euthanasia).

# Results

## 3.1 Part I: "Is miR-132 within the PVN Involved in Oxytocin-induced Anxiolysis?"

*Declaration: The miRNA Deep Sequencing briefly mentioned within this chapter was performed by Stefanie Martinetz (Doctoral thesis, University of Regensburg, Germany) in collaboration with Prof. Dr. Gunter Meister (University of Regensburg, Germany). I contributed by detailed analysis and in vitro, as well as in vivo validation during experiments of my Master thesis, which were finalized in the present doctoral thesis.*

### 3.1.1 microRNA Expression within the Rat PVN in Response to central OXT Treatment

Hypothalamic miRNAs have repeatedly been shown to crucially regulate expression of neuroendocrinologically essential genes (Meister et al., 2013; Taouis, 2016). Moreover, a previous miRNA Deep Sequencing revealed that numerous rat hypothalamic miRNAs are altered in response to icv OXT treatment. I further validated transcript levels of selected miRNAs within the hypothalamic PVN of male and female rats 90 min and 3 hrs after icv OXT infusion (figure 6 A). Moreover, intra-PVN levels of miR-132-3p of male rats pre-treated with an OXTR-A and further OXT application was evaluated to assess whether miR-132-3p undierlies OXTR signaling (figure 6 B). Although miR-132-3p, miR-212-3p, and miR-134-5p were among the non-significantly changed miRNAs of the miRNA Deep Sequencing, in the present thesis I´ll only focus on the *in vivo* validation of these three auspicious miRNAs since miR-132-3p and miR-134-5p were the only ones found to be regulated by OXT treatment, and the miR-132-3p family member miR-212-3p served as control miRNA. Results of the evaluation of other miRNAs will not be shown here.

Within PVN-enriched tissue samples of male ($p = 0.0459$; figure 8 A) and female (by trend; $p = 0.0457$; figure 8 B) rats, miR-132-3p transcript levels were significantly increased 3 hrs after icv OXT infusion, whereas miR-212-5p and miR-134-5p remained unchanged in both genders. Moreover, miR-132-3p levels within the PFC and amygdala of male rats 3 hrs post OXT infusion were comparable to Veh-treated animals (figure 8 D). To reveal whether the detected rise in miR-132-3p levels is mediated by activation of OXTRs, a central blockade of OXTRs by icv infusion of the antagonist (desGly-NH2, d(CH2)5[Tyr(Me)2,Thr4]OVT (Manning et al., 2008)) prior to icv OXT application was performed. This OXTR antagonism prevented the OXT-mediated transcriptional rise by trend ($p = 0.0749$ Veh/OXT vs Veh/Veh) in relative miR-132-3p level within the PVN of male rats (Figure 8 C), suggesting that transcriptional activation of miR-132-3p is downstream of OXTR activation. In contrast, 90 min after icv OXT infusion, miR-132-3p remained unaltered within the

PFC, PVN, and amygdala of male rats treated with OXT when compared to Veh-treated animals (figure 8 E).

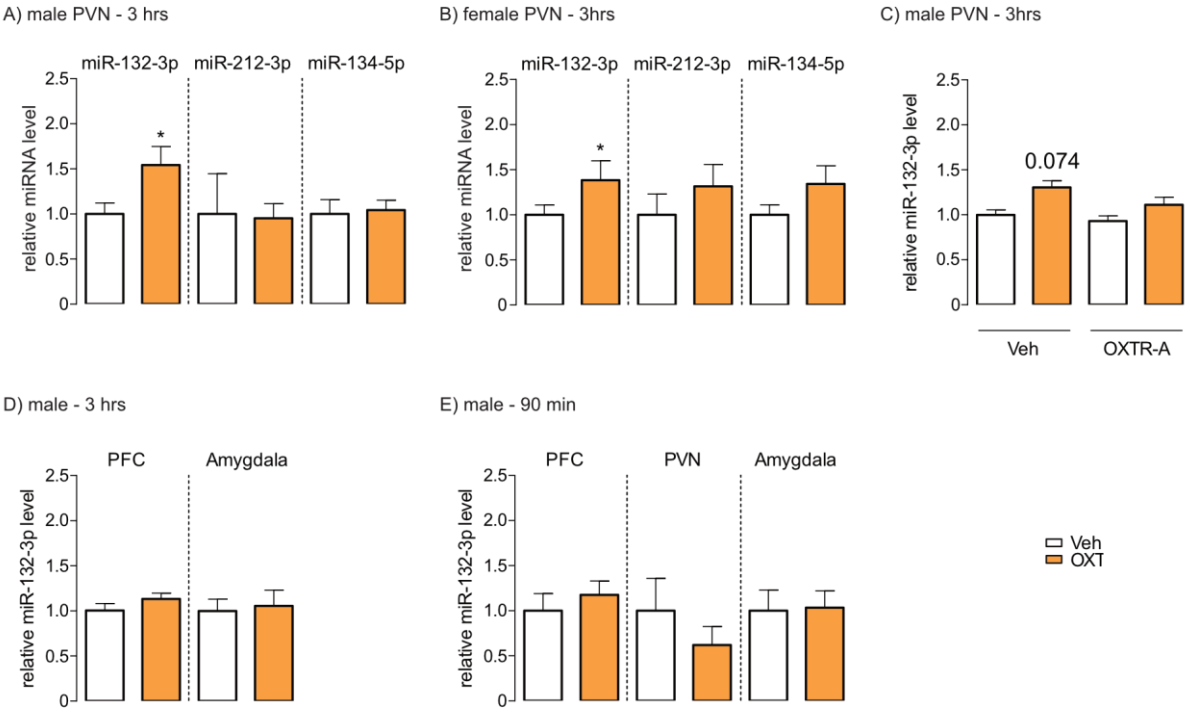


**Figure 8 – miRNA expression within the PVN of male and female rats in response to oxytocin receptor activation or blockade**. A) miR-132-3p, miR-212-5p, and miR-134-5p expression in the paraventricular nucleus (PVN) of male rats 3 hrs post intracerebroventricular (icv) oxytocin (OXT) infusion (0.1 µg / 5 µl) compared to vehicle (Veh) treatment. B) miR-132-3p, miR-212-5p, and miR-134-5p expression in the PVN of female rats 3 hrs after icv OXT application in comparison to Veh-treated rats. C) miR-132-3p transcript levels within the PVN 3 hrs after oxytocin receptor antagonism (OXTR-A; 0.75 µg / 5 µl) and subsequent OXT infusion (0.1 µg / 5 µl). D) miR-132-3p level in the prefrontal cortex (PFC) and amygdala of male rats 3 hrs after icv Veh or OXT treatment. E) miR-132-3p level within the PFC, PVN, and amygdala of male rats 3 hrs post icv Veh or OXT infusion (data represent mean + SEM; * $p \leq 0.05$, OXT vs Veh; A-B: n = 9-12/group; C: n = 6-10/group; D: n = 5-7/group; E: n = 10-13/group).

Statistics:

| | | |
|---|---|---|
| **Male – PVN – 3 hrs** (Status: OXT vs Veh; independent student t-test; two-tailed; figure 8 A) | | |
| miR-132-3p | T (18) = 2.1440 | p = 0.0459* |
| miR-212-3p | T (18) = 0.2264 | p = 0.8234 |
| miR-134-5p | T (16) = 0.1649 | p = 0.8711 |
| **Female – PVN – 3 hrs** (Status: OXT vs Veh; independent student t-test; two-tailed; figure 8 B) | | |
| miR-132-3p | T (18) = 2.1460 | p = 0.0457* |
| miR-212-3p | T (20) = 1.4890 | p = 0.1521 |
| miR-134-5p | T (22) = 0.9390 | p = 0.3577 |
| **Male – PVN – 3 hrs** (Two way ANOVA followed by Bonferroni *post-hoc*; figure 8 C) | | |
| Factor interaction | Factor treatment OXT/Veh | Factor treatment OXTR-A/Veh |
| F (1, 33) = 10.6852; p = 0.2413 | F (1,33) = 10.39; p = 0.0028** | F (1, 33) = 3.3029; p = 0.0911 |

| | | |
|---|---|---|
| Separate statistics: (Status: Veh/OXT vs Veh/Veh; independent student t-test; two-tailed) | | |
| miR-132-3p | T (9) = 2.0130 | p = 0.0749 |
| **Male – 3 hrs – PFC, amygdala** (Status: OXT vs Veh; independent student t-test; two-tailed; figure 8 D) | | |
| PFC | T (12) = 1.2750 | p = 0.2265 |
| Amygdala | T (21) = 0.2427 | p = 0.8106 |
| **Male – 90 min – PFC,PVN, amygdala** (Status: OXT vs Veh; independent student t-test; two-tailed; figure 8 E) | | |
| PFC | T (9) = 0.7220 | p = 0.4885 |
| PVN | T (9) = 0.1170 | p = 0.9093 |
| Amygdala | T (11) = 0.9520 | p = 0.3614 |

### 3.1.2 microRNA Expression Alterations within the PVN of Lactating Rats

During lactation, the brain OXT system is highly activated (Insel, 1986; Jurek et al., 2012; Knobloch et al., 2012; Meddle et al., 2007; Neumann et al., 1993b; Slattery and Neumann, 2008), making lactating rodents an effective model to study intracellular effects of a chronically activated endogenous OXT system. Surely, one has to consider that the OXT system is not the only neuroendocrine system, which is in an activated state during lactation, but effects can be specified to the OXT system by application of an OXTR-A, which abolishes OXTR-mediated signaling. Recently, miRNAs have been highlighted to post-transcriptionally regulate neuroendocrine gene expression in the postpartum brain (Gammie et al., 2016). Although the PVN has not been analyzed here, bioinformatical analysis of the published gene expression data did not reveal differential expression of miR-132-3p, miR-212, and miR-134 in the evaluated brain regions, such as the medial preoptic area (MPOA), LS, and medial PFC.

I evaluated PVN miRNA transcript levels of rat dams on LD 5 and compared them between lactating rats in the presence of their pups and those after removal of the pups 4 hrs or 24 hrs prior to euthanasia, as well as virgin female rats (figure 6 C). As a further indirect proof for central OXT release, dams were observed for their maternal behavior and occurrence of milk ejection reflexes. All lactating rats, which remained undisturbed with their pups showed regular milk ejection reflexes during the last 4 hrs prior to sample preparation. Under these conditions, I did not find the hypothesized increase in miR-132-3p and miR-134-5p (figure 9 A) transcript levels within the PVN, when compared to virgin rats. The same accounted for lactating dams from whom pups have been removed 24 hrs prior to euthanasia and brain dissection for miRNA expression analysis. In contrast, removal of pups 4 hrs prior to sample preparation resulted in significantly increased miR-132-3p ($p = 0.0337$), but not miR-134-5p transcript levels within the PVN when compared to virgin females. Hence, the physiological state of lactation did not alter miR-132-3p level within the rat PVN, but possible contribution of acute stress by removal of the pups can be suggested, which was not a

focus of the present study. To further assess, if miR-132-3p underlies OXTR signaling during lactation, an OXTR-A was applied icv in lactating rats (figure 6 D). However, icv antagonism of the OXTR decreased ($p = 0.0426$; figure 9 D) miR-132-3p transcript level within the PVN of lactating rats, thereby indicating that the highly active brain OXT system during lactation seems to modulate gene expression to some extent *via* miR-132-3p. To confirm equal intracellular activation upon OXTR activation during lactation, I further evaluated OXTR mRNA levels within the PVN of virgin and lactating rats (figure 9 B). No significant expression differences between virgin and lactating rats independent of the presence of their pups were found, revealing that the observed differences in miR-132-3p transcript level do not originate from physiological alterations of the central OXT system during lactation. miRNAs have repeatedly been shown to be present in the CSF and blood plasma when bound to AGO or high density lipoprotein, as well as when packed into exosomes (Chen et al., 2012; Tonge and Gant, 2016; Turchinovich et al., 2012) and thereby even affect behavior (Mathew et al., 2016). Especially miR-132-3p is known to be packed in exosomes, transported via the CSF, and released into the peripheral blood stream (Xu et al., 2017). Therefore, I also evaluated fluctuating miR-132-3p levels within the CSF of virgin females compared to lactating dams with and without (only 4 hrs) pups present prior to sample extraction. Interestingly, the found miR-132-3p regulation within the CSF was comparable to that observed in the PVN: Lactation itself did not result in increased miR-132-3p transcripts, but removal of the pups 4 hrs prior to sample extraction lead to increased CSF miR-132-3p transcript level ($p = 0.04149$, figure 9 C) compared to virgin females.

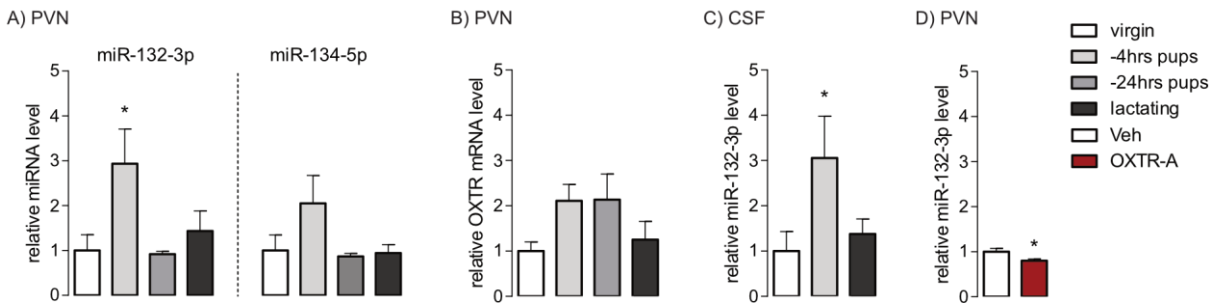


**Figure 9 – miRNA and oxytocin receptor mRNA expression within the paraventricular nucleus and cerebrospinal fluid of virgin and lactating rats.** A) Relative miR-132-3p and miR-134-5p transcript levels in the paraventricular nucleus (PVN) of virgin and lactating rats with and without removal of their pups 4 hrs (-4hrs pups) and 24 hrs (-24hrs pups) prior to expression analysis. B) Relative oxytocin receptor (OXTR) mRNA expression within the PVN of virgin and lactating rats. C) miR-132-3p transcript levels in the cerebrospinal fluid (CSF) of virgin and lactating rats with and without removal of their pups (-4hrs). D) relative miR-132-3p transcript levels within the PVN of lactating rats 3 hrs after intracerebroventricular vehicle (Veh) or OXTR antagonist (OXTR-A; 0.75 µg / 5 µl) infusion (data represent mean + SEM; ** $p \leq 0.01$, * $p \leq 0.05$ vs virgin; n = 5-12/group).

Statistics:

| | | | |
|---|---|---|---|
| **PVN** (Status: -4hrs pups vs virgin; one way ANOVA followed by Bonferroni post-hoc; figure 9 A) | | | |
| | miR-132-3p | F (3, 29) = 3.4580; p = 0.0291* | p = 0.0337* |
| | miR-134-5p | F (3, 29) = 1.9340; p = 0.1461 | |
| **PVN** (One way ANOVA followed by Bonferroni post-hoc; figure 9 B) | | | |
| | OXTR | F (3, 29) = 2.5840; p = 0.0724 | |
| **CSF** (Status: -4 hrs pups vs virgin; one way ANOVA followed by Bonferroni post-hoc; figure 9 C) | | | |
| | miR-132-3p | F (2, 18) = 5.4430; p = 0.0142* | p = 0.0149* |
| **PVN** (Status: OXTR-A vs Veh; independent student t-test; two-tailed; figure 9 D) | | | |
| | miR-132-3p | T (18) = 2.183 | p = 0.0426* |

### 3.1.3 Functional Involvement of miR-132 in Anxiety-related Behavior and Cued Fear

Intra-PVN infusion of OXT has repeatedly been shown to be anxiolytic (Blume et al., 2008; van den Burg et al., 2015; Jurek et al., 2012; Martinetz et al., 2019), however the specific involvement of miRNAs in OXT-induced anxiolysis, especially of miR-132-3p, has not been investigated yet. Since miR-132-3p knockout as well as overexpression both increase anxiety-related behavior (Aten et al., 2019), I investigated the effect of local LNA-mediated miR-132-3p inhibition within the PVN on OXT-induced anxiolysis in male rats in a pilot experiment (figure 6 E). The following treatment groups were analyzed: Scrambled control LNA and Veh (Scr/Veh), scrambled control LNA and OXT (Scr/OXT), miR-132-3p inhibitor LNA and Veh (Inh/Veh), as well as miR-132-3p inhibitor LNA and OXT (Inh/OXT) (figure 10 A).

Although not significant (low n-number), Scr/OXT application within the PVN provoked the expected anxiolytic activity of OXT in the LDB – an increased time spent in the light box (figure 10 B). Moreover, Inh/OXT treatment abolished this tendency of a higher time spent in the light compartment of the LDB. None of the treatments affected locomotor activities of the animals (figure 10 C). Contrastingly, no clear anxiolytic effect of local OXT infusion was seen in the percentage of time spent on the open arm (figure 10 D) and the latency to enter the open arm (figure 10 E) of the EPM, thereby no prevention of the anxiolytic effect *via* intra-PVN miR-132-3p inhibition without affecting locomotor activity depicted by the number of closed arm entries (figure 10 F) was found. However, intra-PVN OXT infusion seemed to reduce the latency to enter the open arm, an effect, which was abolished in Inh/OXT treated rats. Even though the miR-132-3p mediated impairment of the OXT-induced anxiolysis was only seen in the LDB, but not EPM, miR-132-3p within the PVN seems to be a crucial factor downstream of the OXTR signaling pathways. Certainly, the low statistical power due to minimal animal numbers did not reveal any significant effects.

