Molecular and neuronal correlates of social fear in mice

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Contents

Abstract

Fear is a basic adaptive emotional response to threatening environmental stimuli. From an evolutionary standpoint, presence and efficient functionality of the neural substrates of fear are imperative for an organism survival. Human anxiety disorders are caused by the impaired functionality of systems within the brain that code for and regulate our responses to fearful and anxiogenic stimuli. Anxiety and fear-based psychopathologies include social anxiety disorder (SAD), generalized anxiety disorder, panic disorders, obsessive-compulsive disorders. SAD is characterized by excessive fear and avoidance of social situations and severely deteriorates the quality of life of the afflicted individual. Treatment for SAD is mainly phenomenological which is mainly caused by the sparse understanding of the neural and molecular underpinnings of this disorder. Another problem is that although these psychopathologies are twice as prevalent in women in comparison to men, most of the current research uses males as primary subjects. To reveal the molecular and neuronal underpinnings of SAD, we have established a model of social fear using a Social Fear Conditioning (SFC) paradigm in male mice which resembles SAD in humans. Using this model we were able to show that local infusion of neuropeptide oxytocin (OXT) which is known for its prosocial and anxiolytic properties into the lateral septum (LS) reverses social fear in male mice. Social fear conditioned (SFC$^+$) mice showed an increase in OXT receptor (OXTR) binding in the LS which normalized after social fear extinction, while local OXT release in response to social stimuli was found to be blunted in LS of SFC$^+$ mice.

In lieu of these findings, and to address the abovementioned issues I used the SFC paradigm to: (1) Reveal the role of endogenous OXT system in the regulation of social fear in female mice, and (2) assess the contribution of epigenetic mechanisms in the regulation of social fear memory in male mice.

In order to study the endogenous OXT system in females, I chose the state of lactating mice which have an activated brain OXT system as a model. SFC$^+$ lactating mice did not show any SFC-induced fear in comparison to virgin females. This lack of SFC-induced social fear could

be reinstated by intracerebroventricular (icv) infusion of OXTR antagonist (OXTR-A). Conversely, icv infusion of OXT reversed SFC-induced social fear in virgin females. cFos immunohistochemistry revealed increased activation of the LS in SFC$^+$ virgin mice in comparison to the SFC$^-$ controls, and this returned to baseline levels after extinction, whereas LS-activity remained dampened throughout SFC in lactating mice. I also found an increased in the number of OXT-positive fibers within the LS of lactating mice along with increased OXT release in the LS of lactating mice in response to the extinction of social fear. Moreover, calbindin staining of OXTR-Venus mice revealed most of the OXTR-expressing neurons within the LS to be GABAergic interneurons. Corroborating this, local-LS application of the OXTR-A revived, and OXT reversed SFC-induced social fear in lactating and virgin mice respectively implicating LS-OXT system in the reversal of SFC-induced social fear in lactating mice. In line with the pharmacological manipulations, AAV mediated activation of the OXTR-positive neurons within the LS facilitated extinction of social fear whereas constitutive genetic knockdown of OXTR in the mouse brain impaired extinction of social fear. Finally, I was also able to show that specific chemogenetic silencing of magnocellular OXTergic SON afferents to the LS completely blocked social contact in lactating mice.

In the second half of my project, I focused on delineating the epigenetic mechanisms which could underlie the formation of social fear and social fear extinction memory. cFos immunohistochemistry revealed increased activity within the LS of SFC$^+$ male CD1 mice post-acquisition of social fear which reverted to baseline after extinction while such an effect was absent in the case of cued fear conditioning. Following this, I checked for mRNA expression of class I *Hdacs* and found an increase in *Hdac1* in SFC$^+$ mice which again went back to baseline after the extinction of social fear. Pre-extinction pharmacological blockade of HDAC1 within the LS using MS275 led to facilitation of extinction only in the case of social fear. Finally, I performed a microarray to identify the set of genes which are differentially expressed in the LS of SFC$^+$ and SFC$^-$ mice. Cross-referencing these genes with the set of putative HDAC1

regulated genes led me to a final set of genes which could underlie the HDAC1-mediated regulation of social fear extinction.

Taken together, my data show that molecular mechanisms within the LS are crucial for regulation of traumatic events associated with a social context in male and female mice. In the case of female mice, I was able to convincingly show that endogenous OXT-mediated activation of OXTR-positive GABAergic neurons within the LS is essential for countering SFC-induced social fear. In the case of males, I was able to show that HDAC1 regulates social fear extinction memory formation within the LS. Such molecular and neuronal mechanism probably help define the emotional disposition of an individual and form the neuronal correlates of social fear in mice. Thus, their better understanding might help us develop better therapeutic strategies for emotionally crippling psychopathologies such as SAD.

Abstract

Introduction

1.1 Nature of Emotions

"Emotions are passions of a short duration which are intimately linked to organic life"

The above-mentioned statement by a Charles Lerourneau *(Physiologie des Passions*, 1878) states that the link to organic life is a key feature of every emotion. Indeed, it is emotions, whether positive or negative, that make human life meaningful and an implicit assumption made by most studies is that emotions are intrinsic to evolution. In human and non-human primates, emotions have a large cognitive component, which is formed by the ability of these beings to learn and remember the benefits of certain emotions such as love and the negative aspects of others such as fear. A neurobiological approach towards understanding emotions investigates the ability of an organism to perceive emotionally salient cues from the environment, process their valence in accordance with its own survival and then generate an appropriate adaptive behavioural response to cope with the concerned cue. The varied nature of environmental cues warrants development of specific response directed towards each unique cue which quite often leads to one single emotion. Hence, each emotion can be thought of as an agglomeration of several specific behavioural and autonomic responses that manifests as one single, coherent, higher order entity which helps an organism to cope with varied situations.

1.2 Anxiety and Fear

Anxiety and fear are emotions that are often conflated with each other and used interchangeably in lay terms. Ethologically, both are highly adaptive responses that are very intense and essential for an organism's healthy survival, as they are the part of their normal emotional repertoire (McNaughton and Zangrossi, 2008). For the purpose of the present thesis, fear is defined as the behavioural response to real and clear threatening stimuli, whereas anxiety is defined as the behavioural response to potential or ambiguous threats. Both these emotions are intense, and their presence until the real or potential threat is over, seems to be intrinsic for their proper

functioning. Anxiety and fear are both coping strategies which could vary depending on the situation at hand. Active coping strategies are deployed in cases where escape is possible, and they are mediated primarily by activation of the sympathetic nervous system leading to hypertension and tachycardia (Cannon 1915, Olds 1956). On the other hand could be passive coping strategies are used in situations where escape is not possible, and it is usually accompanied by autonomic inhibition, i.e. hypotension and bradycardia along with neuroendocrine changes such as activation of the hypothalamo-pituitary-adrenal (HPA) axis (Engel and Schmale 1972). Having said that, the persistence of these emotional response in the absence of a threat is detrimental to other pro-survival behaviours such as mating, food procurement, reproduction, normal social interactions, and self-care amongst others. Such conflict was beautifully demonstrated by Estes and Skinner (1941) in their work wherein rats that were fear conditioned (See section 1.4.1) to a tone, stopped pressing a food supplying lever in the presence of the tone. Such inappropriate over-activation of circuits involved in fear and anxiety leads to anxiety disorders which are extremely debilitating in nature (Gray and McNaughton 1996, Hazen, Stein et al. 1996).

1.3 Anxiety disorders

Anxiety disorders usually result in significant reduction in the quality of the afflicted individual's life and have been estimated to have a lifetime prevalence of 30% (Andrade, Caraveo-Anduaga et al. 2003, dsDemyttenaere, Bruffaerts et al. 2004, Kessler and Wang 2008, Neumann and Slattery 2016). Maladaptation of anxiety and fear responses leads to various phobias, panic disorder, obsessive-compulsive disorder, social anxiety disorder (SAD), post-traumatic stress disorder (PTSD) and general anxiety disorder (GAD) all of which together fall under the spectrum of anxiety disorders (Neumann and Slattery 2016). SAD is characterized by intense fear and avoidance of social situations (Turner et al., 1992; Faravelli et al., 2000), PTSD-afflicted patients suffer from flashbacks of their respective traumatic incident (Nemeroff

et al., 2006), and GAD is characterized by chronic apprehension and anxiety, which is not focused on a specific environmental stimulus (Kessler et al., 1994). Albeit the neuronal aberrations which occur alongside anxiety disorders have been studied extensively (Deckersbach, Dougherty et al. 2006, Tovote, Fadok et al. 2015), there are many gaps in our understanding of how much these circuits actually contribute towards generating the states of fear and anxiety and how their dysregulation leads to anxiety disorders. Such lack of understanding forms the most daunting hurdle in the development of effective treatment strategies to counter specific subtypes of anxiety disorders. This situation is only worsened by the fact that most of the current descriptions of anxiety disorders is based on their phenomenology and not their neurobiology (DSM-V, American Psychiatric Association, 2013). From this point on, the discussions will be limited to SAD and PTSD (with respect to phenomenology), as obtaining a better understanding of their molecular and neurobiological underpinning formed the primary framework for this thesis.

1.3.1. SAD

In principle, all of us have felt the fear of being judged by others or of making an appearance in front of a group. This stems from a fear that we will end up underperforming, and thus be

(Photo credit: Shawn Coss)

excluded from the group. For our ancestors belonging to or being included in a group was something that increased their chances of their survival, and hence we have evolved to compete for attractiveness (Gilbert 2001) in a manner which would ideally lead to inclusion. SAD originates from the dysregulation of this normal evolutionary fear of exclusion from the group.

SAD is characterised by intense fear and avoidance of social situations (Kessler et. al., 2005) and is the second most common anxiety disorder with a lifetime prevalence of 12.1% (Alonso, Petukhova et al. 2011, Kessler, Petukhova et al. 2012). Approximately 60% of patients afflicted with SAD are females, although there seems to be an overrepresentation of men when it comes to seeking treatment (Xu, Schneier et al. 2012). DSM-V has recognised 2 subtypes of SAD, namely generalised SAD and non-generalized SAD. In generalised SAD, patients fear most social situations (Vriends, Becker et al. 2007, Kerns, Comer et al. 2013). It is much more debilitating than non-generalized SAD and could be co-morbid with other anxiety disorders (Stein and Chavira 1998). On the other hand, non-generalized SAD manifests as a fear of specific situations including performance situations such as public speaking or situations with normal social interaction like dating (Vriends, Becker et al. 2007, Bogels, Alden et al. 2010). It is less debilitating in nature, but could still lead to significant reduction in the quality of the patient's life (Hazen, Stein et al. 1996). Studies have

found that people with SAD often (approximately 86.9%) fear more than one social situations like public speaking (89%) being the most commonly feared social situation followed by entering a room occupied by others (63.1%) and meeting strangers (47.3%) (Faravelli, Zucchi et al. 2000, Lecrubier, Wittchen et al. 2000). The symptoms of SAD which include avoidant behaviour is often considered as the biggest hindrance towards extinction and reversal of social fear (Stangier, Esser et al. 2006). SAD usually has an early onset at the age of 5 to 15 and is often comorbid with secondary disorders such as depression (Schneier, Johnson et al. 1992, Stein and Chavira 1998), agoraphobia (Magee, Eaton et al. 1996), or substance abuse (Schneier, Foose et al. 2010, Buckner, Heimberg et al. 2013). Having said that, lack of social contact in SAD patients due to the fear of negative evaluation is the primary symptom and often precedes symptoms of co-morbid conditions like major depressive disorders (Beesdo, Jacobi et al. 2010, Beesdo, Pine et al. 2010).

1.3.2. PTSD

(Photo credit: Shawn Coss)

Every organism constantly faces situations which are a threat to its survival. Thus, there needs to be an adaptive neurophysiological system which responds to such a situation and furthermore which encodes that threatening experience to aid survival. These threatening or "traumatic" experiences often leave a lasting impression leading to flashbacks, nightmare, avoidance behaviour even in the absence of a real threat and hyper-arousal which are the classical behavioural symptoms of PTSD. The earliest descriptions of traumatic experiences in the aetiology of anxiety disorders can be traced back to Sigmund Freud's "Theory of Seduction" wherein he stated that during childhood an individual is exposed

to varied types of traumas which could have a distressing effect on an individual's ego and lead to neurosis. The contemporary definition of PTSD has its origin from the World War I syndrome of 'shell shock' which was thought to be due to the actual concussion producing the effect of heavy artillery. Soldiers during this time period were shown to have increased stress response, when exposed to reminders of the wartime period (Southwick et al, 1994). Since then, numerous clinical studies which included not only war veterans, but also people who suffered from industrial accidents, Nazi concentration camps and fire hazards (Kinzie and Goetz 1996, Brady, Pearlstein et al. 2000) amongst others have led to the change in our current understanding of PTSD from its definition as a 'gross stress reaction' in DSM-I (American Psychiatric Association, 1952) to an anxiety disorders in DSM-III (American Psychiatric Association, 1980). This has been recently modified, and PTSD is now categorised as trauma- and stressor- related disorder in DSM-V (American Psychiatric Association, 2013). To satisfy this DSM-V criterion, an individual has to be exposed to trauma which involves "exposure to actual or threatened death, serious injury or sexual violence".

Epidemiologically, PTSD is known to have a lifetime prevalence of 1.3% (Creamer, Burgess et al. 2001), however studies with specific sample sets such as Vietnam war veterans or female rape victims have reported a lifetime prevalence as high as 30% (Andrews, Brewin et al. 2000, Andrews, Brewin et al. 2003, Brewin, Andrews et al. 2003). Studies discussing gender differences in the etiology

y of PTSD find that PTSD is more prevalent in women (10.4%) than in men (5.0%) (Boney-McCoy and Finkelhor 1996, Perkonigg, Kessler et al. 2000, Tolin and Foa 2006), although these differences could be caused by inherent gender differences in perception and definition of trauma (Breslau and Kessler 2001). Just like SAD, PTSD is known to have a very high level of comorbidity with GAD (53%) followed by specific phobias (50%), depression (37%) and substance abuse (31%) (Helzer, Robins et al. 1987, Breslau, Davis et al. 1991, Davidson and

Foa 1991, Kessler, Sonnega et al. 1995). However, in the case of PTSD, it is not clear as to whether PTSD precedes or is preceded by any of the above-mentioned disorders (Kessler, Sonnega et al. 1995).

1.4 Treatment of Anxiety disorders

Most of the currently used treatment options for anxiety disorders are very non-specific and used to treat not only all the categories of anxiety disorders, but also certain comorbid psychopathologies such as depression. Behavioral and psychological therapy for anxiety disorder includes evidence-based therapies like cognitive-behavioral therapy (CBT), which involves creating a personalized coping strategy for each patient, and exposure-based therapies, which includes exposure to anxiogenic stimuli in a graded and controlled manner for systematic desensitization (Choy, Fyer et al. 2007, Singewald, Schmuckermair et al. 2015, Stangier 2016). Alternatively, pharmacotherapy is also used to treat anxiety disorders. In this regard, commonly prescribed medication includes selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs) and benzodiazepines, although the use of the later class of anxiolytics has reduced due to its considerable side effects (Bruce, Vasile et al. 2003). Other drugs, which are seldom used to treat anxiety disorders, includes tricyclic antidepressants (e.g. imipramine) and monoamine oxidase inhibitors (e.g. phenelzine). Response rates of 50-55% are commonplace even in the case of the most ideally designed pharmacotherapeutic regime, and this statistic becomes even more daunting in the face of remission rates, which are as low as 25-30% (Holmes, Heilig et al. 2003, Stein and Seedat 2004). Thus, pharmacotherapy is often combined successfully with psychotherapy in order to achieve better remission rates (Gould et al., 1997; Federoff and Taylor, 2001). Low response and remission rates combined with a high rate of relapse just go on to signify the need for the development of better and subtype-specific anxiolytics. However, this endeavour requires a better understanding of the

molecular aetiology of these disorders and thus effective modelling of anxiety in animals is essential.

1.5 Modelling anxiety in rodents

When fear and anxiety are viewed from an evolutionary standpoint, it seems logical that the neural and hormonal systems that control behaviour will contain components that are conserved and are likely to have homologous counterparts in other species (McNaughton and Zangrossi, 2008). Indeed, a basic assumption made while developing an animal model is that the neuronal and behavioural responses to human anxiety can be recreated in a rodent by eliciting a threat to its survival. In this regard, the three basic criteria that an animal model needs to fulfil to be deemed useful are the following:

a. Face validity: A behaviour in the animal appears to be analogous to the behaviour in humans.

b. Predictive validity: Refers to the capacity of a model to predict the outcome of a specific manipulation.

c. Construct validity: Refer to the capacity of an animal model to recruit the same neurobiological substrate as its respective disorder in humans.

Even if the above criteria are met, it is almost impossible to develop an animal model that fully mimics any psychiatric syndrome in its entirety and hence the only criteria that need to be met by an animal model is that of the purpose for which it was developed. Considering this, it makes sense to develop models that cater to specific subtypes of anxiety disorders. Behavioural tests for animals such as the elevated plus-maze (EPM) (Lister 1987), the open field test (OFT) (Stanford 2007) or the light-dark box (LDB) (Bourin and Hascoet 2003), which utilize the innate conflictive drive in rodents of exploring novel spaces versus avoiding areas that are open, at an elevation or brightly illuminated, are often used to measure general innate anxiety. Other more complicated models based on associative learning like the Pavlovian fear conditioning

(Pavlov, 1927) or operant fear conditioning (Skinner, 1938) are often used to study neurobiological mechanisms underlying learned fear. In the following section, we will discuss the EPM and specific fear conditioning paradigms, which were used extensively in my thesis.

1.5.1. Measuring general anxiety in rodents

The EPM is designed for rats and mice (Pellow) and offers the subject a simple choice of exploring open, elevated areas or closed protected areas. This test which was originally developed by Handley and Mithani has been one of the most popular ways for testing anxiety for the last two decades. The EPM consists of two open and two closed arms placed at an elevation. The animal is placed in the closed arm of the plus-maze. Reduction in the novelty of the arm coaxes the animal to explore other parts of the maze and at this point, it is presented with a genuine choice of 2 open arms and 1 closed arm, all with equal novelty. The choice made by the animal at this point indicates the level of anxiety (which is inversely proportional to the time spent exploring the open arms). In the present thesis, EPM was used to measure preconditioning anxiety of lactating, virgin, and male mice.

1.5.2. Cued Fear Conditioning (CFC)

Pavlovian fear conditioning is a process that uses associative learning mechanisms to generate an adaptive response to environmental stimuli. The CFC paradigm uses this powerful, rapid and long-lasting effect of Pavlovian fear conditioning to generate a fear response to a non-threatening cue (light of a particular intensity or sound of a particular frequency). In the CFC paradigm used by us (described in detail in materials and methods), a neutral stimulus (tone) called the conditioned stimulus (CS) is paired with an aversive stimulus (foot shock) called as the unconditioned stimulus (US) and presented to the mice. Through associative learning, the previously neutral tone will acquire aversive properties and the animal will now exhibit freezing (called conditioned response) on the presentation of the tone alone. Presentation of the CS

during extinction without the US leads to a gradual decline of freezing to a point where the animal is no longer fearful of the CS in a process called fear extinction which is akin to exposure therapy in humans (Myers and Davis 2007). The CFC paradigm generates anxiety-like behaviour as the animal expects a threat (US) on CS-presentation and thus it is a way of studying the neural and molecular substrates that underlie the emotions of anxiety and fear in a general context, making it an apt animal model for anxiety disorders like PTSD and GAD.

1.5.3. Social Fear Conditioning (SFC)

Though CFC is a good model to study anxiety disorders in a general context, the presence of a voluntary social component makes SAD more complicated and, thus, it cannot be satisfactorily modelled using CFC. Situations such as a party or any group activity which involve social contact (positive reinforcement) are heavily rewarding stimuli for humans and, therefore, they are motivated to be in social situations over activities performed in isolation (negative reinforcement). SAD patients, on the other hand, try to avoid all kinds of social contact, when presented with a choice, to avoid punishment. Thus, this legitimate conflict of acceptance versus avoidance, when presented with a social situation needs to be considered while developing an animal model which generates symptoms similar to SAD.

Recently, such a mouse model for SAD was developed by Toth et al, in 2012, which uses the SFC paradigm (explained in detail in materials and methods) (Toth, Neumann et al. 2012). The SFC paradigm is based on operant fear conditioning principles, wherein a foot shock (punishment, consequence) is paired with a social stimulus during the process of fear acquisition to induce social fear (avoidance of social stimulus, behaviour) in mice. However, during fear extinction, mice are presented with different social stimuli in their home cage, where they must make a choice to avoid or approach the respective social stimulus. In this case, the mouse usually avoids the social stimulus (behaviour) at first, but the realisation of the absence of a foot shock while making social contact leads to extinction of SFC-induced social fear over multiple exposures to social stimuli. The SFC paradigm is unique, as it generates social anxiety-

like symptoms in mice without other confounding symptoms from co-morbid disorders such as depression or other subtypes of anxiety disorders (Toth, Neumann et al. 2012). Social fear induced by SFC is generalised unlike the other animal models of social avoidance like an acute social defeat (Lukas, Toth et al. 2011, Toth and Neumann 2013, Zoicas, Menon et al. 2016) and lasts for at least 2 weeks (Toth, Neumann et al. 2012). These interesting features of the SFC paradigm put it above the other animal models of social avoidance used in the field of neuroscience to study the molecular psychopathology of SAD.

1.6 Neurocircuitry of fear and anxiety

Conceptually, fear and anxiety are extremely similar emotions, and there is considerable overlap between the neuronal circuits involved in our behavioural response to fearful or anxiogenic stimuli (Davis, Walker et al. 2010, Chen, Wardill et al. 2013, Grupe and Nitschke 2013). Although the anticipatory nature of an anxiogenic stimulus makes it more complicated, development of advanced pharmacogenetics and optogenetic techniques offer much higher spatial and temporal resolution and have helped us functionally characterize individual elements of neuronal circuits and their higher order brain-wide interaction partners (Tye and Deisseroth 2012, Sternson and Roth 2014, Tovote, Fadok et al. 2015).

The basic neurocircuitry involved in our response to fearful and anxiogenic situations includes, but is not limited to, the amygdala, medial prefrontal cortex (mPFC) and the hippocampus (Hip) (Tovote, Fadok et al. 2015). Although, specific brain regions have been touted to have specific functionality in regulation of fear and anxiety, this view is being challenged in the past decade by studies which implicate novel brain regions such as the bed nucleus of the stria terminals (BNST) which is considered to be a part of the extended amygdala and the septal nuclei (part of the septohippocampal system) in the intricate regulation of an entire repertoire of behaviors from learned fear to innate anxiety.

Most of our knowledge about neurocircuitry regulation anxiety comes from the fear conditioning studies and hence from here on forward, we will focus our discussion of circuits involved in processing acquisition and consolidation and extinction conditioned fear. Fig 1 is a representation of neuronal circuits known to be involved in conditioned.
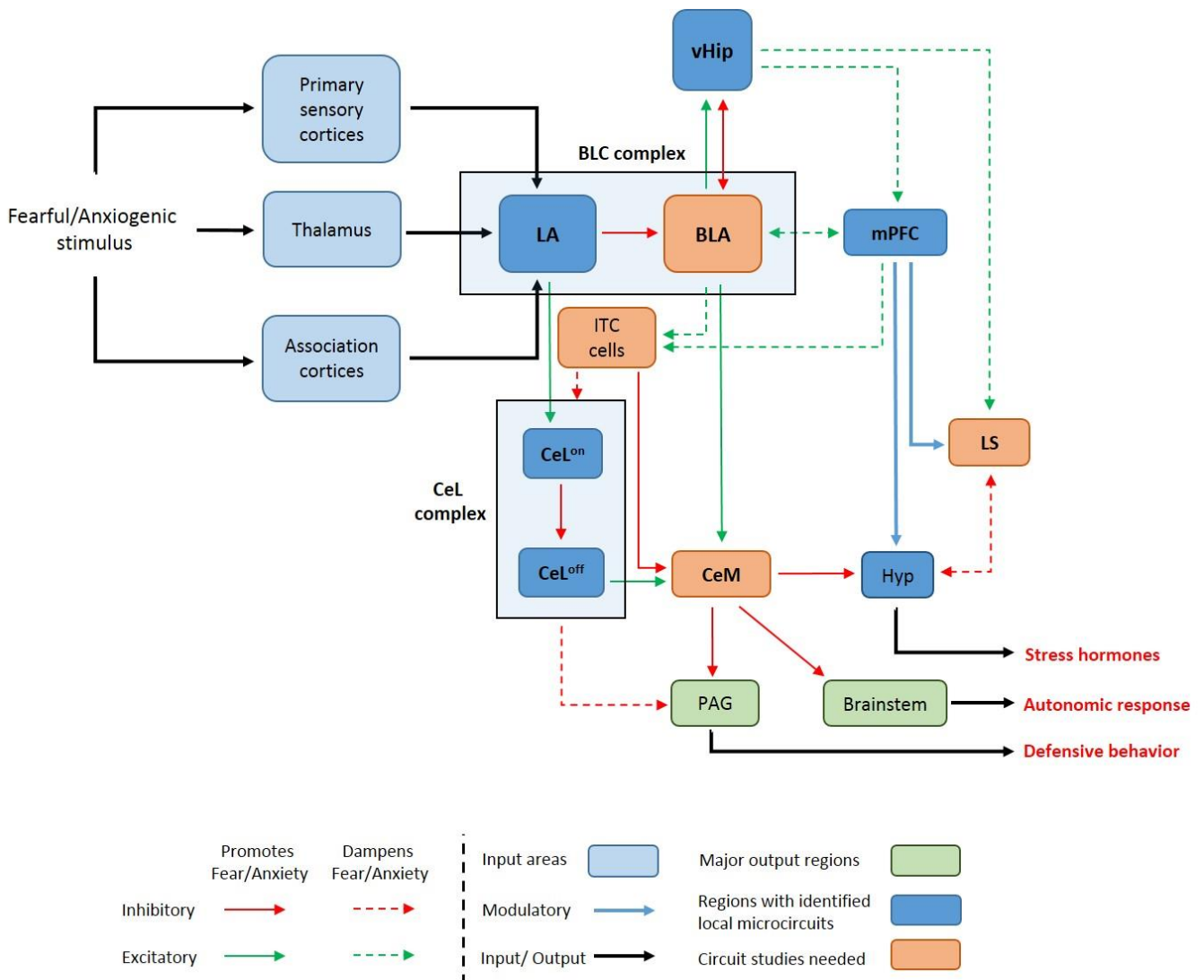
Fig 1. *Basic neuronal circuits involved in fear and anxiety. Regions with a major role in fear and anxiety and heavily emphasized in this thesis are in stated in bold letters. LA (lateral amygdala); BLA (basolateral amygdala); BLC (basolateral amygdaloid complex); mPFC (medial prefrontal cortex); LS (lateral septum); vHip (ventral hippocampus); CeL (lateral nucleus of central amygdala); CeM (medial nucleus of central amygdala); ITC (Intercalated cells); Hyp (hypothalamus); PAG (periaqueductal grey). CeL^{on} and CeL^{off} cells are described in detail in the section 1.6.1. Fig has been adapted from (Tovote et al., 2015).*