It was shown that OXT applied centrally prior to acquisition of cued fear facilitates extinction, whereas application of OXT prior to extinction of cued fear impairs extinction (Toth et al., 2012a). To analyze the functional involvement of miR-132-3p in the PVN in OXT-mediated impairment of cued fear extinction in a pilot experiment, animals were pre-treated with the abovementioned miR-132-3p inhibitor LNA within the PVN prior to bilateral PVN OXT infusions, which were carried out 10 min prior to extinction of cued fear (figure 6 E). In order to reduce the number of experimental animals, rats that underwent the analysis of the functional involvement of miR-132-3p in OXT-mediated anxiolysis were reused after an experimental break of 2 days. During acquisition of cued fear, Scr/Veh infused rats showed higher percent of freezing upon exposure to the second CS-US pairing ($p = 0.0082$) compared to Scr/OXT infused animals (figure 10 G). Since the OXT infusion was carried out prior to extinction training on day 2, the observed effect on acquisition of cued fear might be artificially caused by the small group size analyzed. During the first ($p = 0.0099$) and second ($p = 0.0001$) CS presentations during extinction of cued fear, Scr/Veh-treated animals expressed higher percent of freezing compared to Scr/OXT-treated males, revealing an effect contrastingly to what has been found previously (figure 10 H): Icv OXT infusion 10 min prior to behavioral assessment leads to impairment of cued fear extinction (Toth et al., 2012a). Moreover, Inh/Veh-treated rats showed reduced freezing behavior during exposure to the second CS ($p = 0.0365$) when compared to Scr/Veh-treated conspecifics, illustrating that pre-inhibition of miR-132-3p within the PVN facilitates cued fear extinction. Regarding cued fear retention, rats infused with Inh/OXT infusion spent a reduced time freezing compared to Inh/Veh-treated animals ($p = 0.0284$), which further suggests that OXT within the PVN strengthens cued fear extinction memory consolidation independent of miR-132-3p (figure 10 I). Unfortunately, no pronounced effect of cued fear extinction after local PVN OXT infusions was found, which might also be a result of the repeated OXT infusions during the experimental plan. However, no clear functional involvement of miR-132-3p within the PVN in cued fear acquisition, extinction, and retention, as well as in OXT-mediated impairment of cued fear extinction was revealed.

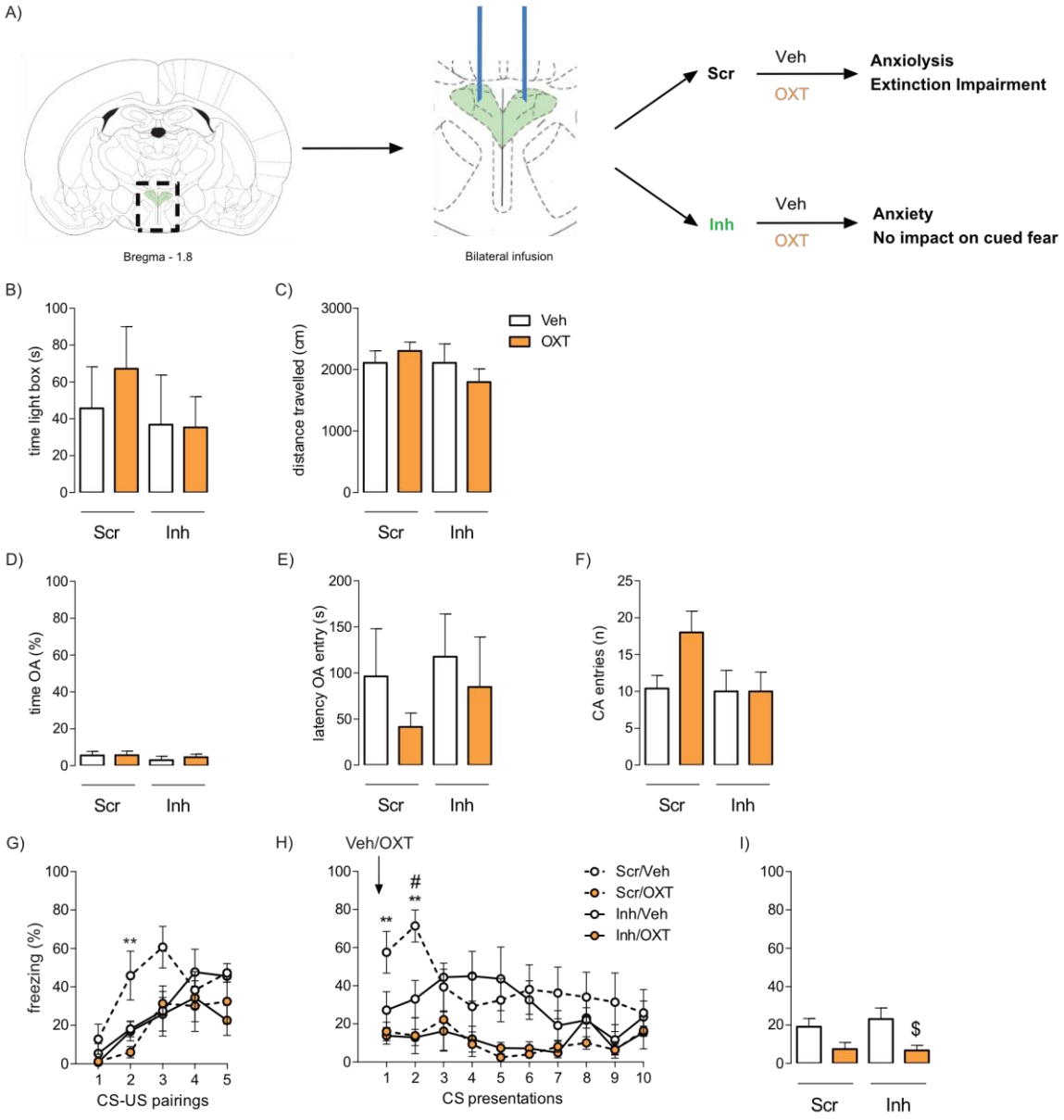


**Figure 10 – Effects of miR-132-3p inhibition within the paraventricular nucleus of male rats on oxytocin-induced anxiolysis and impairment of cued fear extinction.** A) Scheme of scrambled (Scr; 0.5 nmol) and miR-132-3p inhibitor (Inh; 0.5 nmol) locked nucleic acid pre-infusions followed by local vehicle (Veh) or oxytocin (OXT; OXT; 1 µg / 2 µl / hemisphere) treatment in the paraventricular nucleus (PVN) and its effect on anxiety-related behavior and cued fear conditioning. B) Time spent in the light box, and C) distance travelled in the light dark-box. D) Percent of time spent in the open arm (OA), E) latency to enter the OA, and F) number of closed arm (CA) entries on the elevated plus-maze. Percent of time spent freezing while G) CS-US pairing exposure during acquisition, as well as H) CS-presentation during extinction (OXT treatment 10 min prior to extinction), and I) retention of cued fear (CS: conditioned stimulus; US: unconditioned stimulus; data represent mean ± SEM; ** $p \leq 0.01$, Scr/OXT vs Scr/Veh; # $p \leq 0.05$ Inh/Veh vs Scr/Veh; $ $p \leq 0.05$ Inh/OXT vs Inh/Veh; n = 5/group).

Statistics:

| | | | |
|---|---|---|---|
| **LDB** (Two way ANOVA followed by Bonferroni post-hoc; figure 10 A-B) | | | |
| | Factor interaction | Factor treatment Inh/Scr | Factor treatment OXT/Veh |
| Time light box | F (1, 15) = 0.2689; p = 0.6116 | F (1, 15) = 0.8339; p = 0.3756 | F (1, 15) = 0.2016; p = 0.6599 |
| Distance travelled | F (1, 16) = 1.2810; p = 0.2744 | F (1, 16) = 0.0708; p = 0.7936 | F (1, 16) = 1.2850; p = 0.2737 |
| **EPM** (Two way ANOVA followed by Bonferroni post-hoc; figure 10 C-E) | | | |
| | Factor interaction | Factor treatment Inh/Scr | Factor treatment OXT/Veh |
| Time OA | F (1, 16) = 0.1213; p = 0.7322 | F (1, 16) = 0.7962; p = 0.3855 | F (1, 16) = 0.1594; p = 0.6950 |
| Latency OA entry | F (1, 16) = 0.0601; p = 0.8094 | F (1, 16) = 0.5208; p = 0.4809 | F (1, 16) = 0.9644; p = 0.3407 |
| Entries CA | F (1, 16) = 2.1580; p = 0.1612 | F (1, 16) = 2.6370; p = 0.1239 | F (1, 16) = 2.1580; p = 0.1612 |
| **CFC - acquisition** (Two way ANOVA for repeated measures followed by Bonferroni post-hoc; figure 10 F) | | | |
| | Factor interaction | Factor CS-US pairing | Factor treatment |
| | F (12, 60) = 1.28; p = 0.2495 | F (4, 60) = 14.16; p < 0.0001** | F (3, 15) = 3.35; p = 0.0472* |
| | Status: Scr/OXT vs Scr/Veh – CS-US pairing 2 | | p = 0.0082** |
| **CFC - extinction** (Two way ANOVA for repeated measures followed by Bonferroni post-hoc; figure 10 G) | | | |
| | Factor interaction | Factor CS presentation | Factor treatment |
| | F (27, 135) = 1.30; p = 0.1663 | F (9, 135) = 2.13; p = 0.0306* | F (3, 15) = 6.93; p = 0.0038** |
| | Status: Scr/OXT vs Scr/Veh – CS presentation 1 | | p = 0.0099** |
| | Status: Scr/OXT vs Scr/Veh – CS presentation 2 | | p = 0.0001** |
| | Status: Inh/Veh vs Scr/Veh – CS presentation 2 | | p = 0.0365# |
| **CFC - retention** (Two way ANOVA followed by Bonferroni post-hoc; figure 10 H) | | | |
| | Factor interaction | Factor treatment Inh/Scr | Factor treatment OXT/Veh |
| | F (1, 76) = 0.3468; p = 0.5577 | F (1, 76) = 0.1769; p = 0.6752 | F (1, 76) = 12.45; p = 0.0007** |
| | Status: Inh/OXT vs Inh/Veh | | p = 0.0284$ |

## 3.2 Part II: "Does miR-132 Mediate the Oxytocin-induced Reversal of Social Fear?"

*Declaration: The AGO-co-immunoprecipitation and microarray described in this chapter of the thesis were performed in collaboration with Prof. Dr. Gunter Meister, Dr. Daniela Zeitler, and Julia Neumeier (University of Regensburg, Germany), Further bioinformatic analysis was conducted by Dr. Uwe Schwartz (University of Regensburg, Germany).*

### 3.2.1 Analysis of microRNA Expression Levels in the SFC Paradigm in Mice

Generally, transcript levels of miRNAs, especially of miR-132, are known to be induced by neuronal activation and plasticity, which is a consequence of various behavioral paradigms (Aten et al., 2019; Bicker et al., 2014; Nudelman et al., 2010; Ronovsky et al., 2019; Shaltiel et al., 2013; Smalheiser et al., 2011). miR-132 is well known for its plasticity-altering properties (Olde Loohuis et al., 2012), whereas miR-124 has been found to be a crucial non-coding RNA that regulates sociability in mice (Arrant and Roberson, 2014; Gascon et al., 2014), but the specific regulation of both, miR-132 and miR-124, in social fear have not been investigated so far. Since the LS has been repeatedly found to be a crucial brain region for the expression of social fear (Menon et al., 2018; Zoicas et al., 2014), I focused on the analysis of miRNA transcript levels within this region only.

Therefore, I studied whether the expression of miR-132-3p and miR-124-3p within the LS of male mice is altered 30 min, 90 min, and 3 hrs after acquisition or extinction, or 24 hrs after acquisition of social fear (figure 7 A-B). No significant differences in the time spent investigating (% time ± SEM) non-social stimulus 1 to 3 were found 30 min and 3 hrs after extinction of social fear. However, animals used for miRNA expression analysis 90 min post extinction training revealed a significant difference when comparing $SFC^+$ to $SFC^-$ mice ($p = 0.0379$). As expected, no differences in the time spent investigating social stimulus 6 were found between $SFC^-$ and $SFC^+$ mice at all time points. In contrast, the time spent investigating the first social stimulus was significantly lower in $SFC^+$ mice compared to $SFC^-$ animals (30 min: $p < 0.0001$; 90 min:, $p = 0.0038$; 3 hrs: $p = 0.0002$) (table 8).

| Time point | Non-social stimulus (1-3) (% time ± SEM) | | Social stimulus 1 (% time ± SEM) | | Social stimulus 6 (% time ± SEM) | |
|---|---|---|---|---|---|---|
| | $SFC^-$ | $SFC^+$ | $SFC^-$ | $SFC^+$ | $SFC^-$ | $SFC^+$ |
| 30 min | 22.4 ± 4.2 | 23.0 ± 3.4 | 80.3 ± 2.8 | 1.1 ± 0.8 ** | 69.1 ± 12.5 | 54.1 ± 0.8 |
| 90 min | 24.3 ± 3.8 | 12.9 ± 2.6* | 69.9 ± 7.5 | 1.2 ± 0.8 ** | 72.0 ± 3.7 | 63.6 ± 7.8 |
| 3 hrs | 27.3 ± 2.5 | 25.2 ± 2.7 | 63.3 ± 6.8 | 11.7 ± 7.0 ** | 70.9 ± 6.8 | 53.2 ± 10.1 |

**Table 8 – Percent of time investigating the non-social and social stimuli during social fear extinction in male mice corresponding to miRNA expression analysis within the lateral septum.** Mean investigation time (% time ± SEM) of unconditioned ($SFC^-$) and conditioned ($SFC^+$) animals during exposure to the non-social stimulus 1-3, social stimulus 1, and 6 of male mice used for miRNA assessment 30 min, 90 min, and 3 hrs post social fear extinction (data represent mean ± SEM; * $p \leq 0.05$, ** $p \leq 0.01$ $SFC^+$ vs $SFC^-$ of respective stimulus and time; n = 6-10/group).

Statistics:

| | | | |
|---|---|---|---|
| **30 min** (Status: $SFC^+$ vs $SFC^-$; Mann Whitney U-test or independent student t-test; two-tailed; table 8) | | | |
| | Non-social stimulus 1-3 | T (12) = 0.0866 | p = 0.9324 |
| | Social stimulus 1 | T (12) = 26.89 | p < 0.0001** |
| | Social stimulus 6 | U = 10.000; n($SFC^-$) = n($SFC^+$) = 7 | p = 0.0728 |
| **90 min** (Status: $SFC^+$ vs $SFC^-$; independent student t-test; two-tailed; table 8) | | | |
| | Non-social stimulus 1-3 | T (14) = 2.2930 | p = 0.0379* |
| | Social stimulus 1 | T (14) = 3.4640 | p = 0.0038** |
| | Social stimulus 6 | T (14) = 0.9660 | p = 0.3504 |
| **3 hrs** (Status: $SFC^+$ vs $SFC^-$; Mann Whitney U-test or independent student t-test; two-tailed; table 8) | | | |
| | Non-social stimulus 1-3 | T (19) = 0.6345 | p = 0.5333 |
| | Social stimulus 1 | U = 6.000; n($SFC^-$) = 9; n($SFC^+$) = 12 | p = 0.0002** |
| | Social stimulus 6 | U = 41.000; n($SFC^-$) = 9; n($SFC^+$) = 12 | p = 0.3791 |

On one hand, 90 min after acquisition, miR-132-3p was upregulated in $SFC^+$ compared to $SFC^-$ mice (p = 0.0439), whereas 180 min post-acquisition, miR-132-3p transcript levels were restored to basal levels (figure 11 A). Contrastingly, miR-132-3p remained unchanged at all analyzed time points after extinction, suggesting that septal miR-132-3p is involved in fear memory learning and consolidation, but not social fear extinction *per se*. On the other hand, 180 min post-extinction, $SFC^+$ mice showed decreased levels of miR-132-3p within the LS compared to $SFC^+$ mice after acquisition (p = 0.0046), which pinpoints towards reduced miR-132-3p-mediated gene repression as important regulatory effect for social fear extinction learning and consolidation. 30 min after acquisition and extinction as well as 24 hrs after acquisition of social fear, miR-132-3p expression remained unaltered in $SFC^+$ compared to $SFC^-$ mice, revealing a time-limited and highly dynamic transcriptional alteration of miR-132-3p in the LS of mice exposed to the SFC paradigm. Taken

together, the dynamic regulation of septal miR-132-3p in response to acquisition and extinction of social fear indicates it as crucial factor modulating learning and memory-related processes that are required for the animal´s behavioral adaptations.

Regarding miR-124-3p, similar alterations were revealed (figure 11 B): 90 min after acquisition, septal miR-124-3p was found to be upregulated in $SFC^+$ compared to $SFC^-$ mice ($p = 0.0273$), which, comparable to miR-132-3p, pinpoints toward the involvement of miR-124-3p in social fear memory consolidation. Interestingly, $SFC^-$ mice showed a strong upregulation of miR-124-3p levels 90 min after extinction compared to respective unconditioned post-acquisition mice ($p = 0.0011$), an effect that suggests miR-124-3p mediated gene repression as consequence of repeated social investigation during social fear extinction training. Contrastingly, miR-124-3p transcript levels within the LS remained unchanged in $SFC^+$ compared to $SFC^-$ mice at earlier (30 min) as well as later (180 min, 24 hrs) time points after acquisition and extinction of social fear, revealing a highly dynamic regulation of miRNA-mediated gene repression in response to socio-behavioral adaptations.

Importantly, no expression alterations of both, miR-132-3p and miR-124-3p, were found at all analyzed time points when comparing $SFC^-$ mice to further control groups that were either only exposed to the conditioning chamber or received two random shocks when in the chamber.

These data in $SFC^-$ mice suggest a transcriptional increase of miR-124-3p upon repeated social contact. As mentioned in the introduction, social encounters leads to a central release of OXT (Neumann and Slattery, 2016; Neumann et al., 1993b) and, compared to $SFC^+$ animals, $SFC^-$ mice have a higher release of OXT during extinction of social fear (Zoicas et al., 2014). Therefore, the transcript levels of both miRNAs might possibly be altered due to repeated social contact that leads to OXT release and subsequent OXTR activation. To further analyze whether miR-132-3p and miR-124-3p are transcribed as down-stream factors of OXTR activation, transcript levels of both miRNAs within the LS were measured 90 min as well as 3 hrs after icv infusion of Veh or OXT (figure 7 C). For both time points and both miRNAs, no significant alterations were found within the LS (figure 11 D, F). To reveal whether miR-132-3p and especially miR-124-3p transcript levels are increased in response to repeated social contact, mice were exposed to 9 non-social or 3 non-social plus subsequent 6 social stimuli (figure 7 D-E). Independent of the conditioning status, this repeated exposure to social stimuli did not alter both, miR-132-3p and miR-124-3p, levels within the LS when compared to mice exposed to only non-social stimuli (figure 11 C, E).

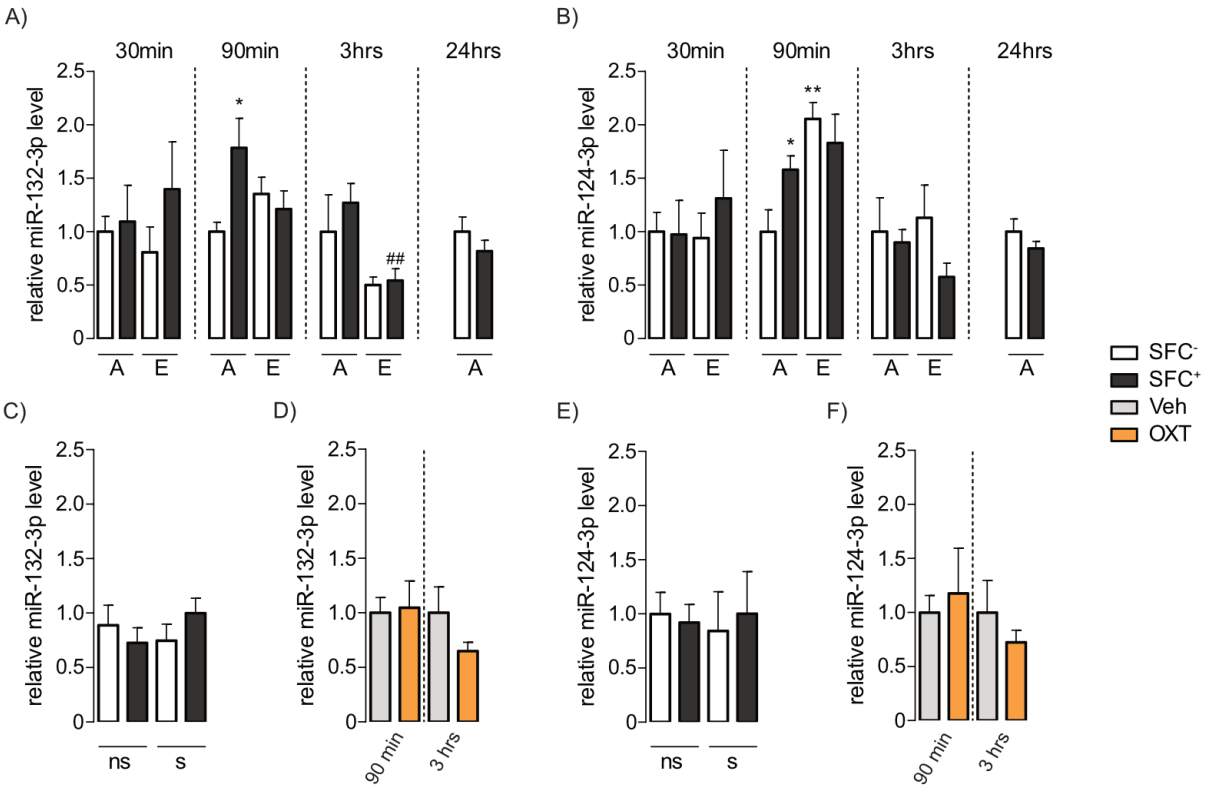


**Figure 11 – miR-132-3p and miR-124-3p expression within the lateral septum of male mice.** A) Relative miR-132-3p transcript level in the lateral septum (LS) of unconditioned ($SFC^-$) and conditioned ($SFC^+$) male mice at different time points (30 min, 90 min, 3 hrs, 24 hrs) after acquisition (A) and extinction (E) of social fear. B) Relative miR-124-3p expression in the LS of $SFC^-$ and $SFC^+$ mice at different time points (30 min, 90 min, 3 hrs, 24 hrs) after acquisition or extinction of social fear. C, E) Relative miR-123-3p and miR-124-3p transcript levels within the LS of $SFC^-$ and $SFC^+$ mice after repeated exposure of to social (s) versus non-social (ns) stimuli. D, F) Relative miR-132-3p (D) and miR-124-3p (F) expression within the LS 90 min as well as 3 hrs after intracerebroventricular oxytocin (OXT; 0.1 µg / 2 µl) or vehicle (Veh) treatment (data represent mean + SEM; * $p \leq 0.05$; ** $p \leq 0.01$ vs $SFC^-$/A of equal time point; ## $p \leq 0.01$ vs $SFC^+$/A of equal time point; n = 6-10/group).

Statistics:

| | | | |
|---|---|---|---|
| **miR-132-3p** (Two way ANOVA followed by Bonferroni post-hoc or independent student t-test; two-tailed; figure 11 A) | | | |
| | Factor interaction | Factor E/A | Factor $SFC^+/SFC^-$ |
| 30 min | F (1, 21) = 0.6713; p = 0.4218 | F (1, 21) = 0.03241; p = 0.8589 | F (1, 21) = 1.2920; p = 0.2684 |
| 90 min | F (1, 24) = 4.5580; p = 0.0432* | F (1, 24) = 0.2580; p = 0.6161 | F (1, 24) = 2.1870; p = 0.1522 |
| | Status: $SFC^+$/A vs $SFC^-$/A (Independent student t-test; two-tailed) | | T (13) = 2.2310; p = 0.0439* |
| 3 hrs | F (1, 22) = 0.63472; p = 0.5617 | F (1, 22) = 10.04; p = 0.0045** | F (1, 22) = 0.6500; p = 0.4287 |
| | Status: $SFC^+$/E vs $SFC^+$/A (Independent student t-test; two-tailed) | | T (11) = 3.5480; p = 0.0046## |
| 24 hrs | | T(14) = 1.0540 | p = 0.3099 |
| **miR-124-3p** (Two way ANOVA followed by Bonferroni post-hoc or independent student t-test; two-tailed; figure 11 B) | | | |
| | Factor interaction | Factor E/A | Factor $SFC^+/SFC^-$ |
| 30 min | F (1, 21) = 0.4234; p = 0.5223 | F (1, 21) = 0.2066; p = 0.6541 | F (1, 21) = 0.3198; p = 0.5777 |
| 90 min | F (1, 26) = 4.1370; p = 0.0523 | F (1, 26) = 10.87; p = 0.0028** | F (1, 26) = 0.8069; p = 0.3773 |

| | | | |
|---|---|---|---|
| | Status: $SFC^+$/A vs $SFC^-$/A (Independent student t-test; two-tailed) | | T (12) = 2.5120; p = 0.0273* |
| | Status: $SFC^-$/E vs $SFC^-$/A (Independent student t-test; two-tailed) | | T (12) = 4.2400; p = 0.0011** |
| 3 hrs | F (1, 22) = 1.027; p = 0.3220 | F (1, 22) = 0.1846; p = 0.6717 | F (1, 22) = 2.1400; p = 0.1577 |
| 24 hrs | | T(14) = 1.1600 | p = 0.2656 |
| **miR-132-3p** (Two way ANOVA followed by Bonferroni post-hoc; figure 11 C) | | | |
| | Factor interaction | Factor s/ns | Factor $SFC^+$/$SFC^-$ |
| | F (1, 26) = 1.7360; p = 0.1991 | F (1, 26) = 0.0863; p = 0.7713 | F (1, 26) = 0.1741; p = 0.6799 |
| **miR-132-3p** (Independentstudent t-test; two-tailed; figure 11 D) | | | |
| 90 min | | T(11) = 0.1593 | p = 0.8763 |
| 3 hrs | | T(16) = 1.3880 | p = 0.1842 |
| **miR-124-3p** (Two way ANOVA followed by Bonferroni post-hoc; figure 11 E) | | | |
| | Factor interaction | Factor s/ns | Factor $SFC^+$/$SFC^-$ |
| | F (1, 27) = 0.1552; p = 0.6967 | F (1, 27) = 0.0156; p = 0.9013 | F (1, 27) = 0.0156; p = 0.9013 |
| **miR-124-3p** (Independent student t-test; two-tailed; figure 11 F) | | | |
| 90 min | | T(11) = 0.3691 | p = 0.7190 |
| 3 hrs | | T(16) = 0.9413 | p = 0.3606 |

### 3.2.2 miR-132-3p Inhibition within the Lateral Septum Impairs Extinction of Social Fear

miR-132 has repeatedly been shown to be involved in synaptic plasticity, memory, as well as fear-related behavior (Aten et al., 2019; Hansen et al., 2016; Hernandez-Rapp et al., 2015; Wang et al., 2013). Moreover, abovementioned alterations in the expression pattern throughout the SFC paradigm highlight miR-132-3p as essential regulator of social fear behavior. To reveal the involvement of miR-132-3p in acquisition and extinction of social fear, miR-132-3p function was specifically inhibited 48 hrs prior to acquisition of social fear *via* LNA microinfusion into the mouse septum (figure 7 F), resulting in the following groups: Scrambled control LNA-infused unconditioned (Scr/$SFC^-$) and conditioned (Scr/$SFC^+$) mice, as well as miR-132-3p inhibitor LNA-treated unconditioned (Inh/$SFC^-$) and conditioned (Inh/$SFC^+$) animals (figure 12 A).

Successful transfection of the septum with the LNA molecules was confirmed by immunofluorescent analysis due to the 5´-FAM labeling of the scrambled and miR-132-3p inhibitor sequence (figure 12 B). During acquisition of social fear, Inh/$SFC^+$ and Scr/$SFC^+$ mice did not differ in the number of CS-US pairings presented to induce social fear, revealing adequate and unaltered learning of social fear (figure 12 C). Similarly, on the next day, there was no group difference in the investigation times displayed when exploring the 3 non-social stimuli during social fear extinction (figure 12 D). As expected and independent of the LNA treatment, $SFC^-$ mice showed significantly higher initial social investigation times compared to $SFC^+$. Moreover, significant differences between miR-132-3p inhibitor and scrambled control-treated mice were evident during extinction

of social fear: As expected, Scr/SFC$^-$ mice showed high investigation of all 6 social stimuli, whereas Scr/SFC$^+$ mice showed a gradual extinction of social fear, which is indicated by rising investigation times from social stimulus 1 to social stimulus 6. Inh/SFC$^-$ mice showed investigation times comparable to respective Scr/SFC$^-$. In contrast, Inh/SFC$^+$ treatment lead to impaired extinction of social fear: Here, the gradual incline of social investigation over the presentation of 6 social stimuli is delayed compared to Scr/SFC$^+$ mice (social stimulus 3: $p = 0.0322$; 4: $p = 0.0076$; 5: $p = 0.0328$). Nevertheless, upon the sixth social stimulus Inh/SFC$^+$ mice showed investigation times comparable to respective Scr/SFC$^+$ mice and both unconditioned groups. On day 3 during extinction recall training (figure 12 E), no differences were found between all four groups, revealing that inhibition of miR-132-3p delays extinction but does not prohibit a successful extinction of social fear.

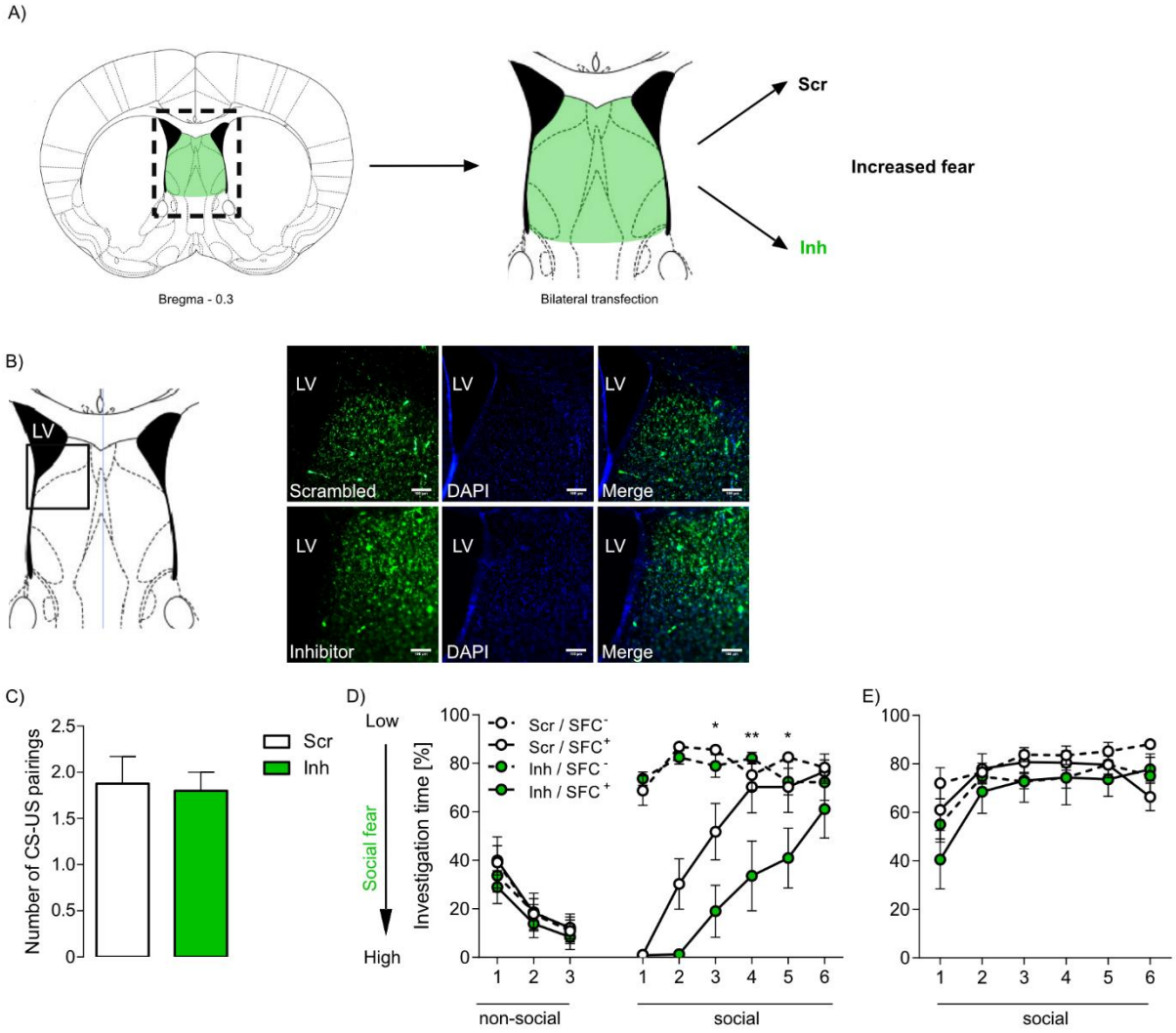


**Figure 12 – Effect of septal miR-132-3p inhibition in male mice on acquisition, extinction, and recall of social fear.** A) Scheme of septal scrambled (Scr; 0.5 nmol) and miR-132-3p inhibitor (Inh; 0.5 nmol) locked nucleic acid infusions and their effect on social fear. B) Scheme of the location and representative immunofluorescent images within the septum for verification of Scr and Inh microinfusions. D) Number of CS-US pairings presented to conditioned mice (SFC$^+$) during acquisition of social fear (continued on next page).

E) Percent of time investigating the stimuli, which were presented during social fear extinction of Scr and Inh-infused $SFC^+$ and unconditioned ($SFC^-$) mice. F) Percent of investigation time presented during social fear extinction recall (scalebar represents 50 µm; data represent mean ± SEM; * $p \leq 0.05$, ** $p \leq 0.01$ Inh/$SFC^+$ vs Scr/$SFC^+$; n = 8-10/group).

Statistics:

| | | |
|---|---|---|
| **Acquisition** (Independent student t-test; two-tailed; figure 12 A) | | |
| | T(16) = 0.2173 | p = 0.8308 |
| **Extinction** (Two way ANOVA for repeated measures followed by Bonferroni *post-hoc*; figure 12 B | | |
| Factor interaction | Factor time | Factor treatment |
| F (24, 232) = 6.4960; p < 0.0001** | F (8, 232) = 48.75; p < 0.0001** | F (3, 29) = 16.28; p < 0.0001** |
| Status Inh/$SFC^+$ vs Scr/$SFC^+$ | Social stimulus 3 | p = 0.0322* |
| | Social stimulus 4 | p = 0.0076** |
| | Social stimulus 5 | p = 0.0328* |
| **Recall** (Two way ANOVA for repeated measures followed by Bonferroni *post-hoc*; figure 12 C) | | |
| Factor interaction | Factor time | Factor treatment |
| F (15, 110) = 0.9437; p = 0.0.5191 | F (5, 110) = 9.0140; p < 0.0001** | F (3, 22) = 1.8400; p = 0.1695 |

Since conflictive effects of supra- and infra-physiological levels of miR-132 on anxiety-related behavior are known (Hansen et al., 2016; Hernandez-Rapp et al., 2015), I investigated whether miR-132-3p inhibition specifically affects social fear without altering general anxiety or locomotor activity. Mice were tested for their general anxiety-related behavior and locomotion in the OFT (figure 13 A-C) and NOI test (figure 13 D-F). In both tests, no differences in the time spent in the center zone as well as distance travelled were found independent of the animal´s treatment and SFC-status, illustrating no effects of septal miR-132-3p inhibition of general anxiety-related behavior or locomotor activity. Taken together, these results demonstrate that miR-132-3p is a crucial and highly specific factor for the extinction of social fear since its inhibition within the septum impairs extinction of social fear without affecting general anxiety and locomotion.

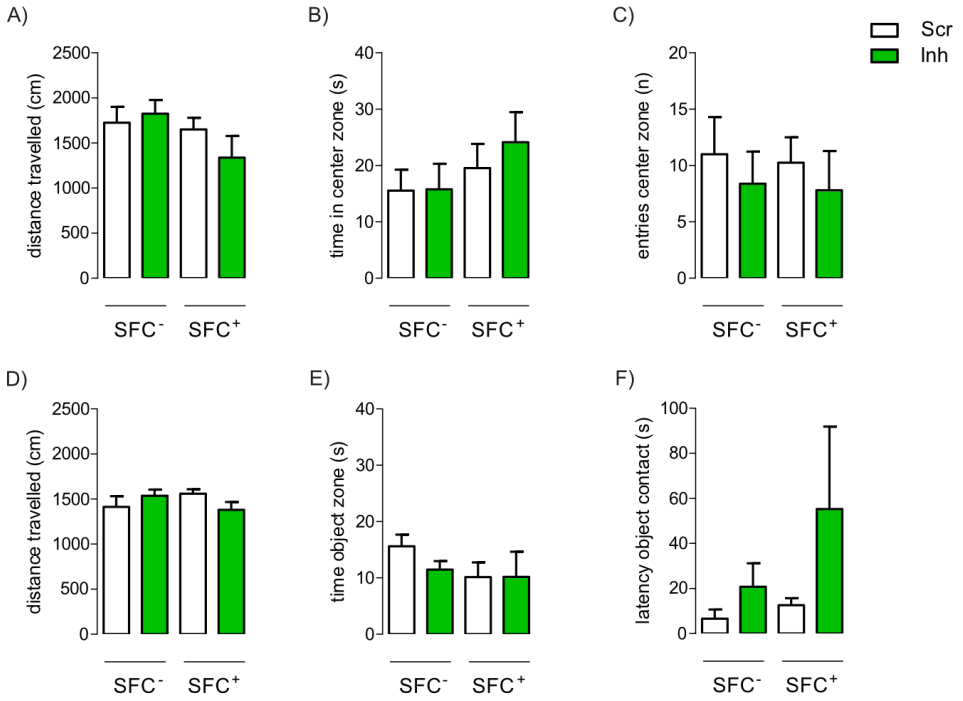


**Figure 13 – Effect of septal miR-132-3p inhibition in male mice on general anxiety-related behavior and locomotor activity.** A) Distance travelled, B) time spent in the center zone, and C) entries into the center zone of the open field test in conditioned ($SFC^+$) and unconditioned ($SFC^-$) mice with septal miR-132-3p inhibitor (Inh, 0.5 nmol) or scrambled control (Scr; 0.5 nmol) locked nucleic acid infusion. D) Distance travelled, E) time spent in the object zone, and F) latency to show contact to the object of the novel object investigation-test in mice infused with the Scr or Inh locked nucleic acids within the septum (data represent mean + SEM; n = 4-6 /group).