1.6.1 Amygdala

Various studies in humans, mice and rats have implicated the amygdala, an almond-shaped cluster of nuclei located ventromedially within the temporal lobe of complex vertebrates, as a center for regulation of fear and anxiety (Allman and Brothers 1994, Wolff, Grundemann et al. 2014, Penzo, Robert et al. 2015, Marcinkiewcz, Mazzone et al. 2016, Mendez-Bertolo, Moratti et al. 2016, Zhu, Liu et al. 2016). The amygdala can be divided into two main sub-areas – the basolateral amygdaloid complex (BLC), which is mostly glutamatergic, and the central amygdala (CeA), which is composed mostly of γ-aminobutyric acid (GABA)-ergic neurons. In a pathway often referred to as the "low road" of fear response, any new fearful or anxiogenic stimulus (auditory, visual and somatosensory) generates sensory information within the thalamus and other sensory cortical regions, which is in turn conveyed to and terminates in the lateral amygdala (LA) (a subnuclei of the BLC) (Johansen, Hamanaka et al. 2010, Pessoa and Adolphs 2010, LeDoux 2014). This activates excitatory glutamatergic projections from the LA towards the lateral nucleus of CeA (CeL) which in turn activates the CeL^{on} neurons, that are characterised by a lack of protein kinase C-delta ($PKC\delta^-$). The CeL^{on} neuron inhibits the CeL^{off} neurons, which are characterised by the presence of protein kinase C-delta ($PKC\delta^+$). The CeL^{off} neurons exercise an inhibitory control over the medial part of the CeA (CeM), which is the main output region of this network and projects towards the periaqueductal grey (PAG), brainstem and hypothalamic regions that regulate downstream defensive behavior such as freezing (Swanson and Petrovich 1998, Tovote, Fadok et al. 2015). Thus, the disinhibition of CeM by a fear-generating stimulus leads to defensive behaviours via a complex pathway involving the different amygdaloid subcircuits (Ciocchi, Herry et al. 2010, Tye, Prakash et al. 2011, Maroun and Wagner 2016). These results are also complemented by studies showing altered GABA receptor levels within the CeA of mice strains bred for high anxiety (Tasan, Bukovac et al. 2011). In addition to the above-mentioned nuclei, a cluster of GABAergic cells that lie at the interface of CeA and basolateral amygdala (BLA, subnuclei of the BLC) called the intercalated

cells (ITC). The ITC cells gate the information flow between BLA and CeA and are thought to be activated by fear extinction procedures (Likhtik, Popa et al. 2008, Tovote, Fadok et al. 2015). The BLA on its own sends projections to the hippocampus to control anxiogenesis (discussed in detail in the section 1.6.3). The so called "high road" to anxiogenic stimulus involves the cortex (discussed in detail in section 1.6.4).

1.6.2 Septum
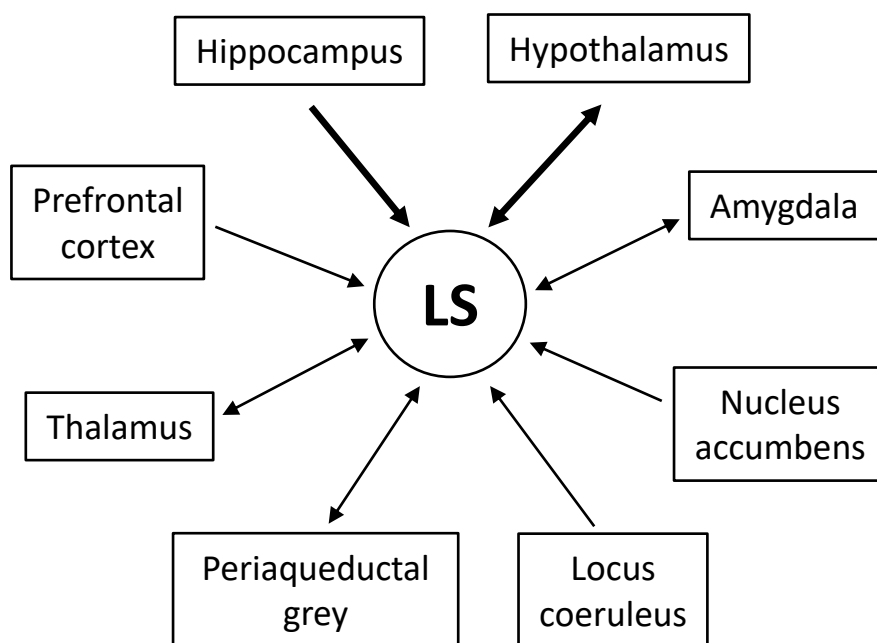
Fig 2. *A schematic for the afferent and efferent connections of the lateral septum (LS). Thick arrows indicate strong connections and double sided arrows indicate reciprocal connections (Fig adapted from Sheehan et al, 2004)*

The septum or septal nuclei is a subcortical forebrain structure, which is located rostrodorsal to the hypothalamus and in between the lateral ventricles in rodents. It can be divided into two functionally, neurochemically and anatomically distinct nuclei namely the lateral septum (LS) and medial septum (MS). MS sends ascending inputs to the hippocampus which are mostly GABAergic and cholinergic in nature and, along with the hippocampus and diagonal bands of broca, it forms the "septo-hippocampal system", which has been heavily implicated in operant

reward learning (Vega-Flores, Rubio et al. 2014) and spatial memory formation (Durkin 1994). LS is thought to serve as an essential converging point for cognitive information from the cortex and hippocampus and affective information coming in from amygdala and hypothalamus which it relays to downstream regions to control the behavioural output in response to varied environmental stimuli (Deller, Leranth et al. 1994, Gray and McNaughton 1996). It comprises mostly of GABAergic projection neurons, (Gallagher, Zheng et al. 1995, Sheehan, Chambers et al. 2004) and has been a region of high interest with regards to stress response and aggression. LS receives strong glutamatergic inputs from the hippocampus and is in turn reciprocally connected (not all) to LS.

cFOS studies have found a negative correlation between aggression and LS activity, and consequently, septal lesions are known to induce a typical "septal rage" phenotype (Potegal, Blau et al. 1981, Goodson, Evans et al. 2005, Lee and Gammie 2009). This is complemented by a gain of function studies showing an increase in LS activity to be correlated with reduced aggression (Wong, Wang et al. 2016). Thus, LS is a key regulation of aggression, which essentially is an enhanced form of social contact. Studies have shown that LS is involved in active stress coping and exerts an inhibitory effect on the HPA axis activity (Herman, Prewitt et al. 1996, Singewald, Rjabokon et al. 2011). These and other studies have led to the prevailing opinion that LS is a region, whose activity could be linked to dampening of anxiety (Sheehan, Chambers et al. 2004). This view has been contested in the past decades by studies which implicate different sub-population of neurons within the LS, for example, the cells expressing corticotropin-releasing factor (CRF) receptor 2 (CRFR2) in the promotion of stress-induced anxiety (Radulovic, Ruhmann et al. 1999, Anthony, Dee et al. 2014).

1.6.3 Hippocampus

The hippocampus (Hip) is a subcortical region located within the medial temporal lobes, which were traditionally thought to be involved in the processing of declarative memory irrespective

of its emotional content. But over the past two decades, studies have shown another side of this interesting brain region, one that is much more plastic and closely linked with emotionality and stress response. Many studies have ascertained a role of the Hip in contextual fear learning and retrieval in rodents (Strekalova, Zorner et al. 2003, Chang and Liang 2017). Reduced hippocampal volumes have been both used as a marker and reported as a consequence of PTSD (Bremner 2002, Gilbertson, Shenton et al. 2002). For example, ventral hip (vHip) lesions have been shown to impair contextual fear conditioning (Kjelstrup, Tuvnes et al. 2002). BLA – vHip connectivity is known to regulate basal anxiety-related behaviour. Optogenetic activation of monosynaptic, glutamatergic BLA projections to the CA1 pyramidal neurons of the ventral Hip (vHip) exerts an anxiogenic effect (Felix-Ortiz, Beyeler et al. 2013). vHip-mPFC synchronicity within the context of anxiety is discussed in section 1.6.4. The septo-hippocampal system, which has been previously mentioned, is known to regulate stress-induced anxiety. Thus, the role of the hippocampus as a regulator of stress-induced anxiety and region which complies context-related information is well documented. Hippocampus also regulates social memory, as shown by a recent study, wherein genetic silencing of the dorsal CA2 pyramidal neurons of the hippocampus impaired social memory in mice (Hitti and Siegelbaum 2014). Considering these results, understanding the role of dorsal Hip (dHip) in coding for a social context during SFC acquisition and retrieval is of importance.

1.6.4 Prefrontal cortex

Higher order brain structures such as the mPFC is key brain structure mediating top-down regulation on brain regions such as the amygdala and thus helping organisms discern safety from danger in a more perceptive response to anxiogenic or fearful stimuli called the "high road" (Pessoa and Adolphs 2010, LeDoux 2014). Theta mPFC input into the BLA is known to provide a safety signal, thereby reducing innate anxiety (Likhtik, Stujenske et al. 2014). Interestingly, disinhibition of CeM by the lack of functional mPFC – CeA connectivity was

hypothesised to be a plausible reason for increased anxiety in humans and monkeys by a recent study (Birn, Shackman et al. 2014). Increase synchronicity in vHip – mPFC connections have also been implicated in anxiety-like behaviour in mice by a study using extracellular in vivo recording especially in the mPFC neurons which encoded an anxiogenic context (Adhikari, Topiwala et al. 2010). Extinction of fear generated by Pavlovian fear conditioning paradigms is known to activate the infralimbic mPFC (IL-mPFC), which sends glutamatergic projection to the BLA activating the GABAergic interneuronal population within the BLA, thus diminishing its excitatory output to the CeA leading to a suppression of the fear response.

1.7. Molecular basis of conditioned fear

As stated before, molecular mechanisms underlying most complex emotions including anxiety and fear are poorly understood. However, the dysregulation of various system implicated in the psychopathology of anxiety disorders have been studied rigorously, and most of the available mechanistic data available to this end come from studies involving fear conditioning paradigms. Hence, like in the previous section, I will keep the discussion in this part of the introduction limited to molecular mechanisms relevant to fear conditioning paradigms.

One could confer almost all mechanistic aspects of fear learning and relearning onto changes in synaptic plasticity that affect intracellular signalling cascades, which ultimately affect changes in gene expression via basic transcriptional and epigenetic mechanisms to mediate long-term memory formation. This information is concisely presented in Fig 3.
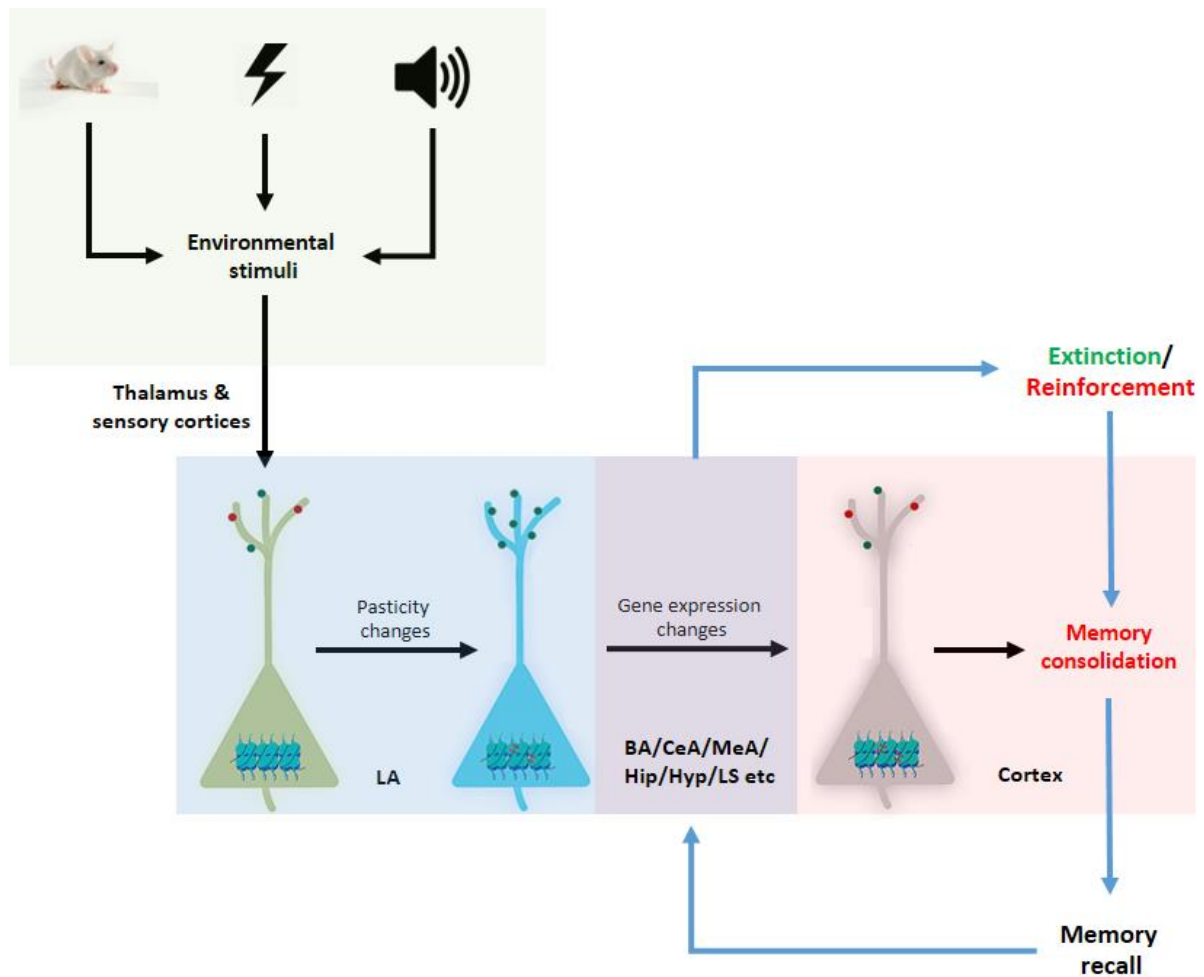
Fig 3. Schematic representation of the molecular processes involved in fear conditioning, fear memory consolidation and extinction. LA (lateral amygdala); BA (basal amygdala); CeA (central amygdala); MeA (medial amygdala); Hip (hippocampus); Hyp (hypothalamus); LS (lateral septum).