Statistics:

| | | | |
|---|---|---|---|
| **OFT** (Two way ANOVA followed by Bonferroni post-hoc; figure 13 A-C) | | | |
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor Inh/Scr |
| Distance | F (1, 32) = 2.7860; p = 0.1049 | F (1, 32) = 5.1330; p = 0.0304* | F (1, 32) = 0.7412; p = 0.3957 |
| Time center | F (1, 32) = 0.5063; p = 0.14819 | F (1, 32) = 4.0100; p = 0.0538 | F (1, 32) = 0.6051; p = 0.4423 |
| Entries center | F (1, 32) = 0.0013; p = 0.9710 | F (1, 32) = 0.1086; p = 0.7439 | F (1, 32) = 1.5190; p = 0.2267 |
| **NOI** (Two way ANOVA followed by Bonferroni post-hoc; figure 13 D-F) | | | |
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor Inh/Scr |
| Distance | F (1, 28) = 6.8290; p = 0.0143* | F (1, 28) = 0.007390; p = 0.9321 | F (1, 28) = 0.2319; p = 0.6339 |
| Time object | F (1, 28) = 0.9023; p = 0.3503 | F (1, 28) = 2.3490; p = 0.1366 | F (1, 28) = 0.8707; p = 0.3587 |
| Latency object | F (1, 28) = 0.7324; p = 0.3994 | F (1, 28) = 1.4800; p = 0.2340 | F (1, 28) = 2.9230; p = 0.0984 |

### 3.2.3 Septal miR-132 Overexpression Facilitates Extinction of Social Fear

Based on the impaired extinction of social fear after inhibition of septal miR-132-3p, I hypothesized that septal miR-132-3p overexpression facilitates extinction of social fear. Therefore, a miR-132-3p mimic was overexpressed within the septum 3 weeks prior to acquisition of social fear using an AAV-mediated approach (figure 7 I). The following treatment groups were evaluated: Control AAV-infused unconditioned (Ctrl/SFC$^-$) and conditioned (Ctrl/SFC$^+$) mice, as well as miR-132-3p mimic expressing AAV-treated unconditioned (Mimic/SFC$^-$) and conditioned (Mimic/SFC$^+$) animals (figure 14 A).

Successful infection of the septum was confirmed by immunofluorescent analysis since the AAV also lead to eGFP expression (figure 14 B). During acquisition of social fear, Mimic/SFC$^+$ mice did not differ in the number of CS-US pairings compared to Ctrl/SFC$^+$ animals, representing normal social fear learning capacities upon miR-132-3p overexpression (figure 14 C). On day 2 during extinction of social fear, mimic-treated mice showed similar investigation times of the non-social stimulus independent of their conditioning status (figure 14 D). Moreover, Mimic/SFC$^-$ mice showed social investigation times comparable to Ctrl/SFC$^-$ animals. However, Mimic/SFC$^+$ mice showed an accelerated gradual incline in social investigation compared to Ctrl/SFC$^+$ animals (social stimulus 4: $p = 0.0086$; 5: $p = 0.0296$), revealing a social fear extinction facilitating effect of septal miR-132-3p overexpression. Social fear extinction recall has not been evaluated in those mice.

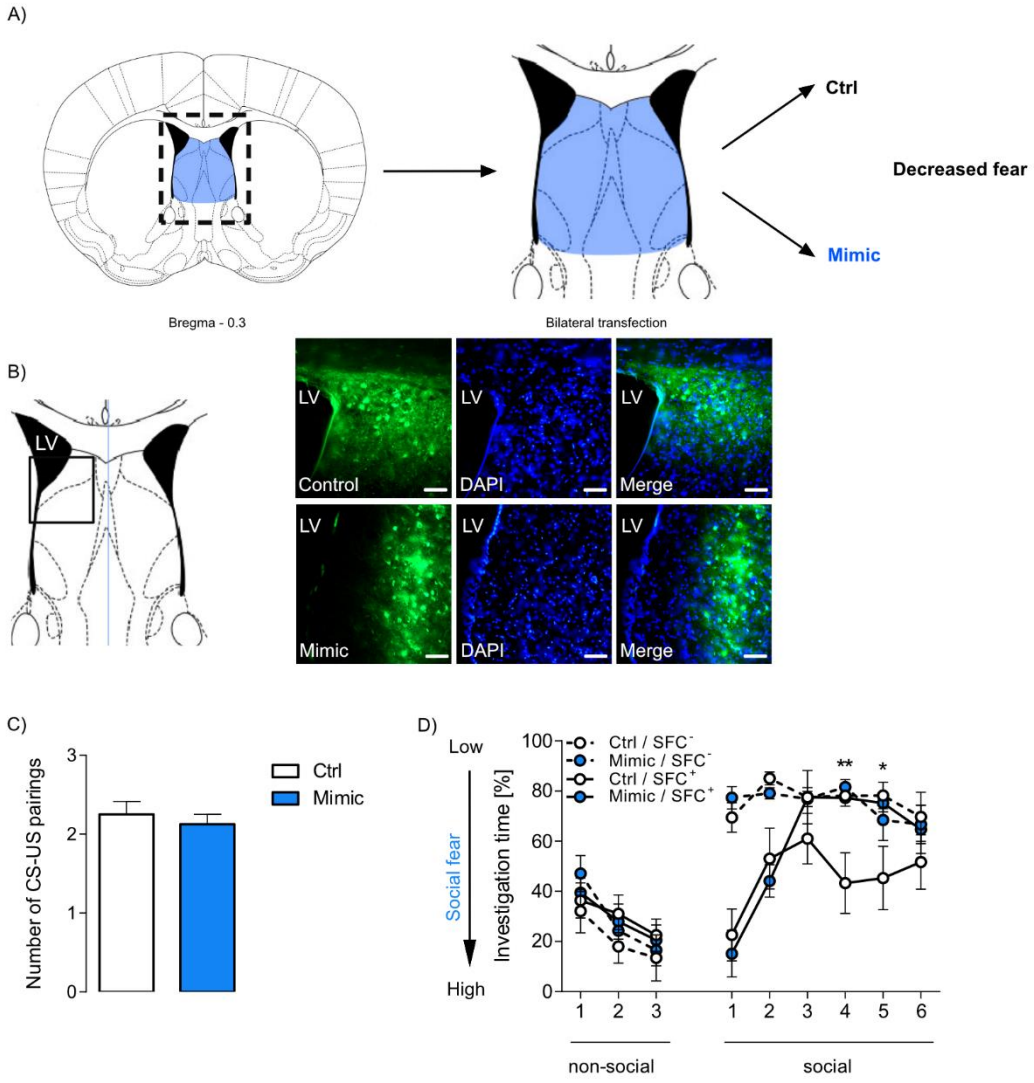


**Figure 14 – Effect of septal miR-132-3p overexpression in male mice on acquisition, extinction, and recall of social fear.** A) Scheme of viral miR-132-3p mimic (Mimic; 4.9 x $10^3$ GC) or scrambled control (Ctrl; 4.9 x $10^3$ GC) infusions into the septum of unconditioned ($SFC^-$) and conditioned ($SFC^+$) male mice and their effect of social fear extinction. B) Representative immunofluorescent images of septal miR-132-3p overexpression induced by an adeno-associated virus. C) Number of CS-US pairings presented to control and mimic infused $SFC^+$ mice during acquisition of social fear. D) Extinction of social fear after viral infection of the septum leading to expression of a control nucleotide sequence or a miR-132-3p mimic (scalebar represents 50 µm; ** $p \leq 0.01$, * $p \leq 0.05$ $SFC^+$/Mimic vs $SFC^+$/Ctrl; data represent mean ± SEM; n = 8-12/group).

Statistics:

| | | |
|---|---|---|
| **Acquisition** (Mann Whitney U-test; two-tailed; figure 14 C) | | |
| | U = 28.00; n($SFC^-$) = n($SFC^+$) = 8 | p > 0.9999 |
| ***Extinction*** *(Two way* ANOVA for repeated measures followed by Bonferroni *post-hoc*; figure 14 D) | | |
| Factor interaction | Factor time | Factor treatment |
| F (24, 192) = 4.4580; p < 0.0001** | F (8, 192) = 39.02; p < 0.0001** | F (3, 24) = 2.9780; p = 0.0516 |
| Status Mimic/$SFC^+$ vs Ctrl/$SFC^+$ | Social stimulus 4 | p = 0.0086** |
| | Social stimulus 5 | p = 0.0296* |

To further reveal whether the facilitating effect of septal miR-132-3p overexpression is specific for social fear extinction, animals were tested for general anxiety-related behavior, and locomotor activity. Here, septal miR-132-3p overexpression did not significantly influence anxiety-related behavior as well as locomotion in the OFT (figure 15 A-C), LDB (figure 15 D-F), and EPM (figure 15 G-I). This demonstrates that miR-132-3p overexpression specifically facilitates extinction of social fear without affecting general anxiety, and locomotor activity.

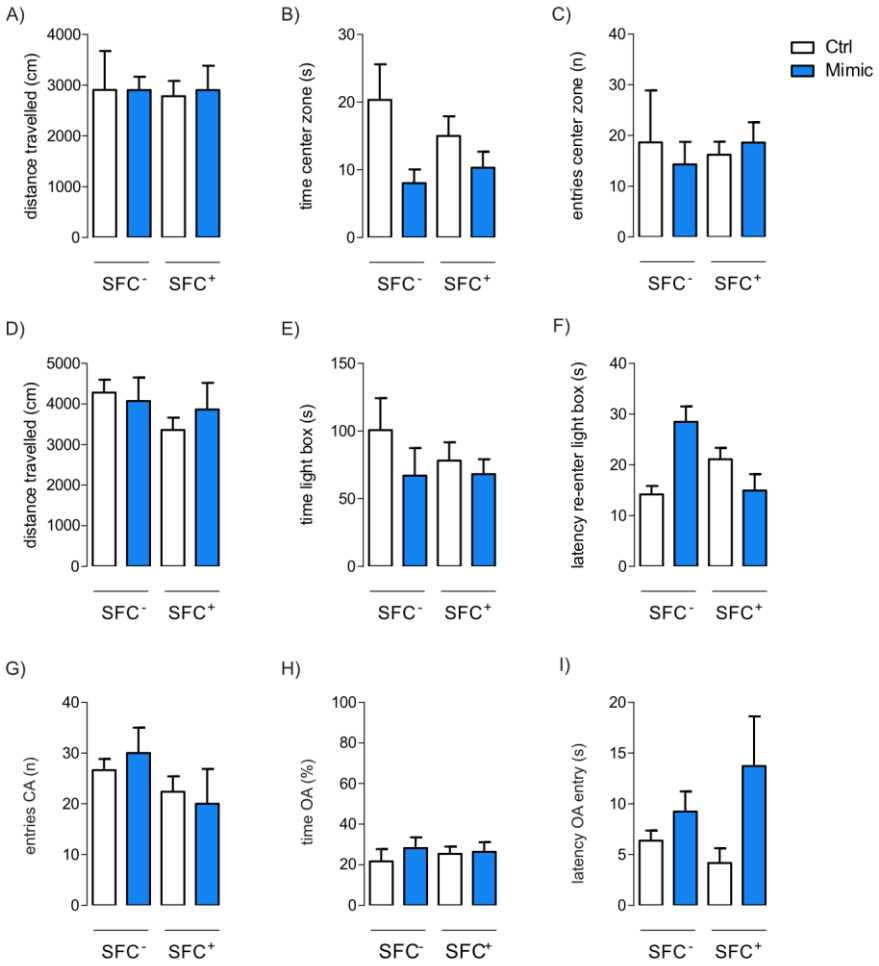


**Figure 15 – Effect of septal miR-132-3p overexpression in male mice on general anxiety-related behavior and locomotor activities.** A) Distance travelled, B) time spent in the center zone, and C) entries into the center zone of the open field test after scrambled control (Ctrl; 4.9 x $10^3$ GCs) or miR-132-3p mimic (Mimic; 4.9 x $10^3$ GCs) virus transduction within the septum of unconditioned ($SFC^-$) and conditioned ($SFC^+$) mice. D) Distance travelled, E) time spent in the light box, and F) latency to re-enter the light compartment of the light dark-box in Mimic and Ctrl virus infused mice. G) Entries into the closed arms (CA), H) percent of time spent in the open arms (OA), and I) latency to enter the OA of the elevated plus-maze after septal mimic or Ctrl infusion (data represent mean + SEM; n = 6-10/group).

Statistics:

| | | | |
|---|---|---|---|
| **OFT** (Two way ANOVA followed by Bonferroni *post-hoc*; figure 15 A-C) | | | |
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor Mimic/Ctrl |
| Distance | F (1, 28) = 0.7130; p = 0.4056 | F (1, 28) = 0.4593; p = 0.5035 | F (1, 28) = 0.7717; p = 0.3872 |
| Time center | F (1, 28) = 2.5710; p = 0.1201 | F (1, 28) = 0.2621; p = 0.6127 | F (1, 28) = 0.2205; p = 0.6423 |
| Entries center | F (1, 27) = 0.0517; p = 0.8217 | F (1, 27) = 0.4917; p = 0.3467 | F (1, 27) = 0.0472; p = 0.8296 |
| **LDB** (Two way ANOVA followed by Bonferroni *post-hoc*; figure 15 D-F) | | | |
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor Mimic/Ctrl |
| Distance | F (1, 28) = 0.3105; p = 0.5818 | F (1, 28) = 0.1039; p = 0.7496 | F (1, 28) = 0.0408; p = 0.8413 |
| Time LB | F (1, 28) = 1.1340; p = 0.2960 | F (1, 28) = 1.8190; p = 0.1883 | F (1, 28) = 0.0335; p = 0.8561 |
| Latency re-enter LB | F (1, 28) = 0.8729; p = 0.3582 | F (1, 28) = 2.3220; p = 0.1388 | F (1, 28) = 0.0065; p = 0.9361 |
| **EPM** (Two way ANOVA followed by Bonferroni *post-hoc*; figure 15 G-I) | | | |
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor Mimic/Ctrl |
| Entries CA | F (1, 28) = 0.1359; p = 0.7152 | F (1, 28) = 0.4253; p = 0.5196 | F (1, 28) = 0.0394; p = 0.8441 |
| Time OA | F (1, 28) = 0.3148; p = 0.5792 | F (1, 28) = 0.4032; p = 0.8573 | F (1, 28) = 0.5851; p = 0.4507 |
| Latency OA | F (1, 28) = 2.0810; p = 0.1602 | F (1, 28) = 1.6970; p = 0.2032 | F (1, 28) = 1.8020; p = 0.1903 |

### 3.2.4 Septal miR-132-3p Inhibition Prevents Oxytocin-mediated Reversal of Social Fear

To further analyze, whether miR-132-3p inhibition is involved in OXT-mediated reversal of social fear, mice were pre-treated with the miR-132-3p inhibitor within the septum 48 hrs prior to exposure to the SFC paradigm and were additionally infused with either Veh or OXT in the LS 10 min prior to social fear extinction (figure 7 G). The following treatment groups were analyzed: Scrambled control LNA with additional local Veh (Scr/Veh) or OXT (Scr/OXT) infusion, as well as miR-132-3p inhibitor LNA with Veh (Inh/Veh) or OXT (Inh/OXT) application (figure 16 A).

In line with the previous experiment, septal inhibition of miR-132-3p did neither influence acquisition of social fear in $SFC^+$, mice represented by a similar quantity of CS-US pairings compared to Scr-infused mice on day 1 of SFC (figure 16 B), nor social investigation time in $SFC^-$ mice (figure 16 C). No alterations in the time investigating the 3 non-social stimuli during social fear extinction were found between treatment groups. Although not significant, Scr/OXT infusion in $SFC^+$ mice lead to higher investigation times compared to respective Scr/Veh-treated animals. This confirms previously published results of the OXT-mediated reversal of social fear (Zoicas et al., 2014). Independent of Veh or OXT treatment and in concordance with the previous experiment, septal miR-132-3p inhibition resulted in lower investigation times during extinction of social fear, representing impaired extinction of social fear. Moreover, significantly lower investigation times were displayed by conditioned Inh/OXT-treated mice compared to respective Scr/OXT-infused animals (social stimulus 1: p = 0.0104; 4: p = 0.0070; 6: p = 0.0172). This demonstrates that septal

miR-132-3p inhibition abolished the social fear reversing effect of OXT. On day 3 during social fear extinction recall (figure 16 D), Inh/OXT-treated mice showed lower social investigation times compared to Scr/OXT-infused animals only during exposure to social stimulus 4 ($p = 0.0216$), which might be an artificial effect and can not be explained by the given treatment. Moreover, investigation times of both, Inh/Veh and Inh/OXT-treated groups, depict low investigation times throughout all 6 stimulus presentations. This reveals a possible long-lasting social fear-enhancing effect of septal miR-132-3p inhibition in combination with local guide cannula placement and infusion, which needs further investigation (not provided in this thesis).

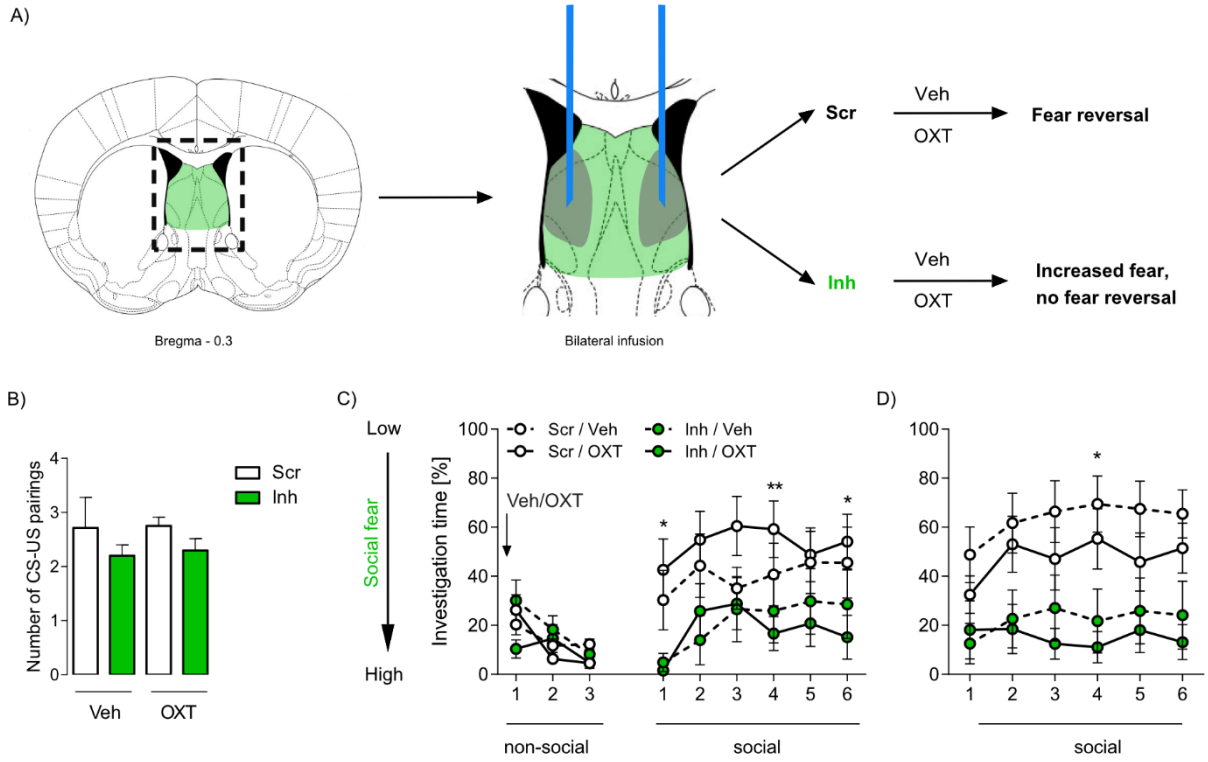


**Figure 16 - Effect of septal miR-132-3p inhibition in male mice on OXT-mediated reversal of social fear.** A) Scheme of septal pre-infusion of a miR-132-3p inhibitor (Inh; 0.5 nmol) or scrambled control (Scr; 0.5 nmol) locked nucleic acid in combination with local vehicle (Veh) or oxytocin (OXT; 5 ng / 0.2 µl / hemisphere) infusions into the dorsal lateral septum of socially fear-conditioned mice, and their effects on social fear. B) Number of CS-US pairings of all four treatment groups during acquisition. C) Percent of social investigation times during extinction training and D) during extinction recall (* $p \leq 0.05$, ** $p \leq 0.01$ Inh/OXT vs Scr/OXT; data represent mean ± SEM; acquisition and extinction: n = 11-12/group; recall: n = 8-9/group).

Statistics:

| **Acquisition** (Two way ANOVA followed by Bonferroni post-hoc; figure 16 B) | | |
|---|---|---|
| Factor interaction | Factor OXT/Veh | Factor Inh/Scr |
| $F(1, 31) = 0.0121$; $p = 0.9131$ | $F(1, 31) = 0.0539$; $p = 0.8179$ | $F(1, 31) = 2.7230$; $p = 0.1090$ |
| **Extinction** (Two way ANOVA for repeated measures followed by Bonferroni post-hoc; figure 16 C) | | |

| | | |
|---|---|---|
| Factor interaction | Factor OXT/Veh | Factor Inh/Scr |
| F (24, 240) = 62.2140; p = 0.0014** | F (8, 240) = 11.21; p < 0.0001** | F (3, 30) = 2.6700; p = 0.0654 (*) |
| Status Inh/OXT vs Scr/OXT | Social stimulus 1 | p = 0.0104* |
| | Social stimulus 4 | p = 0.0070** |
| | Social stimulus 6 | p = 0.0172* |
| **Recall** (Two way ANOVA for repeated measures followed by Bonferroni post-hoc; figure 16 D) | | |
| Factor interaction | Factor time | Factor treatment |
| F (15, 150) = 1.3130; p = 0.2011 | F (5, 150) = 3.7320; p = 0.0033** | F (3, 30) = 4.8470; p = 0.0072** |
| Status Inh/OXT vs Scr/OXT | Social stimulus 4 | p = 0.0216* |

### 3.2.5 Downregulation of miR-132 within OXTR-expressing Neurons Impairs Social Fear Extinction

To further verify that miR-132-3p specifically within OXTR expressing neurons of the mouse septal region is involved in the modulation of social fear behavior, an AAV expressing a shRNA, which downregulates pre-miR-132 transcripts, was infused into the septum of male OXTRCre mice 3 weeks prior to acquisition of social fear, resulting in the following treatment groups (figure 7 H): Scrambled control AAV-infused unconditioned (Scr/SFC$^-$) and conditioned (Scr/SFC$^+$) animals, as well as pre-miR-132 shRNA expressing AAV-treated unconditioned (shRNA/SFC$^-$) and conditioned (shRNA/SFC$^+$) mice (figure 17 A).

Successful transfection of the mouse septum was confirmed by constitutive mCherry expression with further Cre-dependent GFP labeling of all pre-miR-132 shRNA or scrambled shRNA expressing OXTR positive neurons. Downregulation of pre-miR-132 did not affect acquisition of social fear, since the number of CS-US pairings in shRNA/SFC$^+$ animals was equal to those seen in Scr/SFC$^+$ mice (all animals 2 schocks; figure 17 B). Moreover, the percent of time spent investigating the non-social stimuli during social fear extinction did not differ between all four groups (figure 17 C). Independent of the treatment, SFC$^-$ mice displayed significantly higher initial social investigation times compared to the respective SFC$^+$ animals. Interestingly, and in concordance with the results obtained by septal inhibition of miR-132-3p (figure 12), shRNA/SFC$^+$ treatment resulted in an impaired social fear extinction compared to Scr/SFC$^+$-treated animals as seen by the decelerated gradual incline in the percent time investigating the 6 social stimuli, especially stimulus 4 ($p = 0.0051$) and 5 ($p = 0.0098$). Upon exposure to the 6$^{th}$ social stimulus, no group differences were detected, implicating a successful extinction of social fear. Social fear extinction recall has not been evaluated in those mice. Moreover, it has to be declared that no immunofluorescent validation of the shRNA-mediated knockdown of pre-miR-132 has been performed yet. However, the accurate localization of viral transduction was verified in these animals. In summary, repression of miR-132 transcript levels

within septal OXTR expressing neurons of male mice seemed to impair extinction of social fear, further highlighting the crucial contribution of the neuropeptide OXT within this brain region in extinction of social fear.

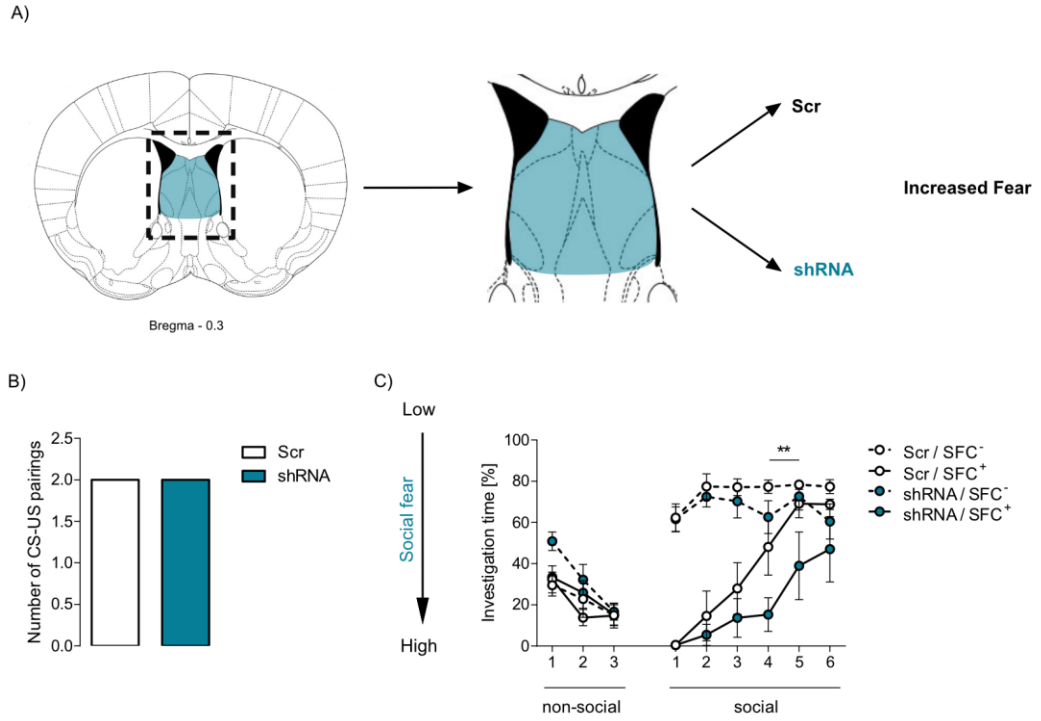


**Figure 17 - Effect of septal miR-132-3p downregulation within oxytocin receptor expressing neurons of the lateral septum of male mice on social fear.** A) Scheme of septal infusion of viral shRNA-mediated downregulation of pre-miR-132-3p (shRNA; 9.4 x $10^6$ GC) or scrambled control (Scr; 9.4 x $10^6$ GC). B) Number of CS-US pairings of Scr and shRNA-infused animals during acquisition of social fear. C) Percent of social investigation time during social fear extinction training (** $p \leq 0.01$ shRNA/SFC$^+$ vs Scr/SFC$^+$; data represent mean ± SEM; n = 6-10/group).

Statistics:

| | | |
|---|---|---|
| **Acquisition** (Mann Whitney U-test; two-tailed; figure 17 C) | | |
| | U = 18.00; n(shRNA/SFC$^+$) = n(Scr/SFC$^+$) = 6 | p > 0.9999 |
| ***Extinction*** *(Two way* ANOVA for repeated measures followed by LSD *post-hoc*; figure 17 D) | | |
| Factor interaction | Factor time | Factor treatment |
| F (24, 224) = 46.936; p < 0.0001** | F (8, 224) = 28.57; p < 0.0001** | F (3, 28) = 16.51; p < 0.0001** |
| Status shRNA/SFC$^+$ vs Scr/SFC$^+$ | Social stimulus 4 | p = 0.0051** |
| | Social stimulus 5 | p = 0.0098** |

### 3.2.6 miR-132-3p Target Gene Analysis

To decipher single target genes and down-stream pathways of miR-132-3p within the septum, an AGO-IP with subsequent microarray hybridization (Affymetrix Clariom mouse S) was performed. Therefore, the miR-132-3p inhibitor or scrambled LNA was infused into the LS of male mice and all AGO-bound target mRNAs were pulled for subsequent Microarray analysis 48 hrs post-infusion. In theory, only mRNAs, which are regulated by any miRNA are enriched after AGO2-IP when compared to the respective input samples. Moreover, mRNAs regulated by miR-132-3p are downregulated in miR-132-3p inhibitor samples after the AGO-IP, since the LNA inhibitor covalently binds to miR-132-3p containing AGO proteins, thereby hindering target mRNA binding.

The principal component analysis revealing the principle abundance variation of the calibrated, summarized data, unfortunately did not result in a clear clustering of the experimental groups. Nevertheless, 164 genes were significantly altered in their expression when comparing the miR-132-3p-inhibitor-treated samples with the scrambled controls (figure 18 A). Further clustering by use of a k-means algorithm revealed three clusters (1, 4, 10) including 44 regulated mRNAs, which follow the expected expression pattern (figure 18 B): They show enrichment in the scrambled control IP samples compared to the respective input sample and these mRNAs are downregulated in the inhibitor-treated samples in comparison to scrambled-treated mice. Additionally, 52 genes incorporated in the clusters 3, 5, 6, 7, and 8 show a downregulation after miR-132-3p inhibitor treatment compared to scrambled control, but no enrichment after scrambled IP compared to the input. Hence, these genes were also considered for the following pathway analysis.

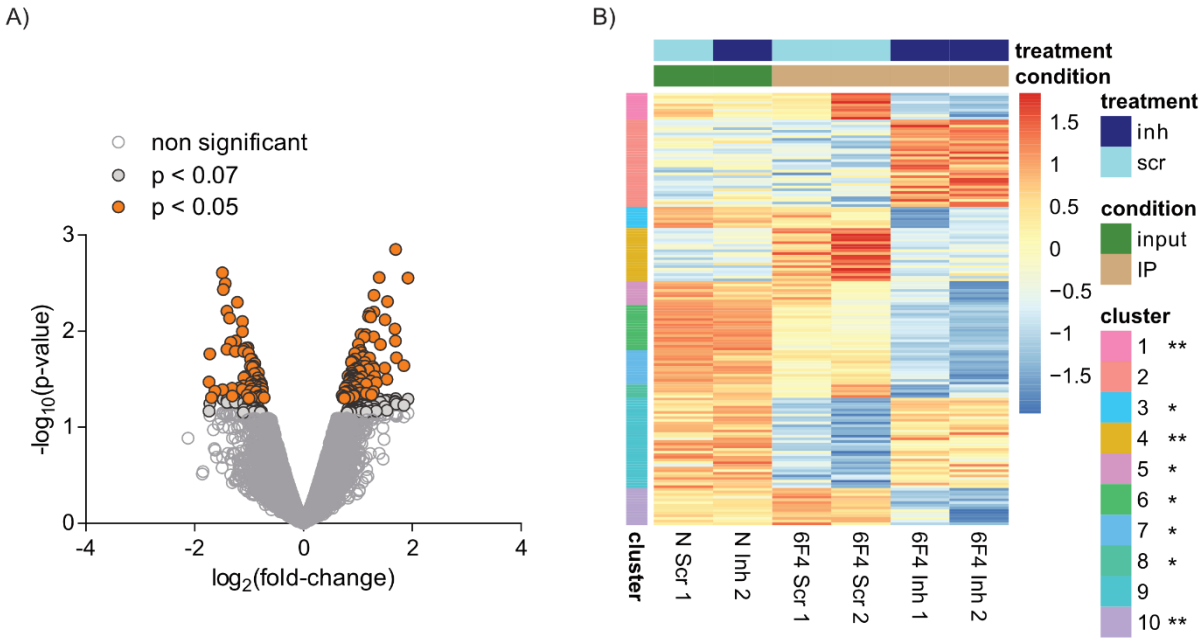


**Figure 18 – Microarray analysis after septal miR-132-3p inhibition in male mice and Argonaute-co-immunoprecipitation.** A) Volcano plot illustrating the fold-change in dependence of the p-value of all genes detected *via* microarray analysis after septal miR-132-3p inhibitor (Inh; 0.5 nmol) or scrambled control (Scr; 0.5 nmol) locked nucleic acid infusion. B) K-means clustering of significantly altered genes after microarray analysis of input (N) or immunoprecipitated (IP) samples of the Argonaute-co-immunoprecipitation (6F4) of septal Scr or Inh infused mouse tissue. Clusters marked with ** represent those three mRNA target clusters, wherein genes follow the expected expression pattern: enrichment in IP compared to N samples and downregulation in Inh-treated samples compared to Scr treatment, whereas * highlights clusters wherein mRNAs do not show an enrichment after IP, but a downregulation after septal Inh compared to Scr treatment (n = 1/N; n = 2/IP).

Pathway analysis by means of Enrichr (Chen et al., 2013; Kuleshov et al., 2016) and WikiPathways 2019 Mouse (Slenter et al., 2018) including all genes from clusters 1, 3, 4, 5, 6, 7, 8, and 10 indicated that the majority of regulated genes are GPCRs or components of the MeCP2 pathway, as well as factors involved in serotonin, interleukin, cytokine, EGFR, Wingless (Wnt), Notch, and MAPK signaling. The most interesting and promising mRNA candidates amongst the genes found regulated in this microarray were paternally expressed 3 (Peg3), peroxisome proliferator activated receptor gamma (Pparg), 5-hydroxytryptamine receptor 2C (Htr2C), disheveled segment polarity protein 3 (Dvl3), nuclear receptor coactivator 1 (Ncoa1), and roundabout guidance receptor 4 (Robo4). All these six mRNAs have been found to possess at least one miR-132-3p binding site by means of online target alignment tools (miRMap (Vejnar and Zdobnov, 2012) and miRWalk2.0 (Dweep and Gretz, 2015)). Regulation of septal transcript levels of all 6 putative miR-132-3p target mRNAs have been further analyzed 90 min after acquisition and extinction in male $SFC^-$ and $SFC^+$ mice (figure 19). Only Dvl3 expression was upregulated in $SFC^-$ mice 90 min post extinction when compared to post acquisition levels in $SFC^-$ mice ($p = 0.0155$). However, this modulation of septal Dvl3 transcript

levels does not follow the expected expression patterns of a target regulated by miR-132-3p. Unfortunately, none of the other evaluated target mRNAs showed a differential expression 90 min post acquisition and extinction of social fear, making further investigation on possible downstream pathways modulated by miR-132-3p and involved in expression of social fear inevitable.

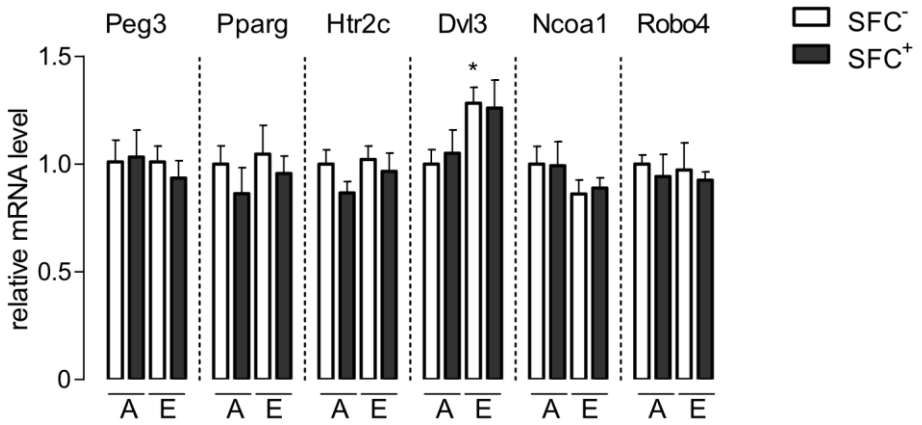


**Figure 19 – Analysis of putative miR-132-3p targets within the septum of male mice exposed to acquisition and extinction of social fear.** Paternally expressed 3 (Peg3), peroxisome proliferator activated receptor gamma (Pparg), 5-hydroxytryptamine receptor 2C (Htr2C), disheveled segment polarity protein 3 (Dvl3), nuclear receptor coactivator 1 (Ncoa1), and roundabout guidance receptor 4 (Robo4) transcript level 90 min after acquisition (A) and extinction (E) training in the septum of unconditioned ($SFC^-$) and conditioned ($SFC^+$) male mice (* $p \leq 0.05$ $SFC^-$/E vs $SFC^-$/A; data represent mean + SEM; n = 7-8/group).