Here, I will firstly examine the role of mechanisms modulating synaptic plasticity during fear memory formation, which will be followed by an introduction of the role of mechanisms regulating gene expression regulation in consolidation and extinction of conditioned fear.

1.7.1. Environment to the neurons: synaptic plasticity and fear acquisition

As was shown in Fig 1, most of the sensory information about the CS and US in a fear conditioning paradigm from thalamic and cortical areas converges at the LA and the BLA making them key regions for the acquisition of fear (Quirk, Armony et al. 1997, Lin, Yeh et al. 2003). This idea is complemented by studies, which show that conditioning-induced plasticity within the LA precedes freezing behaviour and thus seems to be a prerequisite for coding of fear memory (Repa, Muller et al. 2001). Most of the current evidence suggests that associations between the CS and US require Hebbian-plasticity (Hebb., 1949) provoked by US-mediated depolarization within the LA (Johansen, Cain et al. 2011). Most of these plasticity-modulating environmental stimuli lead to the glutamatergic transmission, which functions by increasing the intracellular Ca^{2+} concentration in post-synaptic neurons (Johansen, Cain et al. 2011). A number of glutamatergic receptors including the post-synaptic N-methyl-d-aspartate receptors (NMDARs) – both ionotropic and non-ionotropic – are thought to mediate plasticity within the LA (Rodrigues, Schafe et al. 2001). This, in turn, leads to autophosphorylation of the Ca^{2+}/Calmodulin (CaM) dependent protein kinase II (CaMKII), which has been shown to increase the dendritic spine density and activate fear memory formation via both direct and indirect mechanisms (Silva 2003). One example of the indirect mechanism could be that autophosphorylation and thus activation of CaMKII could lead to phosphorylation of the serine 831 residues of α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid-type glutamate receptor (AMPAR) GluA1 subunit which leads to its translocation and insertion into the cell membrane. Such insertion increases the synaptic strength and is known to aid in short-term memory formation by activation of the so called 'silent synapses' (Malinow and Malenka 2002, Rumpel, LeDoux et al. 2005, Johansen, Cain et al. 2011). Metabotropic glutamate receptors (mGluRs), which are also stimulated by such CS- and/or the US- induced glutamate release, spark acquisition of contextual fear, but are seldom involved in consolidation. Indeed, activation of the group I mGluR5 within the LA and the BLA is known to aid acquisition of

conditioned fear. Such mGluR5 activation within the LA could, in turn, activate the NMDARs and also increase intracellular Ca^{2+} levels via the inositol 1,4,5-triphosphate pathway and thereby activate the downstream CaMKII leading to further enhancement of fear memory formation (Rodrigues, Bauer et al. 2002, Rudy and Matus-Amat 2009, Johansen, Cain et al. 2011).

Evidence from several pharmacological studies has implicated plasticity changes within the GABAergic interneurons within different amygdalar subnuclei in fear memory modulation (Ehrlich, Humeau et al. 2009) (also see section 1.6.1). Quite a few studies have implicated activation of the GABA-synthesizing interneurons in inhibition of projection neurons within the LA leading to modulation of fear memory formation and expression (Bissiere, Humeau et al. 2003, Tully, Li et al. 2007). Although the exact function of GABAergic interneurons within the LS is not clearly known, few studies have been able to shed light on general GABA function in the context of fear conditioning paradigms. Activation of the $GABA_A$ receptor was shown to reduce cued fear acquisition, and this is complemented by studies showing a transient reduction in LTP and GAD65 expression following fear conditioning (Muller, Corodimas et al. 1997, Johansen, Cain et al. 2011). Interestingly, pharmacological blockade of the GABA receptor α1 subunit increases cued fear learning. Although the later result is against a popular opinion which is that GABAergic signalling leads to impairment of fear learning, the reason for such results could be because of increase in GABA availability for receptors which lack this subunit (Wiltgen, Godsil et al. 2009).

1.7.2. From synapse to nucleus: Cell signalling molecules and transcription factors involved in coding of fear

Most of the signalling cascades involved in fear memory formation or extinction memory formation function by increasing intracellular Ca^{2+}. Such increase leads to a barrage of molecular changes within the neuron including the activation of CaMKII and CaMKIV which

seems to be a prerequisite for strong memory formation following fear conditioning as mentioned before. Ca^{2+} also interacts with and activates the Ca^{2+}/ phospholipid-dependent protein kinase (PKC) and cAMP-dependent protein kinase (PKA) which eventually leads to gene expression changes within the nucleus and long-lasting changes in the disposition of a neuron via LTP. Involvement of PKA and PKC in fear memory formation are supported by many studies. Pharmacological blockade of PKA with the specific inhibitor RP-cAMPS within the LA inhibits long-term memory formation, while short-term memories stay intact indicating that signalling cascades involving PKA are involved in fear memory consolidation (Schafe and LeDoux 2000). Even studies with transgenic mice expressing an inactive form of PKA within the hippocampus show reduced LTP and impaired contextual memory formation thus corroborating the role of PKA in the formation of fear memory. PKMζ, a PKC isoform, was shown to be upregulated on induction of hippocampal LTP and is necessary and sufficient for it (Ling, Benardo et al. 2002). CaMKII, PKA and PKC act upon the mitogen-activated kinase (MAPK) signalling cascade, which ultimately leads to the regulation of gene transcription leading to fear memory consolidation and long-term memory formation. Activation of MAPK could lead to subsequent activation of 7 different signalling cascades in vertebrates (Adams and Sweatt 2002). Synchronous activation of CaMKII, PKA and PKC leads to eventual activation of extracellular regulated kinase (ERK) 1 and 2 via a pathway involving MAPK/ERK kinase (MEK) activation. ERK1 and ERK2 translocate to the nucleus and lead to a variety of changes ultimately leading to changes in gene expression and increase in synaptic strength (Wu, Deisseroth et al. 2001, Orsini, Yan et al. 2013). Ample data from studies involving pharmacological and genetic modulation of members of the MAPK signalling cascade have cemented our understanding of their role in formation of fear memories (English and Sweatt 1996, Brambilla, Gnesutta et al. 1997, Merino and Maren 2006)

1.7.3. Changes within the neuronal nucleus: consolidation and formation of long-lasting fear memories

Within the context of fear memories, most signalling cascades converge within the nucleus on the cyclic-AMP (cAMP)/ Ca^{2+} response element-binding protein (CREB). Indeed, CREB activation by MAPK and PKA signalling within the LA seems to be the key for regulation of fear memory formation and consolidation (Hernandez and Abel 2008). ERK1 activates a downstream kinase RSK within the nucleus and this mediates phosphorylation of CREB at the ser133 residue (Impey, Obrietan et al. 1998, Orsini and Maren 2012, Naqvi, Martin et al. 2014). Ca^{2+} dependent activation of CAMKIV also activates CREB. An important difference between the two modes of CREB activation is that CAMKIV-mediated CREB activation is more of an activity-dependent rapid activation, whereas ERK/RSK activation exerts a slower and prolonged effect (Thomas and Huganir 2004). However, the most substantial work in the role of CREB in fear memory formation comes from the work of Sheena Josselyn's lab. Over the last decade, Josselyn and colleagues have shown that neurons expressing an activated form of CREB are preferentially included in the fear memory trace and that CREB over-activation within the LA leads to long-lasting fear memories (Han, Kushner et al. 2007). This study also showed that mice deficient for CREB showed impaired auditory fear conditioning, an effect that could be completely rescued by transfection of the LA with a herpes simplex virus based CREB overexpressing vector even though the vector transfected only 15% of LA neurons (Han, Kushner et al. 2007, Han, Yiu et al. 2008, Yiu, Mercaldo et al. 2014). Once activated CREB binds to its partner - the CREB binding protein (CBP)/p300 and its target DNA binding site, i.e. the CREB response element (CRE) leading to activation of more than 100 downstream target genes including many immediate early genes (IEGs) (Lonze and Ginty 2002). These IEG can be classified as activity-induced regulatory transcription factors (RTF) which include *cFos, zif268* etc and effector IEGs such as *bdnf, arc, homer1a* etc (Chaudhuri 1997). Overexpression of most of these IEGs has been previously correlated with enhanced fear memory formation

and strength by studies utilising contextual and auditory fear conditioning paradigms. For example, an elevated cFos expression has been observed in the ventral part of LA after auditory fear conditioning which fit with results from another study that reveals that neurons in this region exhibit increased activity during fear training and extinction (Radulovic, Kammermeier et al. 1998, Ressler, Paschall et al. 2002, Ploski, Park et al. 2010). An interesting gene is Arc/Arg3.1, whose mRNA undergoes nuclear export and is preferentially localised in the dendrites with activated synapses in the rat dentate gyrus in response to LTP (Rodriguez, Davies et al. 2005). Neurotrophic factors such as BDNF, which is also a CREB target and its receptor, the tropomyosin-related kinase B (TrkB) regulate synaptic plasticity. Activity-dependent release of BDNF (pre or post-synaptic) correlates with increased LTP (Schinder and Poo 2000, Messaoudi, Ying et al. 2002, Lessmann, Gottmann et al. 2003). In concert, inhibition of BDNF activity within the LA with either TrkB antagonism or its genetic silencing prevents long-term memory formation (Rattiner, Davis et al. 2004). This is in contrast to a study which shows that ablation of BDNF early during development specifically within the forebrain leads to an enhancement of fear memories.

Once consolidated, fear memories are long-lasting in nature. However, if these associations are recalled, as it happens during fear extinction, these memories become labile and susceptible to modifications. During extinction, prolonged or multiple exposures to the CS in absence of the associated US creates a new memory trace predicting lack of CS aversion and this competes with the original fear evoking memory trace. On multiple or prolonged exposures to the unpaired CS, the new memory gets stronger and eventually overshadows the fear memory. In principle, the neuronal and molecular mechanisms involved in extinction of fear memories are more or less similar to the ones involved in the formation of the original fear memory, and thus, they are not discussed here in more detail. However, what I find important to introduce in the course of my thesis is the modulation of neuropeptide systems and epigenetic mechanisms by

cognitive enhancers to aid in the process of extinction with the final aim of developing effective therapeutic strategies for anxiety disorder.

1.8. Role of neuropeptides in regulation of fear

All the factors introduced in the sections above regulate formation, consolidation, and extinction of fear memories in a very intricate and synchronous manner. Although we know a lot about it, fear seems to be more complicated, enigmatic and above these basic mechanisms. Neuropeptides are defined as 'small proteinaceous substances produced and released by neurons through a regulated secretory route and act on neural substrates' and have been implicated in the regulation of fear and anxiety by several studies. They are one of the most diverse class of neuronal signalling molecules, and there are more than 70 genes within the mammalian genome, which code for neuropeptides and their receptors. However, as the focus of my thesis is centred around the oxytocin (OXT) system, I will only elaborate on OXT-mediated regulation of anxiety and fear.

1.8.1 Neuropeptide oxytocin and its receptor

The ligand: OXT
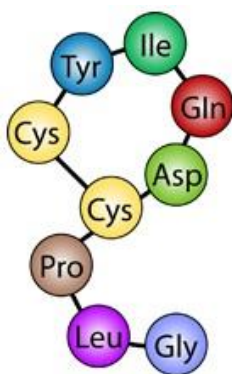
Fig 4. *Neuropeptide Oxytocin*

OXT is a 9-amino acid long neuropeptide, which was one of the first peptide hormones to be structurally characterised and chemically synthesised (Du Vigneaud, Ressler et al. 1953). It is produced within the hypothalamic paraventricular (PVN), supraoptic (SON) and accessory nuclei by magnocellular and parvocellular neurons, which are distinct in morphology, subnuclear location, projections and the amount on OXT produced (Swanson and Sawchenko 1983, Eliava, Melchior et al. 2016). The parvocellular OXT neurons project mainly to the brainstem and spinal cord where they are involved in the control of feeding

behaviour, cardiovascular function, nociception and breathing (Swanson and Sawchenko 1983, Mack, Kc et al. 2002, Eliava, Melchior et al. 2016). The magnocellular OXT neurons project to the posterior pituitary leading to the systemic release of OXT via blood in response to a variety of environmental stimuli. Magnocellular neurons also innervate forebrain regions leading to specific control of behaviour (Bargmann and Scharrer 1951, Knobloch, Charlet et al. 2012). Central release of OXT controls a wide range of socio-emotional behaviors such as maternal behavior (Bosch 2011), sexual behavior (Argiolas and Gessa 1991, Waldherr and Neumann 2007), pair bonding (Bales, van Westerhuyzen et al. 2007), social recognition (Oettl, Ravi et al. 2016), social anxiety (Zoicas, Slattery et al. 2014) among others (for review see: (Landgraf and Neumann 2004)). Local effects of OXT within the SON are more likely due to its dendritic release to attain paracrine effects (Ludwig and Leng 2006).