Statistics:

| **mRNA** (Two way ANOVA followed by Bonferroni *post-hoc*; figure 19) | | | |
|---|---|---|---|
| | Factor interaction | Factor $SFC^+/SFC^-$ | Factor E/A |
| Peg3 | F (1, 26) = 0.2466; p = 0.6236 | F (1, 26) = 0.0705; p = 0.7926 | F (1, 26) = 0.0248; p = 0.6226 |
| Pparg | F (1, 27) = 0.0439; p = 0.8354 | F (1, 27) = 1.0850; p = 0.3068 | F (1, 27) = 0.4130; p = 0.5259 |
| Htr2c | F (1, 27) = 0.3301; p = 0.5704 | F (1, 27) = 1.9130; p = 0.1780 | F (1, 27) = 0.8036; p = 0.3780 |
| Dvl3 | F (1, 27) = 0.1325; p = 0.7187 | F (1, 27) = 0.0202; p = 0.8878 | F (1, 27) = 6.0480; p = 0.0206* |
| | Status: $SFC^-$/E vs $SFC^-$/A (separate student t-test; two-tailed) | | T (13) = 2.786; p = 0.0155* |
| Ncoa1 | F (1, 27) = 0.0458; p = 0.8321 | F (1, 27) = 0.0171; p = 0.8969 | F (1, 27) = 2.2800; p = 0.1426 |
| Robo4 | F (1, 27) = 0.0030; p = 0.9565 | F (1, 27) = 0.3488; p = 0.5597 | F (1, 27) = 0.0609; p = 0.8069 |

# Discussion

## 4.1 General Discussion

Over the last decades, the therapeutic potential of brain neuropeptides as treatment for psychopathologies, such as anxiety disorders, gained attraction (Mathew et al., 2008). As mentioned in the introductory section, the neuropeptide OXT has been extensively described for its pro social and anxiolytic properties. Recently, miRNAs have been extensively postulated to exert modulatory and/or regulatory functions on various behaviors. So far, there is only sparse knowledge about a bidirectional modulatory interaction between the OXT system and miRNAs: On one hand, application of synthetic OXT during labor modulates the expression of an unique subset of myometrial miRNAs (Cook et al., 2015), reinforcing OXT´s role in gene expression regulation during parturition. On the other hand, hypothalamic miR-24 has been shown to regulate OXT and control its transcript and peptide levels (Choi et al., 2013). Moreover, miR-21 is suggested to attenuate OXTR expression in the autism brain, unraveling another brain miRNA, which regulates factors of the OXT system (Mor et al., 2015). However, most of these studies are highly correlative and to date no causal links between the OXT system, regulation of miRNAs, and subsequent behavioral alterations are known. So far, no study postulated brain miRNAs that underlie the OXT signaling pathway in the modulation of anxiety, fear or sociability. Therefore, in my thesis, I aimed to advance the understanding of the regulatory function of miRNAs, especially miR-132-3p, in anxiety-related behavior and social fear in rodents.

First, I described that intra-PVN miR-132-3p expression is increased by icv application of synthetic OXT in rats (figure 8), whereas activation of the endogenous OXT system during lactation did not show similar expression patterns (figure 9). Nevertheless, pretreatment with a specific peptidergic OXTR-A abolished the observed increase of miR-132-3p levels in naïve rats and reduced miR-132-3p level in lactating rats, suggesting a contribution of OXTR-mediated signaling on miR-132-3p transcription within the PVN (figure 8-9). Further, my preliminary data pinpoint towards intra-PVN miR-132-3p as modulator of OXT-induced anxiolysis (figure 10). Second, transcript level assessment revealed miR-132-3p and miR-124-3p within the mouse LS to be regulated upon exposure to acquisition or extinction of social fear (figure 11). Functional analysis utilizing a miR-132-3p inhibitor, virus-mediated overexpression or cell specific knockdown highlighted this specific miRNA as essential regulator of OXT-mediated reversal of social fear (figure 12-17). Last but not least, target mRNAs were evaluated *via* microarray analysis (Figure 18) and verified in the SFC paradigm (figure 19). Taken together, my results suggest miR-132-3p within both, the PVN and LS as parts of

the limbic system, as crucial modulator of OXT-induced anxiolysis and social fear reversal. These results further highlight the brain OXT-miRNA system as potential target to decrypt the development of anxiety disorders.

In the following sections, I will discuss the major findings revealed in the two chapters of the present thesis individually.

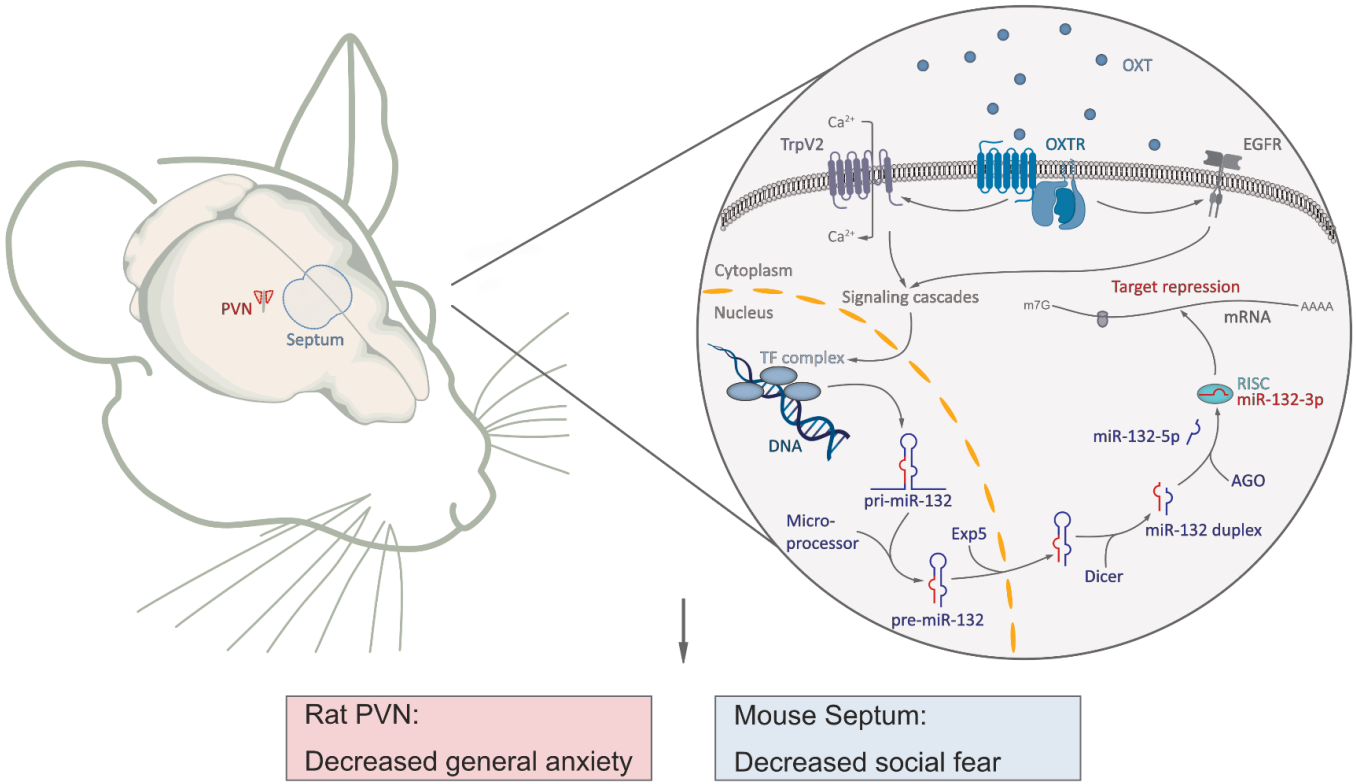


**Figure 20 – Graphical conclusion of the described behavioral effects of miR-132.** Upon activation of the oxytocin (OXT) receptor (OXTR) within the paraventricular nucleus (PVN) of male rats or septum of male mice, transient receptor potential cation channel subfamily V member 4 (TrpV2) channels are incorporated into the cellular membrane and epidermal growth factor receptors (EGFR) are transactivated. Both mechanisms activate numerous signaling cascades, which all converge on the cAMP responsive element binding protein (CREB)-CREB-regulated transcription coactivator (CTRC)/myocyte enhancer factor 2 (MEF-2) transcription factor (TF) complex. Binding of the TF complex to the miR-132/212 gene leads to transcriptional activation and further processing (microprocessor, Dicer) of the primary (pri-miR-132) and precursor (pre-miR-132) miRNAs. As result, miR-132-3p is loaded into the RNA-induced silencing complex (RISC), which contains an argonaute (AGO) protein, and leads to repression of still unknown target mRNAs. miR-132-3p within the rat PVN seems to be involved in OXT-mediated anxiolysis, whereas the same miRNA within the septum of mice mediates the social fear reversing properties of OXT (Exp5: exportin 5; essential for nuclear export).

## 4.2 Part I: "Is miR-132 within the PVN Involved in Oxytocin-induced Anxiolysis?"

To address this question, I assessed intra-PVN miRNA transcript levels in response to central OXT application 90 min as well as 3 hrs post icv infusion in male and female rats (figure 8). Icv OXT treatment lead to increased miR-132-3p within the PVN of male and female rats 3 hrs, but not 90 min (males only) after drug application. Moreover, no expression changes of miR-132-3p were observed in the amygdala or PFC 3 hrs post-infusion. Consistently, prior icv antagonism of the OXTR by pre-infusion of the OXTR-A in male rats prevented the OXT-mediated increase in miR-132-3p. Additionally, pharmacological OXTR blockade in lactating rats *via* the same pharmacological antagonism of the OXTR reduced miR-132-3p within the PVN compared to Veh-treated dams (figure 9).

miR-132/212 is known to be transcribed upon neuronal activation in a highly dynamic manner (Aten et al., 2019; Nudelman et al., 2010; Remenyi et al., 2013; Ronovsky et al., 2019; Shaltiel et al., 2013; Smalheiser et al., 2011) and basal neuronal miR-132 transcript levels are proposed to be stable with a steady turnover (Krol et al., 2010). Hence, the increase of miR-132-3p only 3 hrs, but not 90 min post OXT infusion is expected, although it remained unchanged in the analysis of the preceded miRNA Deep Sequencing. As mentioned above, binding of OXT to its neuronal receptors induces activation of $Ca^{2+}$-dependent intracellular signaling cascades, such as PKC, CaMK I, II, IV, and CaN, as well as the MAPK pathway (Jurek and Neumann, 2018). All these cascades converge on the CREB-CRTC/MEF-2 transcription factor complex resulting in transcriptional activation of target genes. Interestingly, the miR-132/212 promoter sequence features four CREB binding sites and one MEF-2A binding site (TRANSFAC 2019.1, geneXplain GmbH). Upon icv infusion of OXT, intracerebral diffusion, and binding to its receptors within the PVN, the CREB-CRTC/MEF-2 transcription factor complex is activated, can bind to its responsive elements within the miR-132/212 promoter sequence, and thereby trigger transcription of miR-132-3p. Deciphering which of the named signaling cascades downstream of OXTR activation causes the increase on intra-PVN miR-132-3p remains to be determined in future studies. Although miR-132 and miR-212 are members of the same family, which are encoded in the genome in close proximity, and share their promoter sequence, miR-212 did not show the same rise in its expression patterns as miR-132 in the rat PVN 3 hrs post OXT treatment. This discrepancy can be explained by either PTMs on miRNA biogenesis factors, leading to a shift in the preference to process only one of both primary and pre-miRNA transcripts, or co- and post-transcriptional regulation of the miRNA transcripts by RBPs, affecting processing and loading of the processed miRNA into RISC (Treiber et al., 2018).

The OXTR shares high sequence homology to the AVP V1 (~50 %) and V2 (40 %) receptors, leading to common structural features, which play important roles in ligand/receptor recognition and agonist selectivity (Gimpl and Fahrenholz, 2001). OXT binds the V1a receptor with high affinity, hence icv application of an OXTR-A prior to OXT infusions, revealed if the observed effect is specifically mediated by OXTR activation: This combinatorial treatment abolished the increase of miR-132-3p transcript level after OXT infusion, thereby assuring that the miRNA-regulatory effect of central OXT is mediated by signaling *via* the OXTR and most probably not *via* unselective cross-reaction with AVP receptors. For a further proof of concept, a selective OXTR agonist, i.e., TGOT (Manning et al., 2008), or a combinatorial treatment antagonizing AVP receptors prior to OXT application is essential.

I found miR-132-3p within the PVN to be increased in response to icv OXT treatment in male as well as female rats, revealing a gender unspecific regulation of neuronal miR-132-3p. To date, only a limited quantity of human studies focused on gender differences of miRNA expression in healthy or diseased states, revealing inconsistent or even opposite miRNA expression between males and females (Guo et al., 2016, 2017; Tsuji et al., 2017). Of course, miR-132-3p transcript levels within the PVN might differ between male and female rats. However, the increased miR-132-3p expression in response to icv OXT is present in both sexes. Thereby, analysis of putative gender differences remains a separate research topic to be studied in other experimental setups.

When further evaluating the effect of the endogenous OXT system on miRNAs within the PVN, I took advantage of the neuroendocrine changes during lactation, where the endogenous OXT system is highly upregulated (Insel, 1986; Jurek et al., 2012; Knobloch et al., 2012; Meddle et al., 2007; Neumann et al., 1993b; Slattery and Neumann, 2008). In contrast to synthetic icv OXT application in naïve male and female rats, lactating rats did not display altered miR-132-3p transcript levels within the PVN, but a short-term mild stressor (removal of the pups for 4 hrs) resulted in increased intra-PVN miR-132-3p transcript level compared to virgin controls, whereas long-term separation (24 hrs) of dams from their pups did not alter miR-132-3p (figure 9). These data are in concordance with four recent studies evaluating postpartum gene expression in the medial PFC (Eisinger et al., 2014), nucleus accumbens (Zhao et al., 2014), MPOA (Driessen et al., 2014), and LS (Eisinger et al., 2013) of lactating versus virgin rodents *via* microarray, which in a combinatorial analysis of all four data sets demonstrate no expression differences of miR-132 (Gammie et al., 2016). As miR-132 is a prominent non-coding RNA associated with stress coping behavior (Aten et al., 2019), the increased transcript levels are most probably a consequence of stress elicited by removal of the pups from the dams. Nevertheless, antagonizing the OXTR in lactating dams resulted in decreased miR-132-3p expression within the PVN when compared to Veh

infusion, thereby suggesting that miR-132-3p transcript levels within the PVN are at least to some extent dependent on OXTR activation. Importantly, OXTR mRNA levels within the PVN of virgin and lactating (independent of presence of pups) rats did not differ, proving equal initial prerequesites for miR-132 alterations upon receptor activation. During lactation, not only the brain OXT system is altered, but also numerous other important regulatory neuroendocrine pathways that are not always directly involved in maternal behavior or lactation are highly activated. Among those, for example prolactin acting on metabolic pathways, suppression of fertility, and reduction of the neuroendocrine stress response during lactation (Woodside, 2016), or OXT´s sister peptide AVP, which is highly involved in behavioral and molecular adaptations during lactation (Bayerl and Bosch, 2019), are of substantial relevance. Moreover, the hypothalamus, especially the PVN, is a highly plastic and essentially altered brain region during lactation (Averill, 1966). Due to these enormous neuroendocrine adaptations during lactation, numerous compensatory and OXT-contrary effects acting on miRNA expression within the PVN are conceivable, which might result in the unaltered, but stress-responsive miR-132-3p transcript expression within the PVN during lactation when compared to virgin females.

I found miR-132-3p levels to be altered within the CSF of female virgin and lactating rats in a similar manner compared to the respective intra-PVN miRNA analysis (figure 9): Removing the pups 4 hrs prior to euthanasia resulted in increased CSF miR-132-3p in the dams compared to virgins, whereas undisturbed lactating rats had CSF miR-132-3p levels comparable to virgins. Here, removal of the pups 24 hrs prior to euthanasia has not been analyzed. Within body fluids, such as CSF and blood, miRNAs have been found to be present and stable when bound to AGO or high density proteins or when packed into exosomes (Chen et al., 2012; Mathew et al., 2016; Tonge and Gant, 2016; Turchinovich et al., 2012). In the case of miR-132, it has been reported that it is transferred from neurons to endothelial cells *via* secretion of miRNA-containing exosomes to finally maintain brain vascular integrity (Xu et al., 2017). Therefore, the increased miR-132-3p levels in the CSF of lactating rats might originate from secretory neurons of the PVN, where miR-132-3p transcript levels were found to be increased as well. The question, whether the released miR-132-3p-containing exosomes travel to peripheral organs *via* the blood, cannot be answered with the data obtained in this thesis. Therefore, future studies should also focus on plasma miR-132-3p levels to evaluate whether exosomal miR-132-3p targets central and/or peripheral cells. Especially miR-132-3p has been found to regulate steroidogenesis in the ovary, testis, and adrenal gland (Hu et al., 2013, 2017). The found increase in CSF miR-132-3p levels 4 hrs after removal of the pups from the lactating rat could be a neurosecretory communication route between the brain and peripheral organs, such as the adrenals, to regulate the physiological responses to the stressful separation

from the pups. miRNAs are suggested to be essential epigenetic regulators that are transferred from the mother to the offspring *via* the breast milk to orchestrate early programing of the infant (Melnik and Schmitz, 2017), thereby providing another peripheral target for increased neuronal release of miR-132-3p into the CSF of rats. Furthermore, isolation of exosomal RNAs is discussed as critical step, since various isolation methods differ substantially in the obtained RNA yields and RNA size distributions (Eldh et al., 2012; Tang et al., 2017). Therefore, the selected method using the miRCURY™ RNA Isolation Kit for biofluids might not result in an optimal quantification of miR-132-3p levels within the CSF of rats. However, the observed expression patters of miR-132-3p within the CSF are equal to those within the PVN, suggesting that the miRNA isolation method from CSF might be reliable. Since the reason for exosome release as well as the destination for the released exosomes is not analyzed in this thesis, and the selected exosome isolation method might not be optimal, these results have to be interpreted with caution.