Effective modulation of these behaviours requires regulation of OXT synthesis and release, which is realised by different genomic and non-genomic mechanisms acting in a fine-tuned way so as to generate appropriate responses to specific environmental and physiological stimuli. OXT is released both centrally and peripherally in response to various physiological stimuli including stress, suckling during lactation, social interactions, mating etc (Neumann, Russell et al. 1993, Waldherr and Neumann 2007, Zoicas, Slattery et al. 2014). For example, OXT is shown to be released in response to forced swimming in the CeA of male rats, where it presumably supports passive stress-coping (Ebner, Bosch et al. 2005). OXT release is also known to be regulated by other neuropeptides. For example, prolactin is known to inhibit OXT release in virgin rats without interfering with the intracellular signalling cascades and this inhibition is lost during lactation enabling effective milk ejection (Augustine, Ladyman et al. 2017). Another example of such interaction comes from recent unpublished data, which conclusively show that the anxiolytic effects of the neuropeptide S (NPS) within the PVN are mediated via the OXT system (Grund et al, unpublished). At the level of DNA, the OXT promoter contains an oestrogen receptor (ER) response element (ERE) and is activated by

interaction with ERβ (Nomura, McKenna et al. 2002, Shughrue, Dellovade et al. 2002). A genomic mechanism which has recently been highlighted in this regard is that of the DNA methylation of the OXT promoter. A recent study has found that low DNA methylation of the OXT promoter (possibly leading to high OXT expression) in human epithelial cells found in saliva samples to be correlated to high sociability and emotional recognition (Haas, Filkowski et al. 2016).

The OXT receptor (OXTR)

As mentioned before, OXTergic neurons have long-range axonal projections which enable OXT release in different far off brain regions. Within these regions, OXT regulates its central effects by acting through the single OXT receptor (OXTR) which is spread throughout the brain (Sofroniew 1980, Knobloch, Charlet et al. 2012, Dolen, Darvishzadeh et al. 2013, Mitre, Marlin et al. 2016, Otero-Garcia, Agustin-Pavon et al. 2016). Thus, the flipside to this story is the controlled regulation of OXTR expression by a variety of mechanisms, which gives an organism additional control over socio-emotional behaviour in response to relevant physiological and environmental cues. The OXTR is a classical class I G-protein-coupled receptor with 7 transmembrane domains and is expressed in a variety of brain regions including MeA, BLA, nucleus accumbens (NAcc), BNST, MPOA, Hyp, Hip, ventral palladium, PAG, striatum, LS, VTA and the olfactory bulb (Grinevich, Desarmenien et al. 2014, Grinevich, Knobloch-Bollmann et al. 2016) (Fig 5). OXTR expression within these regions can be regulated depending upon ligand-availability. For instance, lack of OXT after SFC in male mice is compensated by an increase in the OXTR binding within the LS (Zoicas, Slattery et al. 2014). Conversely, chronic administration of OXT leads to a downregulation of the OXTR (Peters, Slattery et al. 2014).

At a genomic level, OXTR promoter possesses binding sites for many transcription factors including c-Myc, SP1, nuclear factor kappa B (NFκB), CREB etc (Blanks, Shmygol et al. 2007). OXTR promoter in rodents contain an ERE, and OXTR expression is activated by ERα.

The human OXTR promoter, however, lacks a complete ERE, and thus, may not be directly activated by ER transcription factors (Levin 2015). Interestingly, the SP1 and ERα binding sites on the OXTR promoter lie within a large CpG island on its promoter. Hence, differential DNA methylation can indeed effect OXTR expression as shown by both *in vitro* and *in vivo* studies in mice (Mamrut, Harony et al. 2013, Harony-Nicolas, Mamrut et al. 2014). This effect is supported by studies in humans, which link reduced OXTR promoter methylation to psychiatric disorders such as SAD and postpartum depression (Ziegler, Dannlowski et al. 2015, Kimmel, Clive et al. 2016).

Such intricate regulation at different levels of OXT and OXTR make them crucial nodal points in research aiming to find putative therapeutic options for anxiety disorders. In the following sections, I examine the current state of the art in the field of anxiety and fear research with respect to OXT system.

1.8.2 Neuropeptide oxytocin and regulation of general anxiety

Though initially known for its peripheral effects on parturition and milk ejection reflex (Hawker and Robertson 1957, Ota, Shinde et al. 1965, Boyd 1972), discovery of its central anxiolytic, prosocial and anti-stress properties in the 1990s has made this neuropeptide a prime candidate for treatment of neuropsychiatric disorders (Jezova, Skultetyova et al. 1995, Cushing and Carter 1999, Neumann and Slattery 2016). Interest in central effects of OXT have been further enhanced by an abundance of studies in humans using intranasal OXT, wherein this neuropeptide is shown to have prosocial and anxiolytic properties (Meyer-Lindenberg, Domes et al. 2011, MacDonald and Feifel 2014). Indeed, intranasal OXT has been studied as a mode of OXT delivery for treatment of autism spectrum disorders, PTSD, SAD amongst others (Eckstein, Scheele et al. 2016, Neumann and Slattery 2016, Ooi, Weng et al. 2017).

Ample studies have indicated a definitive role for the OXT system in anxiety and, as a general principle, the OXT is considered to be a strong anxiolytic factor depending on the site of its

infusion. This effect has been confirmed by a number of studies in both male and female rodents with either intracerebroventricular (icv) or local intra-PVN application of OXT, for example in the CeA and PVN, which generates robust anxiolysis as measured using EPM and LDB amongst others (Ring, Malberg et al. 2006, Blume, Bosch et al. 2008). This effect of OXT can be nullified at different levels as centrally blocking the OXTR and the downstream signalling cascade within the PVN with an MAPK inhibitor (Blume, Bosch et al. 2008).

Complementing the abovementioned data obtained using synthetic OXT, studies have shown that an activated endogenous OXT system during lactation (Neumann, Russell et al. 1993, Neumann, Torner et al. 2000, Jurek, Slattery et al. 2012) and sexual activity in both males (Waldherr and Neumann 2007) and females (Nyuyki, Waldherr et al. 2011) has an anxiolytic effect, and in such a situation, blockade of OXTR by OXTR antagonist (OXTR-A) prevents this effect.

1.8.3 OXT and the regulation of fear

The OXTR is expressed in the CeA, MeA, BLA, mPFC, LS, HiP and Hyp suggesting that the OXT system plays a crucial role in the regulation of fear memories. However, the effect of OXT on fear memory formation, consolidation and extinction have been very difficult to dissect. This is because of very temporal, region and context-specific effects of this system on fear regulation.

Recently, it has been shown in rats and mice that icv infusion of OXT before cued fear extinction leads to an increase in fear expression, whereas similar treatment before cued fear acquisition leads to a reduced fear expression during extinction in the CFC paradigm (Toth, Neumann et al. 2012). More region-specific pharmacological approaches have shown that the fear-enhancing effects of OXT are due to its actions on the CeA, as infusion of an OXTR-A ((Thr^4, Gly^7)-OXT) before contextual fear conditioning reduced fear responses (Lahoud and Maroun 2013), and a similar infusion into the BLA led to enhancement in fear (Maroun and

Wagner 2016). In contrast, fear-reducing effect of OXT has been shown within the CeA via the disinhibition of the local GABA circuit (Viviani, Charlet et al. 2011). Interestingly, the CeL^{off} neurons (see section 1.6.1) which are $PKC\delta^+$ also express the OXTR, and specific optogenetic activation of OXTergic inputs to the CeL leads to activation of the CeL^{off} neurons, which in turn inhibits the CeM leading to an attenuation of fear responses within the CFC paradigm (Knobloch, Charlet et al. 2012).

Manipulation of the OXT system within the LS, a region that controls stress, fear and social memory, also, modulates fear in a social context. Studies have shown that genetic overexpression OXTR enhances social defeat-induced social avoidance and subsequently enhanced contextual memory (Guzman, Tronson et al. 2013). This was supported by studies from our lab, which show an increased OXTR binding with a concomitant reduction in OXT release in the LS of mice fear conditioned using the SFC paradigm. In the same study intra-LS infusion of OXT was shown to reverse SFC-induced social fear (Zoicas, Slattery et al. 2014). Thus, OXT within the LS seems to enhance social memory in a valence-independent way. In support of this idea, previous studies from our lab have shown that LS-OXT system mediates social memory in male rats against juveniles and adult females (Lukas, Toth et al. 2013). Another region, where OXT was found to modulate fear memory is the infralimbic-mPFC (IL-mPFC), where OXT infusion before fear memory retrieval enhanced extinction of fear. OXT-mediated induction of LTP, which subsequently leads to a strengthening of glutamatergic synapses within the IL-mPFC could underlie the IL-mPFC-OXT effects on the extinction of fear (Vouimba and Maroun 2011, Lahoud and Maroun 2013, Maroun and Wagner 2016).

1.9 Epigenetics – the molecular bridge between environment and behaviour

Converging evidence from neurological, molecular and genetic studies suggests an important role of epigenetic mechanisms and factors in the aforementioned psychopathologies (Levenson and Sweatt 2005, Tsankova, Renthal et al. 2007, Chahrour, Jung et al. 2008, Ohnishi, Ohnishi et al. 2011, Robison and Nestler 2011). Epigenetics and its associated terms have several different connotations. If one thought of the 'genome' as the DNA and the nucleotide sequence it encodes, then the 'epigenome' could be thought of the genome with the associated histone-assembly and the DNA-methylation patterns, which together form the 3D chromatin structure, which when modified, alters the spatial availability of transcription enhancing or repressing sequences and thus providing an extra level of gene expression. This kind of regulation helps the genome to respond to environmental stimuli in a fine-tuned manner and thus forms the 'molecular bridge' between the environment and genes. Some of the best studied epigenetic mechanisms in the context of neuropsychiatric disorders are histone modification and DNA methylation. Although other epigenetic mechanisms like RNA modification, non-coding RNA-mediated regulation and genomic imprinting are known to regulate emotionality, they are out of the scope of this thesis and will not be discussed here.

1.9.1 Histone modifications and regulation of fear

Chromatin is tightly packed within the nucleus and consists of DNA and proteins, which facilitate its organisation within the nucleus. The most basic unit of chromatin is the nucleosome consisting of DNA sequence with 147 base pairs (bp) in length, which is wound around a histone protein octamer which consists of 2 canonical copies of H2A, H2B, H3 and H4 (Luger, Mader et al. 1997) (Fig 5). The N-terminal tail of the histone monomer projects out of the nucleosomal complex and contains a multitude of positively charged amino acid residues, such as threonine, serine and lysine. These residues are covalently modified by acetylation, methylation, phosphorylation, sumoylation to change the affinity of the histone octamer to the DNA and consequently effecting changes in gene expression. Fig 6 gives a summary of the most well-studied histone modification and proteins involved in their execution.
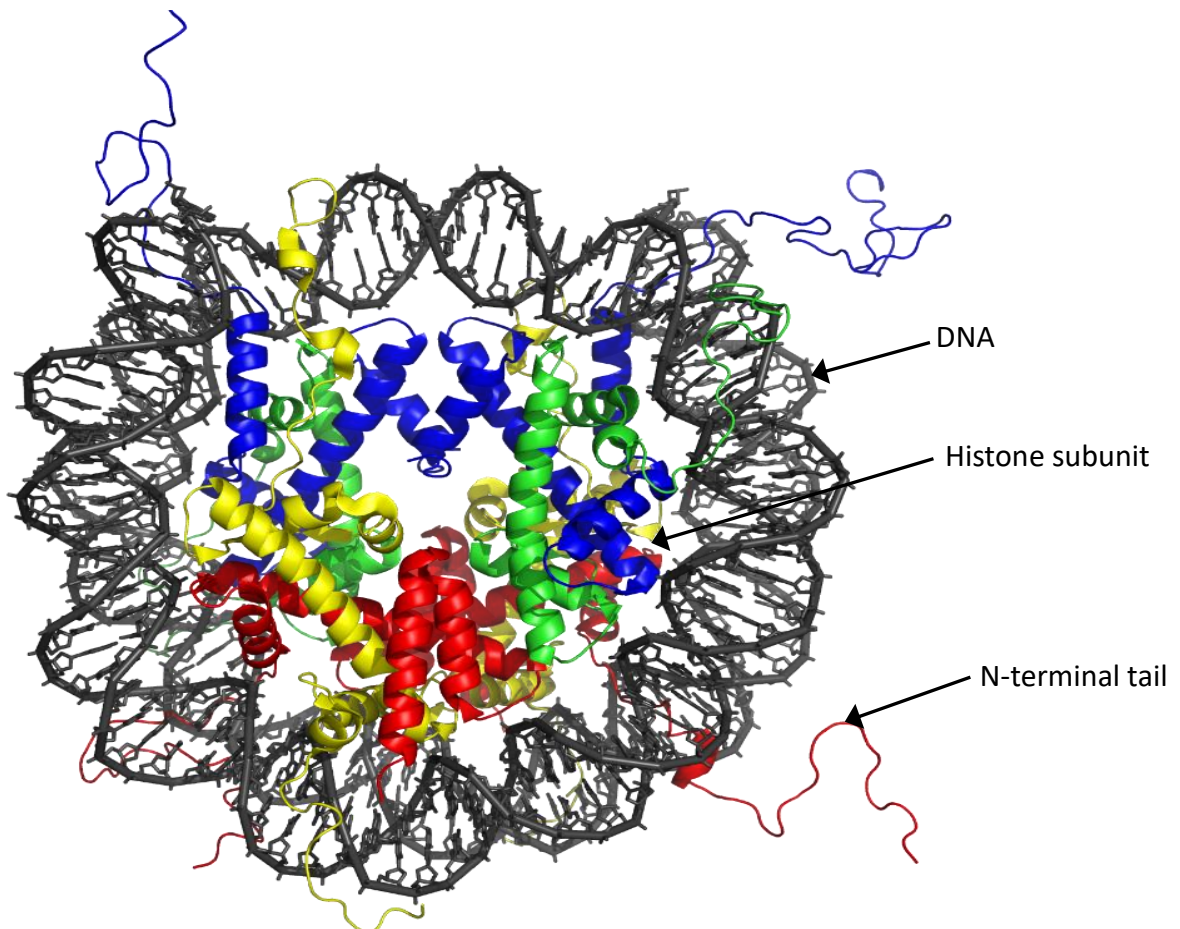
Fig 5. Crystal structure of a nucleosome (Fig modified from protein data bank)

Phosphorylation and dephosphorylation of histones are brought about by histone kinases and phosphatases, respectively (Berger 2010). Contextual fear learning is known to induce histone phosphorylation. In mice, inhibition of histone kinases like mitogen and stress-activated kinase 1 (MSK1) reduces H3 and H4 phosphorylation and impairs contextual fear (Chwang, O'Riordan et al. 2006). However, MSK1's phosphorylation targets also include CREB and NFκB, and this possesses a challenge, which has not been yet resolved (Arthur 2008). Dephosphorylation is known to be intimately linked with histone acetylation, as evidenced by the fact that overexpression of protein phosphatase 1 (PP1) reduces histone deacetylase (HDAC) function and enhances object recognition memory (Koshibu, Graff et al. 2009). Methylation of histones is one of the most enigmatic post-translational modifications. Although it has been implicated
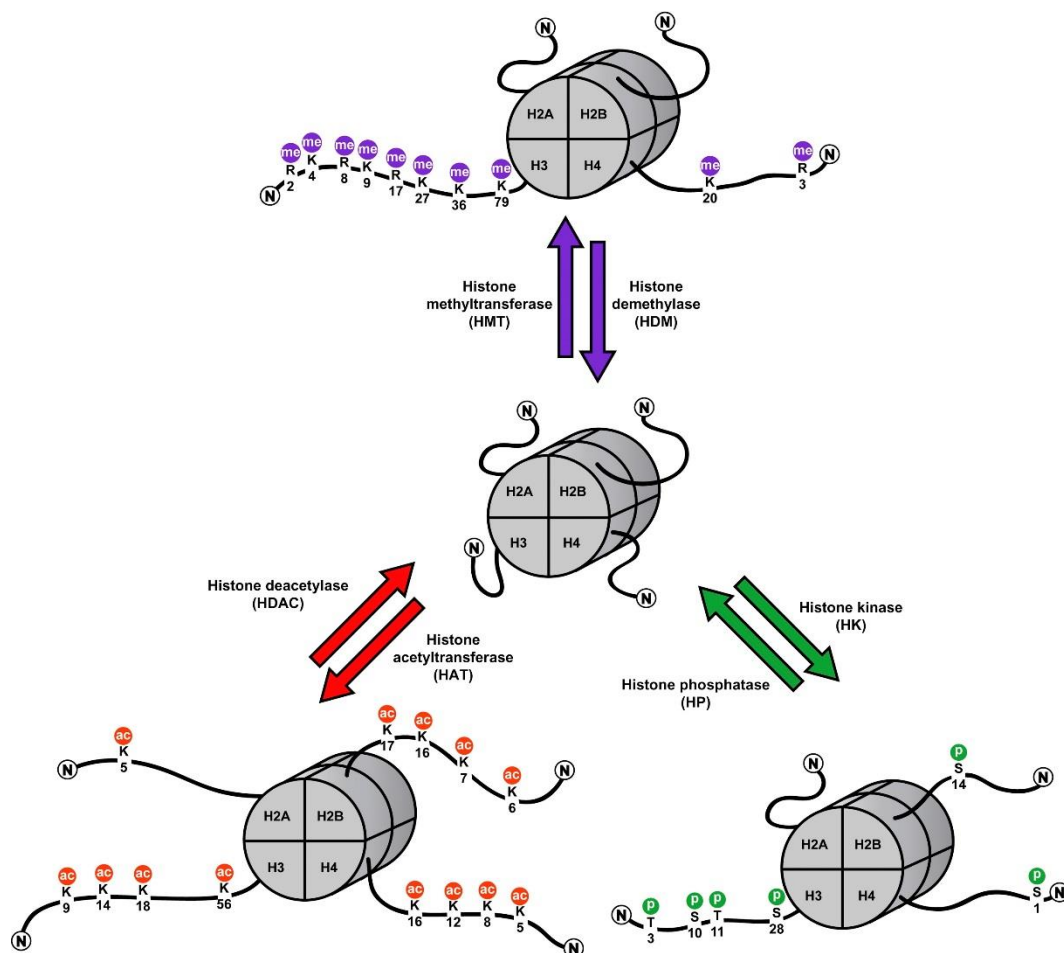
Fig 6. *Post translational histone modifications (K: lysine, R: arginine, S: serine, T: threonine, me: methylation, ac: acetylation, p: phosphorylation and N: nitrogen terminal) (Picture has been modified from: Epigenetic regulation of the nervous system, Sweatt et. al.,).*

in memory formation and CNS function, its diversity makes it a complicated phenomenon. Methylation of histones occurs at both lysine and arginine residues and could have 3 possible variations: monomethylation, dimethylation or trimethylation each leading to a different outcome. H3K4 trimethylation – a mark associated with active transcription – is increased after contextual fear conditioning in the Hip on the promoters of an IEG, *zif68* and the neuronal plasticity factor *bdnf* (Gupta, Kim et al. 2010).

Although it is tempting to introduce the role of histone phosphorylation and methylation in fear memory, keeping the scope of this thesis in view, I will focus on histone acetylation, which is the most well-studied histone modification and is one of the central themes of this thesis. Acetylation of the N-terminal tails of H3 and H4 histones are known to be involved in the maintenance and consolidation of fear memory are discussed in detail (Bredy, Wu et al. 2007, Fischer, Sananbenesi et al. 2007, Peleg, Sananbenesi et al. 2010). The bidirectional catalysis of acetylation marks on histones is brought about by a set of enzymes called histone acetyltransferases (HATs), which catalyse the addition, and histone deacetylases (HDACs), which aid the removal of acetyl groups (Strahl and Allis 2000).

CBP which is a transcriptional coactivator of CREB, has intrinsic HAT activity and is known to be involved in transcription of several memory-related genes. Indeed, both HAT activity and CREB binding activity of CBP is essential for long-term memory formation and LTP (Korzus, Rosenfeld et al. 2004). Inhibition of HDACs is known to augment memory formation, to cause long-term potentiation within the hippocampus, and also to restore the ability to form new associations in mice (Levenson, O'Riordan et al. 2004, Fischer, Sananbenesi et al. 2007, Peleg, Sananbenesi et al. 2010). Class I HDACs, i.e. HDAC1, HDAC2, HDAC3 and HDAC8 have been previously implicated in memory formation and regulation (Yang, Chen et al. 2013, Penney and Tsai 2014, Adler and Schmauss 2016, Nott, Cheng et al. 2016), and specific pharmacological inhibition of different isoforms is known to have specific effects. For example, overexpression of HDAC2 increases spine density and synaptic plasticity, whereas

overexpression of HDAC1 under similar conditions has no effect (Guan, Haggarty et al. 2009). Using a viral knockdown strategy, a recent study also demonstrated that blocking HDAC3 in the hip and BLA enhanced contextual fear memory, whereas blocking HDAC3 within the LA enhanced cued fear (Kwapis, Alaghband et al. 2017). Such differential HDAC activity extends in an isoform-, brain region- and context-specific manner to mouse models of anxiety disorders (Bahari-Javan, Maddalena et al. 2012, Morris, Mahgoub et al. 2013, Graff, Joseph et al. 2014). Multi-layered regulation of acetylation of histones presents us with numerous targets and difficulties in the treatment of anxiety-related disorders. Although research in this field is in its infancy, great advances in the availability of genetic tools and specific pharmacological inhibitors present brand new avenues for development of therapeutic options.

1.9.2 DNA methylation and regulation for fear and anxiety
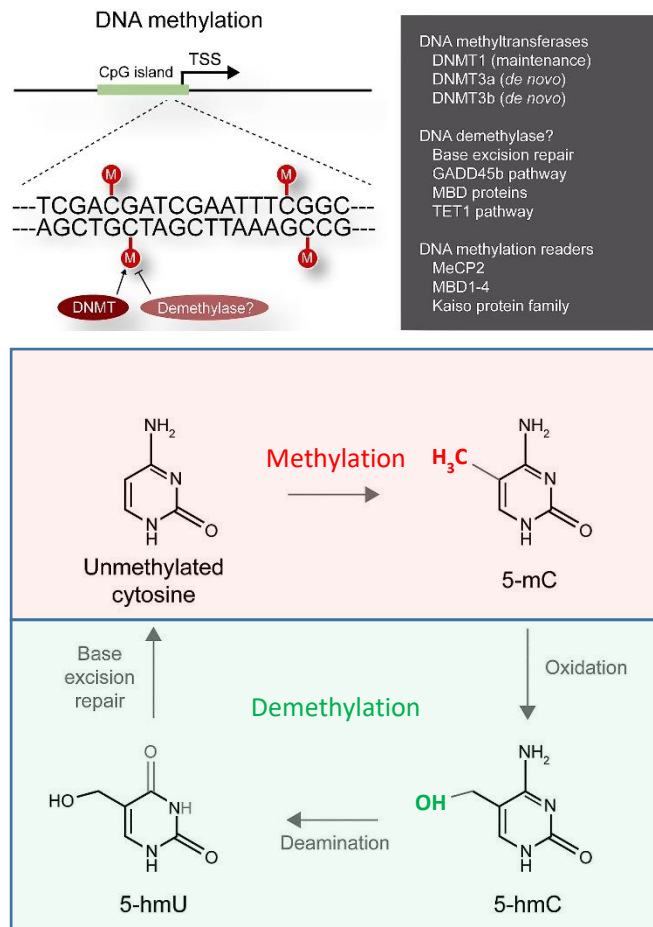
Fig 7. *DNA methylation schematic representation (TSS: transcription start site, M: CH3/methyl group). (Picture has been modified from: Epigenetic regulation of the nervous system, Sweatt et. al.,).*

DNA methylation refers to the covalent modification of the cytosine base in the DNA sequence with the addition of a 5' methyl group. Methylation occurs at regions within the DNA sequence which are rich in cytosine (C) and guanosine (G) nucleotides which are usually underrepresented in the genome and occur in small clusters called labelled 'CpG islands' usually resulting in suppression of gene expression (Cooper and Krawczak 1989).

This view has been challenged with recent discoveries describing methylation of cytosine residues outside the context of CpGs and also methylation leading to transcriptional activation (Chahrour, Jung et al. 2008, Cohen, Zhou et al. 2008). Enzymes that add methyl groups to the cytosine residues are called DNA methyltransferases (DNMTs), and they can be functionally categorised into maintenance DNMT (DNMT1) and *de novo* DNMTs (DNMT3a and DNMT3b). DNMTs are expressed in the brain although the DNMT3b expression is low in the adult CNS (Feng, Zhou et al. 2010). Contextual fear conditioning is known to enhance hippocampal DNMT levels (Miller and Sweatt 2007). Consistent with this notion, inhibition of DNMTs impairs memory formation (Lubin, Roth et al. 2008). Glenn Schafe's group has shown that cued fear conditioning is associated with increased DNMT3A expression within the LA and inhibition of this protein impairs formation of cued fear (Maddox and Schafe 2011). Interestingly, such

learning produces methylation and demethylation at specific sites presenting a much more complicated picture of involvement of DNA methylation in synaptic plasticity and memory formation (Day and Sweatt 2010). The role of DNA demethylation in fear memory formation and maintenance is not clearly understood. Clinical studies have linked differential methylation of various genes with symptoms of anxiety disorders. One of the well-studied system in this regard is the OXT system (as discussed in detail in section 1.8.1)

1.10 Aims of the present thesis

As explained before in section 1.3.1 and 1.5.3, using the SFC paradigm we can generate SAD-like symptoms in mice. Social anxiety generated by SAD is devoid of confounding symptoms of general anxiety and depression-like behavior which plagues most of the well-established models of SAD. This makes the SFC paradigm unique, in that we can specifically study the molecular and neuronal mechanisms underlying social anxiety and avoidance. During my PhD thesis, I focused on the molecular and neuronal adaptations which occur during SFC in male and female CD1 mice. Within the realm of the abovementioned background I focused on answering the following questions:

a. Does endogenous OXT signaling regulate fear in female mice?

b. Do epigenetic mechanisms play a role in regulation of fear memory in male mice?

Thus, to answer the following questions we performed 2 different studies each one of which is described below.

1.10.1 Studying the role of endogenous OXT signaling in regulation of social and cued fear in mice

Many studies in the past have tried to understand the effect of OXT on different forms of conditioned fear. Data obtained over the past decades by us and others have showed temporal and spatial differences in OXT-effects on trauma using cued fear conditioning based models (Knobloch, Charlet et al. 2012, Toth, Neumann et al. 2012, Guzman, Tronson et al. 2013, Zoicas, Slattery et al. 2014). For social trauma, OXT-effects seem to me more straightforward. Using the SFC paradigm, our lab has previously shown that LS-OXT infusion reverses SFC-induced social fear (Zoicas, Slattery et al. 2014). However, most of these studies involve delivery of supraphysiological amounts of OXT (Toth, Neumann et al. 2012, Zoicas, Slattery et al. 2014). Although these studies reveal important information about role of OXT system on conditioned fear, they do not tell us a lot about how endogenous OXT system effect social and

non-social trauma generated by SFC and CFC respectively. Also, most of the studies performed in the field of fear and anxiety research use male rodents as primary subjects of research even though most anxiety disorders are twice as prevalent in females as in males (see section 1.3.1). In lieu of above-mentioned studies my first aim was as follows:

"I aimed to find out the role of activated brain OXT signaling in regulation of fear in female mice"

1.10.2 To delineate the epigenetic mechanisms within the LS the regulate fear in male CD1 mice.

Although widely studied in the context of non-social fear memory formation and extinction, our knowledge about the role of epigenetic mechanisms, i.e., both histone modifications and DNA methylation in the context of social fear remains negligible. In the case of HDACs, current research suggests extreme region and isoform-dependent specificity in HDAC function and, consequently, there is a lack of clarity in our understanding of the role of specific HDACs in mediating molecular and neuronal mechanisms involved in fear learning and extinction. This impedes progress in the development of effective therapeutic options for specific subtypes of anxiety disorders. Therefore, in this part of the thesis, my aim was as follows:

"To identify the role of HDACs' in regulation of fear in male CD1 mice."