For final functional analysis of miR-132-3p in the view of anxiety-related behavior and cued fear, a LNA inhibiting miR-132-3p was infused into the PVN 48 hrs prior to assessment of the anxiolytic and fear-impacting property of local intra-PVN OXT infusions (figure 10; only preliminary data). As expected, local OXT leads to a reduction in anxiety-related behavior assessed on the LDB (Jurek et al., 2012; Martinetz et al., 2019), whereas previous inhibition of miR-132-3p function within the PVN tended to prevent the OXT-mediated anxiolysis. Similarly, intra-PVN OXT infusion seemed to decrease the latency to enter the open arm of the EPM, an effect which was prohibited by prior miR-132-3p inhibition within the PVN. Although in both tests for anxiety-related behavior, namely LDB and EPM, no clear anxiolytic effect after PVN-OXT infusion was found, miR-132-3p seems to be a promising candidate involved in the anxiolytic properties of OXT signaling within the PVN. During assessment of cued fear, results contrastingly to what has been found previously (Toth et al., 2012a) were found. Icv infusions of OXT prior to extinction of cued fear are known to increase the freezing response upon CS presentations during extinction training. Unfortunately, intra-PVN application of OXT compared to Veh treatment in rats pre-infused with a scrambled control or miR-132-3p inhibitor LNA, resulted in facilitation of cued fear extinction. Moreover, intra-PVN inhibition of miR-132-3p prior to acquisition of cued fear facilitated extinction of cued fear. This result is in contrast to the effects found after septal miR-132-3p inhibition prior to SFC in male mice. Thereby, miR-132-3p within the rat PVN might modulate reduction of cued fear, whereas the same miRNA within the mouse septum seems to increase social fear. This differential acquisition and extinction of traumatic experiences in a social versus non-social context could originate from the contrary role of the OXT system in fear, although the brain regions mediating the effect are quite similar (Toth et al., 2012a). OXT neurotransmission within the LS might therefore be involved in development

and/or neural support of social fear, whereas in the case of cued fear, the effects of OXT are time- and region-dependent. However, the specific role of miRNAs, especially miR-132-3p, remains elusive. Taken together, OXT infusion prior to extinction of social fear might strengthen extinction memory consolidation, but further investigation is needed, since no explicit effect of intra-PVN miR-132-3p inhibition on OXT-mediated impairment of cued fear was found. However, the statistical power of this behavioral assessment is yet not high enough to give a definite conclusion, but a clear tendency of intra-PVN miR-132-3p inhibition preventing OXT-mediated reduction of general anxiety-related behavior is visible. Especially miR-132 has been repeatedly associated with the modulation of anxiety-related behavior (Aten et al., 2019; Hernandez-Rapp et al., 2015; Kumari et al., 2016; Malan-Müller et al., 2013). However, both, infra- and supra-physiological levels of miR-132, have been found to cause an anxiogenic phenotype, as shown by studies in knockout mice, as well as mice overexpressing miR-132 (Aten et al., 2019). However, in rats no such miRNA manipulating studies are known yet. Although only preliminary, this thesis is the first study to describe a functional effect of local miR-132-3p inhibition within the PVN on general anxiety-related behavior and cued fear extinction in male rats. As discussed in the introductory section, miR-132-3p is highly involved in neuronal plasticity by promoting dendrite and spine outgrowth (Impey et al., 2010; Wayman et al., 2008). *In vitro* application of the neuropeptide OXT have controversially been found to result in either outgrowth or retraction of neurons, which is suggested to be dependent on the cell type, species, and sex of the cell donor (Meyer et al., 2018). According to the observation of increased miR-132-3p post OXT infusion, neuronal plasticity should be enhanced, which is contradictory to the majority of the mentioned published *in vitro* studies, but in line with the observation that LTP, which is also evoked by OXTR signaling (Froemke and Carcea, 2017), is instrumental for anxiolysis (Glangetas et al., 2017). Therefore, miR-132-3p underlying OXTR activation might lead to increased synaptic plasticity and thereby to the observed anxiolytic properties of OXT *in vivo*. Anyhow, prospective experiments are indispensable to understand the involvement of miR-132-3p in OXT-mediated neuroplasticity alterations and the resulting anxiolysis in detail.

Taken together, miRNAs, especially miR-132-3p, represent powerful novel players in the context of deciphering the molecular underpinnings of OXT-mediated anxiolysis. Herein, I demonstrated that miR-132-3p is reliably and sex-independently altered within the PVN upon central OXTR activation in a time-dependent manner, whereas chronic activation of the OXT system during lactation does not influence transcription levels. These results further indicate that intra-PVN miR-132-3p underlies the OXT-induced anxiolysis and shed further light on the promising possibility to use miRNA manipulation as eminent treatment alternative for anxiety disorders.

## 4.3 Part II: "Does miR-132 Mediate the Oxytocin-induced Reversal of Social Fear?"

miRNAs have repeatedly been revealed as pivotal modulators of memory-related processes and socioemotional behavior. As miRNAs related to regulation of brain plasticity (Olde Loohuis et al., 2012) and based on the studies described in part I of the present thesis, especially miR-132 and miR-124 are promising non-coding RNA candidates to investigate the molecular underpinnings of the social fear reversing effect of OXT in the LS (Menon et al., 2018; Zoicas et al., 2014). A targeted approach to evaluate altered miRNA transcript levels within the LS in a time-dependent manner, indicated miR-132-3p and miR-124-3p as interesting candidates to be functionally involved in acquisition, consolidation, or extinction of social fear (figure 11): Both miRNAs were temporally increased in $SFC^+$ mice 90 min after acquisition compared to unconditioned animals, revealing a putative function in acquisition or consolidation of social fear. At later post-acquisition time points, as well as 90 min after extinction, this effect nullified. Interestingly, $SFC^-$ mice displayed higher septal miR-124-3p 90 min post extinction compared to the respective post-acquisition analysis, suggesting that either repeated social contact during extinction training and/or acquisition or consolidation of social fear extinction memory lead to increased transcription and processing of miR-124-3p. Unfortunately, repeated social contact 24 hrs after acquisition of social fear did not induce increased levels of miR-124-3p and miR-132-3p within the mouse LS of conditioned compared to unconditioned mice, excluding the social component of the SFC as cause for miRNA transcript changes and suggesting the involvement of both miRNAs in social fear memory consolidation. In a frontotemporal dementia mouse model, miR-124-3p was recently revealed to promote social behavior *via* reducing GluA2, a $Ca^{2+}$-impermeable AMPA receptor subunit, in the PFC (Arrant and Roberson, 2014; Gascon et al., 2014). This fascinating observation that a single miRNA is able to affect complex behavior, such as sociability, further strengthens the suggestion of miR-124-3p acting as pro-social non-coding RNA in the case of social fear extinction. In comparison to conditioned mice, social fear extinction training in unconditioned mice results in increased OXT release within the LS (Zoicas et al., 2014). Thereby, local OXTR activation of within the LS might be the cause of increased miR-132-3p and miR-124-3p expression. To investigate this putative causal link, I further analyzed whether transcript levels of both miRNAs are regulated in response to icv OXT infusion. Unexpectedly, central OXT application neither increased miR-132-3p, nor miR-124-3p 90 min, as well as 3 hrs post-infusion. These results are contrary to the rat studies described in part I of the present thesis. However, a possible explanation for this discrepancy is the faster metabolism of mice compared to rats and the infusion site. Since the mouse LS is closer to the icv infusion site than the rat PVN, the intracellular response to OXT binding its receptor thereby regulating miRNA expression might be shifted in its time dynamics and thereby cause differential

results concerning the dynamics of miRNA transcription like the ones obtained in the rat PVN. In any case, this miRNA transcript analysis clearly suggests miR-132-3p and miR-124-3p within the LS as important modulators of acquisition or consolidation of social fear.

For the further functional analysis, I focused on miR-132-3p only. Manipulation of septal miR-132-3p availability *via* infusion of a LNA 48 hrs prior to acquisition (figure 12) or AAV-mediated infection 3 weeks prior to acquisition (figure 14) of social fear did not affect the number of CS-US pairings during acquisition of social fear, but extinction of social fear in conditioned mice: On the one hand, the process of social fear extinction was impaired when functionally inhibiting miR-132-3p within the septum, on the other hand, septal miR-132-3p overexpression facilitated extinction of social fear. Both treatments did not affect general anxiety-related behavior of locomotor activity (figure 13, 15). Although miR-132-3p transcript levels were not altered at all three analyzed time points after extinction of social fear (figure 11), the increased expression 90 min post acquisition of social fear might lead to long-lasting effects on social fear behavior. This phenomenon might be mediated by the impact of miR-132-3p on neuronal plasticity: miR-132-3p mediated post-transcriptional gene regulation results in synaptic outgrowth and increased dendrite length, branching, and spine density as well as width (Olde Loohuis et al., 2012), thereby strengthening synaptic transmission (Edbauer et al., 2010; Impey et al., 2010) and enhancing LTP (Ronovsky et al., 2019). LTP as part of the Hebbian plasticity, has been extensively studied as cellular basis for learning and memory by rapid modification of the efficacy of individual synapses in an input-specific manner (Collingridge et al., 2004; Feldman, 2009; Malenka and Bear, 2004; Neves et al., 2008; Sjöström et al., 2008). Successful and long-lasting memory of aversive associative memories, especially in the case of social events, is of particular importance to protect the organism from future harm by the same or equal situations. miR-132-3p inhibition within the LS might lead to dysfunctions in social learning and memory, as already described for non-social events (Hansen et al., 2016; Ronovsky et al., 2019), thereby causing the impairment in social fear extinction by altering LTP and synaptic plasticity during the memory consolidation phase. Whereas overexpression of septal miR-132-3p might cause enhanced LTP and neuronal plasticity, resulting in facilitated social learning during the social fear extinction memory consolidation phase. Nevertheless, miR-132-3p inhibition did not lead to ineffective extinction of social fear, since no significant differences between the LNA treatments were seen during short-term extinction recall. Although conditioned mice display lower social investigation times during extinction of social fear, which is basically a repeated exposure to social stimuli, the social contact might act as rewarding stimulus in rodents and humans (Krach et al., 2010; Trezza et al., 2011), thereby forming new appetitive memory. Generally, acquired appetitive and aversive memories are independently stored to allow the individual to respond to

environmental changes in a flexible and sensitive manner (Nasser and McNally, 2013). Hence, mammals are suggested to possess an appetitive motivational system, which mediates approach and reward, as well as an aversive motivational system mediating defensive strategies and fear (Amsel, 1962; Rescorla and Solomon, 1967). Although plenty of knowledge is already obtained about the neural substrates of both systems separately (Johansen et al., 2011; Maren and Quirk, 2004; Pickens and Holland, 2004; Rodrigues et al., 2004), only sparse information about their interaction exists (Nasser and McNally, 2013). From the results obtained in this thesis, no definite link between miR-132-3p mediated gene regulation and consolidation of aversive and appetitive learning and memory is possible. To establish such a relation, experiments assessing the long-term consequences of miR-132-3p inhibition and overexpression on social fear extinction learning and extinction memory consolidation are inevitable. Except for social learning as affected function, septal miR-132-3p might also be involved in anxiety-related behavior, which will in turn impact expression of fear. Importantly, neither general locomotor activity, nor general anxiety-related behavior were altered in response to septal miR-132-3p inhibition (figure 13) or overexpression (figure 15), further excluding an effect of miR-132-3p on anxiety-related behavior and confirming a specific effect of septal miR-132-3p manipulation on social learning.

To further assess, which neuronal subpopulation within the septum is responsible for the functional effect of miR-132-3p manipulation on social fear extinction, a pharmacological and Cre recombinase-dependent approach was conducted. Septal miR-132-3p inhibition prior to local OXT application into the LS prevented the expected OXT-mediated reversal of social fear during extinction training (Zoicas et al., 2014), without affecting acquisition and recall of social fear (figure 16). Moreover, shRNA-mediated knockdown of pre-miR-132, thereby also of the mature miRNA, within septal OXTR expressing neurons impaired extinction of social fear without affecting acquisition of social fear (figure 17). Both methods individually confirm that miR-132-3p is a downstream effector of OXTR activation, which is indispensable for the social fear reversing effect of OXT. OXTergic projections to the septum of mice originate in the PVN and SON and release OXT within the LS in response to social stimuli (Menon et al., 2018). Within the mouse septum, OXTRs are located in the MS and ventral LS (Gould and Zingg, 2003; Jurek and Neumann, 2018). Although I targeted to infuse OXT into the dorsal LS, the applied synthetic OXT easily reaches its receptors in the ventral part by diffusion. As mentioned in the discussion of part I, OXTR signaling activates the CREB-CRTC/MEF-2 transcription factor complex, which has its responsive elements present within the promoter of miR-132/212, and might be the putative mechanism to transcriptionally activate miR-132-3p. Ultimately, the social fear reversing effect of OXT was prevented *via* miRNA-mediated target mRNA inhibition. Thus, my results reveal that neurons expressing the OXTR are responsible

for the functional involvement of miR-132-3p in social fear extinction. However, further characterization is warranted to decrypt the molecular pathways in detail.

Substituting the studies on intra-PVN miR-132-3p on anxiety-related behavior in rats (part I of the thesis) with experiments focusing on the involvement of septal miR-132-3p in social fear behavior in mice was based on the fact that the SFC paradigm is established in male and female mice (Menon et al., 2018; Toth et al., 2012b), but not rats. Social memory in mice is superior to that of rats: recognition memory in rats wanes after 30 to 60 min, whereas in mice it lasts for days to weeks (Engelmann, 2009; Engelmann and Landgraf, 1994; Kogan et al., 2000; van der Kooij and Sandi, 2012; Letty et al., 1997; Toth et al., 2012b). This disparity is suggested to be mediated by differences in the olfactory systems between species, in which neurons are less activated in rats compared to mice in response to odor exposure (Noack et al., 2010). Due to the inferior social memory of rats, the analysis of miR-132-3p in social fear was studied in mice only. The rearrangement from rodent anxiety-related behavior to social fear made it further necessary to study the septum in lieu of the PVN, since it is highly implicated in OXT-mediated reversal of social fear (Menon et al., 2018; Zoicas et al., 2014), whereas the PVN accounts for OXT-induced anxiolysis (Blume et al., 2008; van den Burg et al., 2015; Jurek et al., 2012; Martinetz et al., 2019). However, manipulation of miR-132-3p levels and function within the mouse septum and rat PVN revealed that miR-132-3p is involved in both, OXT-mediated reversal of social fear and OXT-induced anxiolysis, respectively. However, the downstream mRNA targets and affected pathways might not be comparable in both species and therefore need further detailed and separate investigation.

Due to its numerous downstream projections, the LS is an important regulatory region of emotional responses including fear-related behaviors. For example, it is known to be highly activated in response to aversive and stressful stimuli, such as forced swimming, immobilization stress, and fear conditioning (Beck and Fibiger, 1995; Pezzone et al., 1992; Singewald et al., 2003). Moreover, the LS is a well-established brain region accounting for the regulation of social behavior. It is directly connected to hippocampal sub regions, such as CA2, which controls social memory (Hitti and Siegelbaum, 2014), CA3 regulating contextual fear (Besnard et al., 2019), or to the anterior hypothalamus to control the stress response (Anthony et al., 2014). Neuroanatomical assessment of the LS showed that most of the OXTR-expressing neurons are GABAergic in nature (Menon et al., 2018) and are suggested to directly or indirectly, e.g., *via* the diagonal band of Broca, project to numerous brain regions such as amygdala or ventral hippocampus, which orchestrate the processing and expression of social fear. However, the precise functional involvement of miR-132-3p in modulation of its downstream targets within these neuroanatomical circuits needs further investigation.

The potential mRNA targets of miR-132-3p within the LS, which were obtained by AGO-IP with subsequent microarray analysis (figure 18), revealed numerous genes involved in the MeCP2 pathway, as well as factors involved in serotonin, interleukin, cytokine, EGFR, Wnt, Notch, and MAPK signaling. Unfortunately, none of the well-established miR-132-3p targets, such as the phosphatase and tensin homolog Pten (Aten et al., 2019; Ronovsky et al., 2019; Wong et al., 2013), MeCP2 (Bijkerk et al., 2018; Hernandez-Rapp et al., 2015; Klein et al., 2007; Lyu et al., 2016; Su et al., 2015), the rho GTPase activating protein 32 p250GAP (Yuan et al., 2016), AGO2 (Leonov et al., 2015), and SIRT1 (Aten et al., 2018, 2019) were found to be regulated in the present microarray, suggesting one or a network of different, still unknown target mRNAs of miR-132-3p, which might be responsible for its functional effect on social fear behavior within the mouse septum. Understandably, the regulation and functional consequence of miR-132-3p target genes needs further in vivo validation on a molecular and behavioral level. Validation of septal mRNA level of six selected putative miR-132-3p targets (Peg3, Pparg, Htr2C, Dvl3, Ncoa1, and Robo4) did not reveal any alterations, therefore further analysis is inevitable (figure 19). Since miRNAs regulate gene expression on a posttranscriptional level, especially *via* affecting translation efficiency, evaluation of the protein levels of these possible targets, as well as miRNA-target mRNA interaction analysis *via* luciferase assays is necessary. Moreover, technical discrepancies have to be taken into account: The selected microarray is proven to quantify all transcript variants of the gene, whereas the majority of primers used for *in vivo* target validation did not detect all transcript variants. Hence, mRNAs found as putative miR-132-3p targets *via* microarray analysis might not be revealed as targets in mice exposed to SFC. In the following paragraphs, possible signaling pathways and behavioral function of Peg3, Pparg, Htr2c, Dvl3, Ncoa1, and Robo4 will be summarized.

One interesting target that I found to be upregulated upon septal miR-132-3p inhibition, is the neuronal Peg3, which is downregulated in response to miR-132-3p inhibition. Recently, the DNA-binding protein Peg3 has been shown to directly bind within the third exon of the OXTR gene, thereby repressing OXTR expression in mammary epithelial cells and the hypothalamus (Frey et al., 2018). Contrastingly, female Peg3 knockout mice show lower OXTR binding within the MPOA and LS (Champagne et al., 2009). Behavioral relevance of Peg3 is mainly characterizes with regards to social behavior: Mutation in Peg3 in female mice causes severe maternal care impairments (Curley et al., 2004; Frey and Kim, 2015; Li et al., 1999), increased maternal aggression (Champagne et al., 2009), and lower exploratory drive, whereas male knockout mice fail to respond normally to sexually receptive females (Swaney et al., 2008). Interestingly, wild type dams also present impairments in maternal care and increased anxiety-related behavior, when cross fostering Peg3 knockout pups (McNamara et al., 2018). This maternal deficit phenotype is suggested to be driven

by the reduced ultrasonic vocalizations elicited by the Peg3 knockout offspring. However, 48 hrs post acquisition of social fear, OXTR binding within the dorsal LS is increased in conditioned compared to unconditioned male mice (Zoicas et al., 2014), an effect that might relay on miR-132-3p mediated transcriptional repression of Peg3. Recently, a circRNA originating from the Peg3 locus, circPeg3, has been identified (Perera et al., 2018), suggesting a self-regulatory negative feedback loop: miR-132-3p is suppressing Peg3 expression to minimize the sponging effect of circPeg3 on the miRNA. Since the Affymetrix Clariom S mouse microarray detects only exon regions of mRNAs and thereby does not provide any information about transcript variants, circPeg3 might be amongst those detected. However, discussing the regulatory feedback of miR-132 and circPeg3 in the light of SFC clearly remains highly speculative.