Materials and Methods

This section describes the techniques used during the course of the current thesis in detail. For greater convenience, the techniques used have been divided into behavioural and molecular techniques. At the end of each method, a list of experiments pertaining to the corresponding results section, wherein these techniques were used, is provided.

2.1. Behavioral techniques

2.1.1 Animals

Male, virgin female or lactating CD1 mice (Charles River, Sulzfeld, Germany, 8-12 weeks of age at the start of experiments) were kept group-housed under standard laboratory conditions (12/12 h light/dark cycle, lights on at 06:00, 22°C, 60 humidity, food, and water *ad libitum*) in polycarbonate cages (16 x 22 x 14 cm) until described otherwise. Age and sex-matched CD1 mice were used as social stimuli in the Social Preference Test (SPT) and the SFC paradigm. All experimental procedures were performed between 08:00 and 15:00 hrs in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the NIH.

2.1.2. SFC paradigm

SFC was performed as previously described on 3 consecutive days (Toth, Neumann et al. 2012).

Social fear acquisition (day 1). For social fear acquisition all 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). After a 30-s adaptation period, they were presented an empty wire mesh cage (7 x 7 x 6 cm) as a non-social stimulus for 3 min, which was replaced with an identical small cage containing an unfamiliar sex and age matched conspecific. Unconditioned (SFC⁻) control mice could freely investigate the social stimulus in the conditioning chamber for 3 min without receiving any foot shocks, whereas social fear conditioned (SFC⁺) mice were

given a 1-s electric foot shock (0.7 mA) each time they investigated the social stimulus. If a mouse received just 1 foot shock, then it was observed for an additional 6 minutes for social contact after which, lack of contact with the conspecific meant that the animal had acquired social fear and was returned to its home cage. However, if the test mouse approached and investigated the conspecific again within this 6 min, indicating a lack of social fear learning, it received another foot shock and was returned to its home cage, when no further social contact was made for 2 min. This was true for any number of foot shocks above 2, and on average, SFC$^+$ mice received 2-3 foot shocks (mean 2.4 foot shocks/mouse)

Social fear extinction training (day 2). One day after SFC, mice were exposed to 3 non-social stimuli (empty cages) in their home cage to assess non-social investigation as a parameter of non-social fear and general anxiety-related behaviour. Mice were then exposed to 6 different unfamiliar social stimuli, i.e. 6 different age and sex matched mice, each in a different small cage to assess social investigation as a parameter of social fear. Each stimulus was placed near a short wall of the home cage and presented for 3 min, with a 3-min inter-stimulus interval. Gradual, increase in the percentage of time spent investigating the social stimulus for the SFC$^+$ mice were a definitive measure of social fear extinction.

Social fear extinction recall (day 3). One day after extinction training, mice were exposed to 6 different, age and sex matched, unfamiliar social stimuli in their home cage, each in a different small cage (see days 1 and 2), for 3 min with a 3-min inter-stimulus interval.

2.1.3. CFC paradigm

CFC was performed as described previously on 3 consecutive days (Toth, Neumann et al. 2012).

Cued fear acquisition (day 1). All mice were placed in the conditioning chamber (context A; transparent Perspex box: $23 \times 23 \times 36$ cm with electric grid floor; cleaned with lemon scented detergent) and, after a 5-min adaptation period, exposed to five conditioned stimulus (CS) – unconditioned stimulus (US) pairings with a 2-min inter-stimulus interval. The CS (80 dB, 8 kHz, 30 s continuous sound) co-terminated with a mild electric foot shock (US; 0.7 mA; pulsed current, 2 s). The animals were returned to their home cage after the last CS-US pairing.

Cued fear extinction training (day 2). On day 2, mice were placed in context B (black Perspex box: $23 \times 23 \times 36$ cm with a smooth floor; cleaned with floral smelling detergent) and, after a 5-min adaptation period, exposed to 20 CS presentations with a 5-s interstimulus interval. They were returned to their home cage after the last CS presentation. These CS presentations were collapsed into ten blocks with the mean freezing percentage during two CS presentations represented in each block respectively.

Cued fear extinction retention (day 3). One day after cued fear extinction training, animals were again placed in context B, and after a 5-min adaptation period, they were exposed to 2 CS presentations with a 2-min inter-stimulus interval. Animals were returned to their home cage after the last CS presentation. These CS presentations were then collapsed into one block with each bar representing the mean freezing percentage during 2 CS presentations.

2.1.4. EPM

 mFor general anxiety-related behaviour, mice were tested on the EPM as previously described (Lister 1987). The apparatus consisted of two open (6 x 30 x 0.2 cm, 100 lx) and two closed (6 x 30 x 16 cm, 30 lx) arms radiating from a central platform (6 x 6 cm) at an elevation of 35 cm above the ground. Mice 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 innate non-social anxiety. The number of entries into the closed arms is an indicator of locomotor activity.

2.1.5. SPT

The test is based on the social approach-avoidance test previously described in mice (Berton et al., 2006; Lukas et al., 2011). All mice were transferred from their home cage to a new observation cage, which was considered as the novel environment. After a 30-sec habituation period, mice were exposed to an empty wire-mesh cage (7 x 7 x 6 cm) placed at one end of the observation cage (like for social fear extinction and recall) for 2.5 min. The empty cage was then exchanged for an identical cage containing an age- and sex- matched conspecific for another 2.5 min. The percentage of time mice spent investigating the non-social versus social stimulus considered to be the measure of social preference.

2.1.6. Hargreaves' Plantar Test

The Hargreaves' Plantar Test was used to test for possible lactation-associated adaptations in pain perception. Mice were trained for 3 consecutive days, twice a day to remain calm in a transparent, bottomless plexiglass box (8 x 6 x 6 cm) with holes for breathing, which was placed on the glass floor of the test apparatus for 10 min. On the test day mice were placed in the Plexiglas box on the glass floor of the test equipment (Ugo Basile model 7371, Monvalle, Italy) 10 min before the test started. Subsequently, a focused thermal heat stimulus was delivered from a fixed distance to the plantar surface of the hind paw, and paw withdrawal latency was measured for up to 15 s. Each of the hind paws was tested thrice. Data represent an average of 6 trials per mouse. Each mouse was tested at 2 different intensities (0.50: 190mW/cm^2; 0.70: 245mW/cm^2) with an inter-test interval of 2 hrs.

2.1.7. Stereotactic implantations

Implantation of guide cannulas (21 G: icv and 23G: local, 8 mm length; Injecta GmbH, Germany) for icv (from Bregma +0.2 mm, lateral +1.0 mm, depth -1.4 mm) or bilateral infusion into the LS (-0.3 mm, ±0.5 mm, -1.6 mm; Paxinos and Franklin, 2001) was performed under

isoflurane anesthesia (Forene, Abbott GmbH, Wiesbaden, Germany) and semi-sterile conditions (Toth, Neumann et al. 2012, Zoicas, Menon et al. 2016). To avoid postsurgical infections, mice received subcutaneous antibiotics (3 µg/30 µl Baytril, Bayer GmbH, LevERKusen, Germany). Lactating mice underwent surgery on LD 1; they showed the complete repertoire of maternal behaviour no later than 1 hr after completion of surgery. After surgery, all mice were repeatedly handled for at least 5 days prior to infusion experiments.

2.1.8. Intracerebral infusions

The following substances were infused in an icv mode prior to behavioural testing using a 27-g infusion cannula inserted into the icv guide cannula:

1. Vehicle (2 µl; sterile Ringer solution)

2. OXTR-A (desGly-NH2, d(CH2)5[Tyr(Me)2,Thr4]OVT; 2 µg/2 µl)

For local pharmacological manipulations within the LS, mice received bilateral infusions of substances mentioned below via a 30-g infusion cannula:

1. Veh (0.2 µl/side)

2. OXTR-A (20 ng/0.2 µl/side)

3. OXT (5 ng/0.2 µl/side)

4. MS275 (375 ng/0.2 µl/hemisphere)

These infusions were made using a to a Hamilton syringe and were performed 30 min before SFC or CFC extinction training for OXTR-A, 10 min before SFC extinction for OXT and 90 min before SFC and CFC extinction for MS275 (an ortho-amino anilide inhibitor of class I HDACs with high affinity for HDAC1). The dose and timing of OXTR-A, OXT and MS275 were selected based on previous studies (Lukas, Toth et al. 2011, Bahari-Javan, Maddalena et al. 2012, Toth, Neumann et al. 2012, Toth, Neumann et al. 2012, Zoicas, Slattery et al. 2014).

The correct infusion site was histologically verified and, accordingly, mice with false hits (due to a misplaced cannula) were excluded from statistical analysis. **Microinfusion of AAV for section 3.1.11 and 3.1.12 were performed by Thomas Grund and hence are not mentioned here.**

2.3. Scoring of behavior

All behavioral parameters except CFC (computerized scoring, TSE fear conditioning software) were manually scored by an observer blind to treatment using JWatcher (1.0, Macquarie University and UCLA).

2.2. Molecular techniques

2.2.1. Tissue isolation and mRNA extraction

Mice were subjected to SFC as mentioned above and left undisturbed in their home cages for 120 min after social fear acquisition on day 1 and social fear extinction on day 2. Mice were then sacrificed, their brains were rapidly removed, flash frozen and stored at -80°C until they were cryo-sliced (300-µm; Bregma: 0.98mm – 0.02mm) to obtain tissue micro punches from the LS, amygdala, vHip and PVN. These micro-punches were homogenised in TRI reagent (Sigma) and stored at -20°C for mRNA isolation. Total RNA was isolated using chloroform extraction followed by precipitation with isopropanol and glycogen before elution into the nuclease-free water. The quality of the isolated total RNA was assessed using nanodrop spectrophotometer.

2.2.2. Analysis of gene expression using quantitative real-time PCR (qRT-PCR)

A Hundred nanograms of isolated mRNA were reverse transcribed into cDNA using Super Script III first-strand synthesis system (Invitrogen). Relative mRNA expression for *Hdac1* (NM_008228), *Hdac2* (NM_008229), *Hdac3* (NM_010411), *Hdac8* (NM_027382), *Oxtr*

(NM_001081147.1), *Npy* (NM_023456.3), *Bax* (NM_007527), *Jun* (NM_010591.2) was measured using SYBR green (Qiagen) where ribosomal protein L13A (*Rpl13A*, NR_073024) and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*, NM_001289726). Primer details for each gene are provided in table 1.

Gene	Accession number	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Amplicon Length
Gapdh	NM_001289726	GCTCCACGCTATTCCGATGA	GGGGAGACACTTGCGCATATC	99
Rpl13A	NR_073024	CTCCTTGTCTCATCCCACTG	CAGGGATGATGTTCTGGGCA	104
Hdac1	NM_008228	ACTACGACGGGGATGTTGGA	ATTGGCTTTGTGAGGACGGT	140
Hdac2	NM_008229	ACTTGAGGGATATTGGTGCTGG	CGCTAGGCTGGTACATCTCC	138
Hdac3	NM_010411	GGGCTGTGATCGATTAGGCT	ATATGTCCAACACCGGGCAA	213
Hdac8	NM_027382	AGGGAAAACGCTATCCTCTGA	AGATTCCCTTTGATGTAGTTGAG	150
Oxtr	NM_001081147.1	ACGTCAATGCGCCCAAAGAAC	TGCACGAGTTCGTGGAAGAGATG	124
Npy	NM_023456.3	TGGCCAGATACTACTCCGCT	TCTTCAAGCCTTGTTCTGGGG	145
Bax	NM_007527	CTGCAGAGGATGATTGCTGA	GATCAGCTCGGGCACTTTAG	174
Jun	NM_010591.2	AAAACCTTGAAAGCGCAAAA	TGTTTAAGCTGTGCCACCTG	77

Table 1. qRT-PCR primer details.

2.2.3. Analysis of gene expression using microarray

Sample preparation for microarray hybridization was carried out as described in the Affymetrix GeneChip WT PLUS Reagent Kit User Manual (Affymetrix, Inc., Santa Clara, CA, USA). In brief, 200 ng of total RNA were used to generate double-stranded complementary (c) DNA. 15 µg of subsequently synthesized cRNA was purified and reverse transcribed into sense-strand (ss) cDNA, where unnatural dUTP residues were incorporated. Purified ss cDNA was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) followed by a terminal labeling with biotin. 3.8 µg of fragmented and labeled ss cDNA were hybridized to Affymetrix Mouse Gene 2.1 ST Array Plates. For hybridization, washing, staining and scanning an Affymetrix GeneTitan system, controlled by

the Affymetrix GeneChip Command Console software v4.2, was used. Summarized probe set signals in log2 scale were calculated by using the RMA (Irizarry, Hobbs et al. 2003) algorithm with the Affymetrix GeneChip Expression Console v1.4 Software and exported into Microsoft Excel. Sample processing was performed at an Affymetrix Service Provider and Core Facility, "KFB - Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany; www.kfb-regensburg.de).

2.2.4. cFos immunohistochemistry

Mice were transcardially perfused with paraformaldehyde under deep anaesthesia. Brains were then removed and processed for cFos immunoreactivity as previously described (Singewald, Salchner et al. 2003). Briefly, cFos immunoreactivity for assessed using a polyclonal primary antibody (cFos: 1:4000; sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 hrs and a biotinylated goat anti-rabbit secondary antibody (1:1500; Vector Laboratories, Burlingame, CA, USA) for 90 min. An avidin-biotin-horseradish peroxidase procedure (Vectastain, ABC-Kit, Vector Laboratories) with 3,3´-diaminobenzidine (DAB) as the chromogen was used to visualize cFos-positive neurons. Cells containing a nuclear brown-black reaction product were considered as cFos-positive. The anatomical localisation of labelled cells was aided by a stereotaxic atlas (Paxinos and Franklin, 2nd edition, 2001). The number of cFos-positive cells was counted bilaterally in 3 to 4 sections in a tissue area of 0.1 mm² by an observer blind to the experimental groups. The average of these cell counts was calculated for each animal.