One important signaling pathway pinpointed by analysis of the microarray is the Wnt/β-catenin pathway. Generally, this pathway has already been investigated in the context of fear, where it is known to be essential for amygdala- and hippocampus-dependent learning and memory (Maguschak and Ressler, 2011; Xu et al., 2015). Dvl3, which is suggested as hub of the Wnt signaling pathway (Gao and Chen, 2010), was found regulated in the AGO-IP microarray analysis. It is highly expressed in neurons, where it is involved in prenatal regulation of β-catenin and thereby modulates adult social as well as stereotypic behavior (Belinson et al., 2016). Supporting the significance of Dvl3 on social behavior, post social defeat stress, Dvl3 is downregulated in the nucleus accumbens of mice and pharmacological blockade of Dvl3 function renders mice more susceptible to social defeat stress (Wilkinson et al., 2011). Moreover, Pparg, which is highly expressed in neurons and microglia, was detected as promising target gene. The Pparg pathway is known to converge with MAPK-ERK signaling and its agonism enhances cognitive capacities in autism (Denner et al., 2012) and improves contextual fear conditioning in rats (Gemma et al., 2004). Most interestingly, Pparg agonism is known to inhibit the Wnt/β-catenin pathway (Vallée et al., 2019), again portending to an involvement of miR-132-3p mediated regulation of the Wnt/β-catenin signaling activity for the modulation of social fear learning and memory, which needs further investigation.

The serotonin system is well-characterized to modulate Pavlovian fear conditioning, fear expression, and fear extinction (Bauer, 2015). One of the serotonin receptors, Htr2C, which is exclusively expressed in CNS neurons and famous for its effects on satiety and sexuality, was revealed as putative miR-132-3p target in the septum of male mice. Knockout of Htr2C causes sociobehavioral deficits (Séjourné et al., 2015) and increased anxiety-related behavior (Heisler et al., 2007a) in mice. Furthermore, pharmacological activation of Htr2C within the amygdala increases anxiety-related behavior in rats (Campbell and Merchant, 2003; de Mello Cruz et al.,

2005). In humans, SNPs within the Htr2C gene are associated with anxiety disorders, depression, and dysregulated stress responses (Avery and Vrshek-Schallhorn, 2016; Grzesiak et al., 2017). Expression of Htr2C from its encoding gene is regulated by circadian signals and the HPA axis (Holmes et al., 1995, 1997) and conversely, Htr2C activates the HPA axis by neuronal depolarization since it is co-expressed on CRH neurons (Heisler et al., 2007b). Exposure to foot schocks, i.e., during acquisition of Pavlovian conditioning, results in a strong activation of the HPA axis (Daviu et al., 2014). Whether there transcriptional alterations are mediated by miR-132-3p remains elusive, but all these physiological and behavioral functions of Htr2C make it a promising candidate for future verification.

Ncoa1, which is highly expressed in neurons and astrocytes, was found as miR-132-3p target *via* the microarray analysis. Ncoa1 is a coactivator, which directly binds to MRs and GRs, thereby modulating the function of proopoiomelanocortin neurons (Yang et al., 2019). Knockout of Ncoa1 results in an attenuated HPA axis , especially CRF, response to chronic, but not acute stressor exposure and glucocorticoid application (Lachize et al., 2009). Moreover, decreased anxiety-related behavior in novel environments, increased exploratory behavior, and deficits in sensorimotor gating compared to wildtype littermates were observed (Stashi et al., 2013). Interestingly, Ncoa1 is able to bind Pparg independent of the presence of a ligand, and coexpression of both factors increased the transcriptional activity of Pparg, revealing that Ncoa1 influences not only the HPA axis, but also the Pparg-mediated signaling pathway (Zhu et al., 1996). miR-132-3p mediated repression of Ncoa1 might thereby dampen the HPA axis response and interfere with the Wnt/β-catenin signaling activity in the LS of mice, a regulatory effect that needs further investigation.

Last, but not least, Robo4, which is mainly expressed in brain endothelial cells, was found to be regulated *via* microarray analysis. Only sparse knowledge about the functional relevance of Robo4 is present: SNPs in Robo4 have been associated with ASD and reflect the social interaction of these patients, suggesting a possible contribution of Robo4 in the pathogenesis of autism (Anitha et al., 2008).

All target mRNAs obtained from the described microarray only showed slight fold-change differences in their expression, and the statistical analysis is only based on the p-value, not on the statistically more powerful adjusted p-value of the multiple comparisons. The abovementioned target mRNAs and pathways arose from different clusters: Either they were enriched over input and downregulated upon miR-132-3p inhibition, or the chosen mRNAs did not show enrichment over input, but were downregulated after septal miR-132-3p inhibition. Surely, the first option is more stringent compared to the second, however, due to the extremely low sample number and

minor differences of transcript levels, both cluster types were taken into account for the analysis. However, this indicates that these results have to be interpreted with caution.

In summary, this is the first study revealing that the neuropeptide OXT regulates one distinct miRNA within the LS and, thereby, affects complex socio-emotional behavior, such as social fear, by repression of one or a network of still unknown target mRNAs.

# Perspectives and Future Directions

All findings described within this thesis lead to several open research questions and hypotheses. Further studies focusing on these, will result in an advanced understanding of the etiology of anxiety disorders and the molecular underpinnings of the anxiolytic and pro-social properties of OXT. Within the discussion of this thesis, several of these hypotheses were mentioned. In the following paragraphs, the 6 major remaining questions are discussed briefly.

***"Which miR-132-3p repressed targets are involved in the anxiolytic effect of OXT?"***

Although only preliminary, I revealed that miR-132-3p inhibition within the PVN might prevent the OXT-induced anxiolysis in male rats. Surely, the experiment needs to be repeated to increase the statistical power. Nevertheless, future investigation should further focus on evaluation of miR-132-3p targets, which might be involved in the anxiolytic effect. These studies will lead to advanced knowledge of the molecular underpinnings of OXT-mediated anxiolysis; knowledge, which is inevitable to develop novel neuropeptide based treatment strategies for GAD.

***"Which transcription factor activated by OXTR signaling induces miR-132-3p expression?"***

I found miR-132-3p to be upregulated within the PVN in response to icv OXT treatment (90 min post infusion) independent of the gender of the rats, whereas icv OXTR antagonism prior to OXT treatment abolished the transcriptional activation of miR-132-3p within the rat PVN. Moreover, miR-132-3p inhibition within the mouse septum prevents the expected OXT-mediated reversal of social fear (Zoicas et al., 2014). Both results suggest that miR-132-3p functions downstream of OXTR activation, however, a causal link has not been established so far. Since all intracellular signaling cascades activated by OXTR signaling converge on the CREB-CRTC/MEF2 transcription factor complex (Jurek and Neumann, 2018) and miR-132 possesses the respective binding sites in its promoter sequence, this transcription factor complex might mediate the activating effect of OXTR signaling on miR-132 transcription. To assess whether CREB, MEF2A, or a combination of both transcription factors is mediating this intracellular effect of OXT, a luciferase assay for the respective responsive elements of the miR-132 promoter can be performed.

***"Does septal miR-132 functionally affect social learning and memory or social fear expression?"***

In the present thesis, I found that septal miR-132-3p is essential for adequate social investigation behavior during social fear extinction, since its inhibition impaired extinction, whereas its overexpression facilitated extinction of social fear. Moreover, inhibiting septal miR-132-3p prevents the OXT-mediated reversal of social fear and preliminary data suggests that shRNA-mediated knockdown of pre-miR-132 in OXTR-expressing neurons impairs extinction of social fear. All these data reveal that miR-132-3p either functionally affects learning and memory of social events, or that it is crucial for adequate social fear extinction behavior *per se*. So far, miR-132 has been found to impair non-social learning and memory (Hansen et al., 2016) and memory acquisition (Wang et al., 2013). Clearly distinguishing learning and memory from fear behavior is in general a difficult neuroscientific task. Effects of septal miR-132-3p manipulation on social learning and memory can be behaviorally examined by short-term and long-term social recognition memory tests (Jacobs et al., 2016) or social discrimination tests (Engelmann et al., 1995), whereas general learning and memory capacities can be measured by tests such as the Morris water maze (Morris, 1984), Barnes maze (Barnes, 1979), object recognition task (Leger et al., 2013), or cued and contextual fear conditioning (Kim and Jung, 2006). Although neither septal miR-124-3p, nor miR-132-3p were altered in unconditioned animals compared to context control or random shock control mice at all time points, a prior training phase might be an important trigger for miRNA transcription in response to exposure to conspecifics. When comparing the effects of septal miR-132-3p manipulation on cued or contextual fear conditioning with its consequences on the abovementioned mazes or the object recognition task, important additional information about the valence of training prior to the behavioral readout will be given. If manipulation of septal miR-132-3p levels alters behavioral phenotypes in those memory-related tasks, the effect seen on social investigation time during social fear extinction is quite likely based on alterations in learning and memory rather than fear extinction. Additionally, inhibition of septal miR-132-3p after successful acquisition, specifically 48 hrs prior to extinction of social fear, will help in deciphering if miR-132-3p is involved in either strengthening of social fear acquisition memory or extinction of social fear.

***"Does miR-132 alter neuronal plasticity and thereby cause the observed behavioral effects?"***

miR-132 is well-characterized as plasticity-regulating miRNA (Aksoy-Aksel et al., 2014; Olde Loohuis et al., 2012). The effect of septal miR-132-3p manipulation on social fear-related behavior I revealed in this thesis might be mediated by miR-132-3p´s effects on neuronal plasticity. Therefore, the

evaluation of neuronal outgrowth and dendritic branching in the LS of unconditioned and socially fear conditioned mice, as well as in OXTR-expressing neurons is inevitable to gain knowledge about a potential plasticity-altering effect of miR-132-3p, which is induced by acquisition of social fear. Moreover, the molecular profiles of septal OXTR expressing neurons can be evaluated *via* infusion of a viral translating ribosome affinity purification (vTRAP) into the septum of OXTR-Cre mice and subsequent isolation and characterization of the OXTR positive neuronal subpopulation (Nectow et al., 2017). Neuronal plasticity is dynamic, but can also be persistent over time. Hence, a traumatic experience, such as the foot shock during acquisition of social fear, might lead to persistent alterations of neuronal plasticity, which might be mediated by miR-132-3p. In addition to the standard SFC procedure, which includes extinction 24 hrs post acquisition as well as extinction recall 24 hrs post extinction, later social fear extinction time points should be considered as well to potentially decipher a mechanism leading to persistence of social fear for at least 14 days post acquisition (Toth et al., 2012b).

***"Which targets of miR-132 are responsible for the social fear extinction facilitating effect?"***

The present data of miR-132-3p regulated targets by AGO-IP with subsequent microarray analysis revealed several promising candidate genes for further analysis. After assessment of their mRNA and protein levels within the LS of unconditioned and conditioned mice, pharmacological or viral inhibition or knockdown and activation or overexpression prior to extinction of social fear might result in the expected behavioral effects. Based on the growing interest in the development of novel therapeutic strategies, which are specific for SAD, extensive knowledge about the molecular and behavioral consequences of brain neuropeptide signaling modulating emotional responses is essential.

***"What is the functional involvement of septal miR-124 in social fear?"***

I demonstrated that miR-124-3p is upregulated in conditioned compared to unconditioned mice 90 min post acquisition, but also in unconditioned mice 90 min post extinction compared to respective levels after acquisition of social fear. Moreover, the rise in miR-124-3p was neither induced by icv OXT application, nor by exposure to repeated social contact. Nevertheless, miR-124-3p might be an essential miRNA for extinction of social fear, since it is known to regulate social behavior (Arrant and Roberson, 2014; Gascon et al., 2014). Functional inhibition, overexpression, or OXTR-neuron specific knockdown of miR-124-3p prior to acquisition of SFC is necessary to reveal a potential

functional behavioral effect. Furthermore, miR-124-3p´s regulatory effect on social behavior is known to be caused by altered AMPA receptor subunit composition (Gascon et al., 2014), revealing a possible mechanism, by which also social fear learning, memory consolidation, or extinction behavior might be regulated via miRNAs. Of course, finding miR-124-3p in conjunction with miR-132-3p as crucial regulator of social fear would suggest that miRNA function in networks to regulate their target genes, which adds another layer of complexity to the development of novel SAD treatment options.

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# Abbreviations

| | |
|---|---|
| 3´UTR | 3´untranslated region |
| AAV | Adeno-associated virus |
| AGO | Argonaute |
| AMPA | Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| AP | Anterior-posterior |
| ASD | Autism spectrum disorder |
| AVP | Arginine vasopressin |
| BDNF | Brain derived neurotrophic factor |
| BNST | Bed nucleus of the stria terminalis |
| CaMK | Calcium/calmodulin dependent protein kinase |
| CaN | Calcineurin |
| CBT | Cognitive-behavioral therapy |
| CCR | Carbon catabolite repressor |
| cDNA | Complementary DNA |
| ceRNA | Competing endogenous RNA |
| CFC | Cued fear conditioning |
| circRNA | circular RNA |
| CNS | Central nervous system |
| CREB | cAMP-responsive element binding protein |
| CRF | Corticotropin-releasing factor |
| CRTC | CREB-regulated transcription coactivator |
| CS | Conditioned stimulus |
| CSF | Cerebrospinal fluid |
| Ctrl | Control |
| CUMS | Chronic unpredictable mild stress |
| DCP | Dipeptidyl carboxypeptidase (mRNA-decapping enzyme) |
| DDX | DEAD Box RNA helicase |
| DDX | DEAD-box helicase 6 |
| DGCR8 | DiGeorge critical region 8 |
| DV | Dorso-ventral |
| Dvl3 | Disheveled segment polarity protein 3 |
| eEF2 | Eukaryotic elongation factor 2 |

| | |
|---|---|
| eGFP | Eukaryotic green fluorescent protein |
| EGFR | Epidermal growth factor receptor |
| EPM | Elevated plus-maze |
| Exp5 | Exportin 5 |
| FAM | 6-carboxyfluorescein |
| GABA | γ-aminobutyric acid |
| GAD | Generalized anxiety disorder |
| GR | Glucocorticoid receptor |
| HPA | Hypothalamus-pituitary-adrenal |
| Htr2C | 5-hydroxytryptamine receptor 2C |
| icv | Intracerebroventricular |
| Inh | miR-132-3p inhibitor |
| IP | Immunoprecipitation |
| $IP_3$ | Inositol-1,4,5-triphosphate |
| LD | Lactation day |
| LDB | Light dark-box |
| LDCV | Large-dense core vesicle |
| LNA | Lockd nucleic acid |
| lncRNA | Long non-coding RNA |
| LS | Lateral septum |
| LTP | Long-term potentiation |
| $m^7G$ | 7-methyl-guanosine-triphosphate |
| MAPK | Mitogen-activated protein kinase |
| MeCP2 | Methyl-CpG binding protein 2 |
| MEF-2 | Myocyte enhancer factor 2 |
| mGluR | Metabotropic glutamate receptors |
| miRNA | microRNA |
| ML | Medio-lateral |
| MOPA | Medial preoptic area |
| MR | Mineralocorticoid receptor |
| MS | Medial septum |
| Ncoa1 | Nuclear receptor coactivator 1 |
| NMDA | N-methyl-D-aspartate receptor |
| NOI | Novel object investigation |

| | |
|---|---|
| NOT | Negative regulator of transcription |
| NPS | Neuropeptide S |
| OFT | Open field test |
| OXT | Oxytocin |
| OXTR | Oxytocin receptor |
| OXTR-A | Oxytocin receptor antagonist |
| PARN | Poly(A)-specific ribonuclease |
| PD | Pregnancy day |
| Peg3 | Paternally expressed |
| PFC | Prefrontal cortex |
| $PIP_2$ | Inositol-4,5-bis-phosphate |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| Pparg | Peroxisome proliferator activated receptor gamma |
| pre-miRNA | Precursor miRNA |
| pri-miRNA | Primary miRNA |
| PTM | Post-translational modification |
| PVN | Paraventricular nucleus |
| qPCR | Quantitative polmerase chain reaction |
| RAGE | Vascular receptor for advanced glycation end-products |
| RBP | RNA binding protein |
| RISC | RNA-induced silencing complex |
| RNAi | RNA interference |
| Robo4 | Roundabout guidance receptor 4 |
| SAD | Social anxiety disorder |
| SCN | Suprachiasmatic nucleus |
| Scr | Scrambled (control sequence) |
| SFC | Social fear conditioning |
| $SFC^-$ | Unconditioned |
| $SFC^+$ | Conditioned |
| shRNA | Short hairpin RNA |
| SIRT1 | Sirtuin 1 |
| SNARE | Soluble N-ethylmaleimide-sensitive factor attachment receptor |
| snoRNA | Small nucleolar RNAs |

| | |
|---|---|
| SNP | Single nucleotide polymorphism |
| SNRI | Serotonin-noradrenalin reuptake inhibitors |
| SON | Supraoptic nucleus |
| SSRI | Selective serotonin reuptake inhibitors |
| TRBP | *Trans*-activation-responsive RNA binding protein |
| TrpV2 | Transient receptor potential cation channel subfamily V member 2 |
| TUT | Terminal uridyl transferase |
| US | Unconditioned stimulus |
| Veh | Vehicle |
| Wnt | Wingless |

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# Curriculum Vitae

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| | |
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## Education

Since 2015 **PhD student** in Neurobiology at the University of Regensburg, Germany,
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2013-2015 **Master of Science in Biology**, University of Regensburg, Germany

**Master thesis** at Department of Behavioural and Molecular Neurobiology
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Topic: Tiny titans – microRNAs regulated by oxytocin

2010-2013 **Bachelor of Science in Biology**, University of Regensburg, Germany

**Bachelor thesis** at Department of Molecular and Cellular Neurobiology
Prof. Dr. Peter J. Flor
Thema: Effects of pharmacological blockade of mGluR5 and genetic ablation of mGluR7 on physiological and behavioral consequences of chronic psychosocial stress in mice

2001-2010 Johannes-Turmair **Gymnasium**, Straubing, Germany

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# List of Publications

**Bludau A**, Royer M, Meister G, Neumann ID, Menon R. **2019**. Epigenetic Regulation of the Social Brain. Trends in Neuroscience 42(7):471-484.

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