2.2.5. Immunofluorescence

a. Characterization of OXTR-positive neurons. Brain sections of LS of OXTR reporter mice (Yoshida, Takayanagi et al. 2009) were co-stained with a calbindin (GABA neuron marker) specific antibody (Zhao, Eisinger et al. 2013). Shortly, brain sections were incubated according

to the similar staining protocol with a cocktail of primary antibodies: chicken anti-GFP (ab13970, Abcam, 1:10000) and rabbit anti-calbindin (CB-38, Swant, 1:5000), and secondary antibodies (Chicken Alexa488 and mouseCy3, VectorLabs, respectively).

b. Characterization of OXT-positive neuronal fibers. The blocks containing the LS were dissected from fixed mouse brains and Vibratom-cut (Leica) into 50-µm free-floating sections. The sections representing 4 planes of rostro-caudal levels of the LS (Bregma: + 0.1, 0.3, 0.6 and 0.9 mm) were chosen for further OXT-immunolabeling. The free-floating sections were blocked in 1% Triton-PBS buffer containing 1% NGS, 0.2 % BSA for 1 hr at room temperature (RT) and incubated with monoclonal anti-OXT antibody (1:2000) (provided by Dr. Gainer, (NIH)) in 1% Triton-PBS buffer for 24 hr at 4°C. After several wash steps in PBS they were incubated for 1 hr at RT with goat anti-mouse Alexa 488 (ThermoFisher) in 1% Triton-PBS buffer. Images from mounted and cover-slipped sections were then acquired with a Leica TCS SP5-2 confocal microscope. Fiber densities were calculated using Fiji online software. A Z-transform of confocal scan stacks was applied and the "flattened" images were then "overlaid" with a digital grid (available as a Fiji plugin). The number of OXT-positive fiber crossings with the grid bars was taken as a main parameter for fiber number estimation.

Protein	Antibody	Protocol
GFP	ab13970, Abcam	1:10000
OXT	Monoclonal anti-OXT (Prof. gainer)	1:2000
Calbindin	CB-38, Swant	1:5000
cFos	Sc-52, Santa Cruz Biotech.	1:4000
HDAC1	PA1-860, ThermoFisher scientific	1:333
NeuN	MAB377, EMD millipore	1:333
GFAP	G3893, Sigma-Aldrich	1:400

Table 2. Immunofluorescence primary antibody details.

2.3 Statistical analysis

For statistical analysis GraphPad Prism version 6.0 for windows (GraphPad software, San Diego California USA, www.graphpad.com) was used. Data were analyzed by Student's t-tests, one-way or two-way Analysis of Variance (ANOVA) for repeated measures, followed by a Bonferroni's post-hoc analysis whenever appropriate. Statistical significance was set at $p<0.05$. Overall statistics have been stated for each experiment separately in the results section.

Results

3.1 Septal oxytocin signaling regulates social fear in female mice.

3.1.1 Summary

I observed that after social fear acquisition, lactating mice did not express social fear, when presented with a conspecific during extinction. Using advanced neuroanatomical techniques, I then go on to show that the state of lactation induces a plethora of changes in the LS-OXT system, which include increased OXT-positive fibers and release within the LS. Finally, using pharmacological, genetic and chemogenetic tools, I amply demonstrate that increased OXT-signaling within the LS caused by magnocellular OXTergic projections from the SON is responsible for the regulation of social fear extinction in female mice. To the best of my knowledge, this is the first study providing evidence for extra-amygdalar circuits regulating social fear in female mice.

3.1.2 Behavioural characterization of lactating mice: general anxiety, social preference, and social fear conditioning

EPM: There was no difference in general anxiety-related behavior and locomotor activity between lactating and virgin mice, as the percentage of time spent on the open arms of the EPM (Fig 8A) and the number of entries into the closed arms (Fig 8B), respectively, were similar in these groups.

SPT: Both lactating and virgin mice displayed a prolonged exploration of the social stimulus (cage with mice) compared with the exploration of the non-social stimulus (empty cage) ($p<0.001$ vs. non-social stimulus; Fig 8C) indicating similar naturally occurring social preference behavior.

SFC: During acquisition of social fear (day 1), lactating and virgin mice showed similar investigation times of the non-social stimulus (small empty cage; data not shown), confirming the similar non-social anxiety-related behavior. Moreover, Lac SFC[+] and Vir SFC[+] mice received a similar number of foot shocks during exploration of the social stimulus, i.e., a conspecific in a small cage (2.4 ± 0.4 foot shocks). During social fear extinction training (day 2), Vir SFC[+] mice showed severely diminished social investigation, which is a measure of social fear, in comparison to all other groups ($p<0.05$ vs. Vir SFC[-], Lac SFC[-], Lac SFC[+]; Fig 8D). In contrast, Lac SFC[+] mice were not different in comparison to their Lac SFC[-] controls already during exposure to the 2nd social stimulus indicating abolished social fear. During social fear recall (day 3), Vir SFC[+] mice showed an overall reduction in social investigation compared to their unconditioned controls ($p<0.05$ vs. Vir SFC[-]; Fig 8E). Social investigation pattern of Lac SFC[+] mice during social fear extinction training on day 2 was not dependent on the presence of pups in the dams' home cage after the social fear acquisition, as no behavioral difference was found between dams of the Lac SFC[+]/pups[+] (separated 2 hr before extinction) and Lac SFC[+]/pups[-] (separated immediately post-acquisition) groups (Fig 8F).

Statistics:

EPM (Lac mice vs Vir mice; Student's T test) General anxiety (Fig 8A) Locomotion (Fig 8B)	Group effect (Status: Vir mice vs. Lac mice) T(11) = 0.2488, p = 0.808 T(11) = 0.3105, p = 0.762	
Social preference test (Lac mice vs Vir mice; 2way ANOVA) SPT (Fig 8C) *SFC (Status: Vir vs. Lac mice; 2way ANOVA)* Social fear extinction (Fig 8D) Social fear recall (Fig 8E) Social fear extinction (Fig 8F)	*Stimulus effect (social vs. non-social stimulus)* $F(1, 34) = 175.5$, $P < 0.05^*$ *Group effect (Group: status x SFC)* $F(3, 44) = 17.80$, $p < 0.05^*$ $F(3, 43) = 4.78$, $p = 0.05^*$ $F(1, 13) = 1.71$, $p = 0.997$	*Group x stimulus effect* $F(1, 34) = 2.709$, $P = 0.109$ *Group x stimulus effect* $F(24, 352) = 5.583$, $p < 0.05^*$ $F(15, 215) = 1.17$, $p = 0.297$ $F(8, 104) = 0.61$, $p = 0.764$
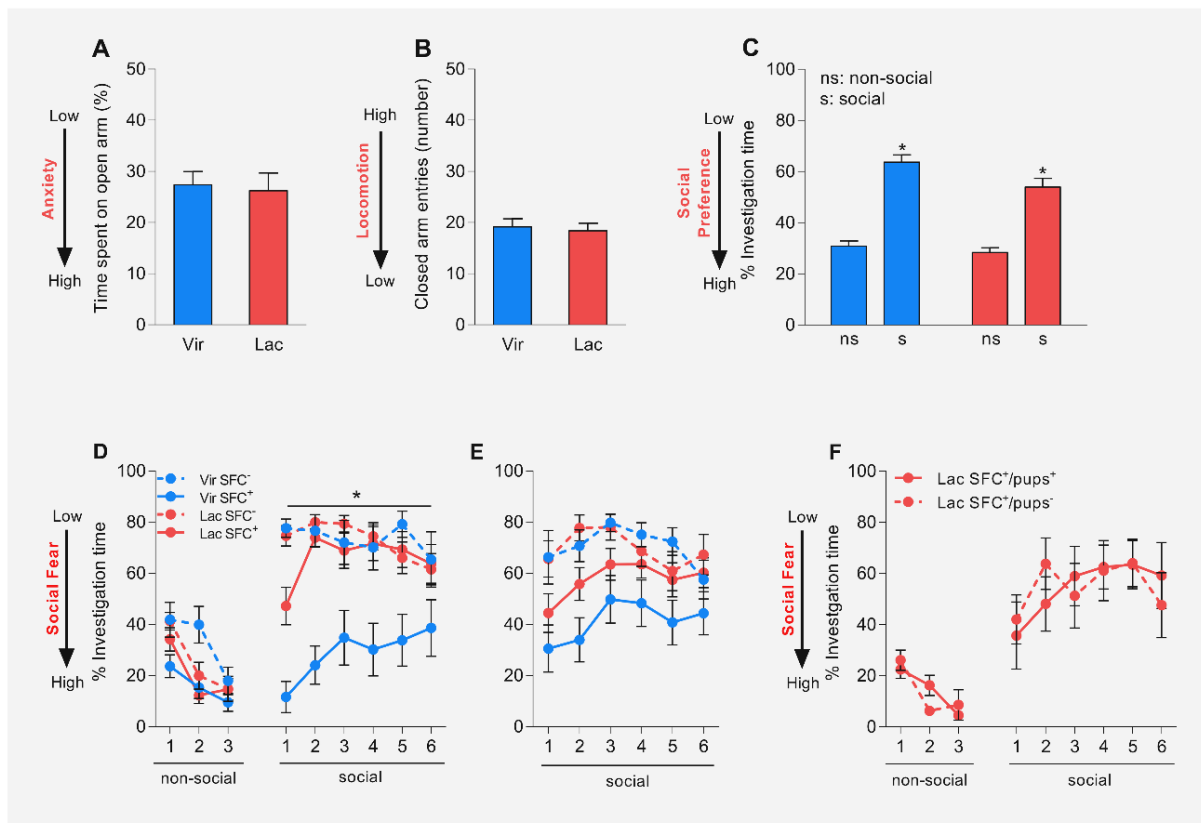
*Fig 8. Lactation has no effect on general anxiety-related behavior (A) and locomotor activity (B) on the elevated plus-maze, and on naturally occurring social preference behavior in the social preference test (C), but counters expression of SFC-induced social fear (social fear extinction: D; recall: E) irrespective of the presence of pups after social fear acquisition (social fear extinction, F). Lactating (Lac; red) and virgin (Vir; blue) mice (n = 11 – 14/ group) were social fear conditioned (SFC⁺) or left unconditioned (SFC⁻) during social fear acquisition (day 1), and the percentage of investigation of 3 non-social (empty cage) and 6 social (cage with a con-specific) stimuli was monitored during social fear extinction (day 2; D, F) and social fear extinction recall (day 3; E). Data represent mean ± SEM. * p<0.05 vs. non-social stimulus (C), Lac SFC⁺ (D), Vir SFC⁻ (D), and Lac SFC⁻(D).*

3.1.3 Icv pharmacological blockade or activation of the OXTR retrieves SFC-induced social fear in lactating mice or reverses SFC-induced social fear in virgin mice.

Although lacking regional specificity, icv administration of substances is a good way to show their general effect on behavior. Previous studies from our lab have shown that SFC-induced social fear in male mice could be reversed by icv infusion of synthetic OXT – an effect which could be blocked *via* prior infusion of the OXTR-A (Zoicas, Slattery et al. 2014). With this in view, we performed an experiment to modulate the OXT system in the brain, firstly by infusing the OXTR-A icv into lactating mice to block the endogenous OXT system, and secondly by infusing synthetic OXT in virgin female mice to activate the OXT signaling within the brain and check for its effects on SFC-induced social fear.

Lactating mice: During social fear extinction, 30 min after icv infusion of either vehicle or OXTR-A, SFC$^+$/OXTR-A lactating mice showed reduced social investigation compared with both SFC$^+$/Veh and SFC$^-$/OXTR-A lactating mice (p<0.05; Fig 9A) indicating the involvement of the brain OXT system in the lack of SFC-induced social fear seen in mice during lactation. Also, confirming our previous results, SFC$^+$/Veh-treated lactating mice displayed a level of social investigation comparable to the SFC$^-$/Veh group (p<0.05; Fig 9A) indicating abolished social fear. During social fear recall, all groups showed increased social investigation (Fig 9B) indicating successful extinction.

Virgin mice: Icv infusion of OXT 10 min prior to social fear extinction completely reversed SFC-induced social fear in virgin mice. While SFC$^+$/Veh virgin mice exhibited reduced investigation of the social stimulus reflecting social fear, SFC$^+$/OXT virgin mice (p<0.05; Fig 9C) showed increased investigation of the social stimulus and were no different from the SFC$^-$ controls reflecting a lack of SFC-induced social fear. Also, SFC$^+$/Veh-treated virgin mice displayed significantly reduced social investigation compared to the SFC$^-$/Veh group (p<0.05; Fig 9C), which was indicative of social fear. During social fear recall, all virgin mice showed

increased social investigation (Fig 9D) indicating successful extinction of social fear. ***This experiment was performed in collaboration with Dr. Iulia Zoicas.***

Statistics:

SFC (Lac mice: Veh vs OXTR-A; 2way ANOVA)	*Group effect (Group: Treatment vs. SFC)*	*Group x stimulus effect*
Social fear extinction (Fig 9A)	$F(3, 30) = 12.6, p < 0.05*$	$F(24, 240) = 3.80, p < 0.05*$
Social fear recall (Fig 9B)	$F(3, 22) = 1.25, p = 0.3145$	$F(15, 110) = 1.14, p = 0.331$
SFC (Vir mice: Veh vs. OXT; 2way ANOVA)	*Group effect (Group: Treatment vs. SFC)*	*Group x stimulus effect*
Social fear extinction (Fig 9C)	$F(3, 26) = 23.5, p < 0.05*$	$F(24, 208) = 6.85, p < 0.05*$
Social fear recall (Fig 9D)	$F(3, 26) = 0.85, p = 0.4764$	$F(15, 130) = 1.875, p = 0.313$
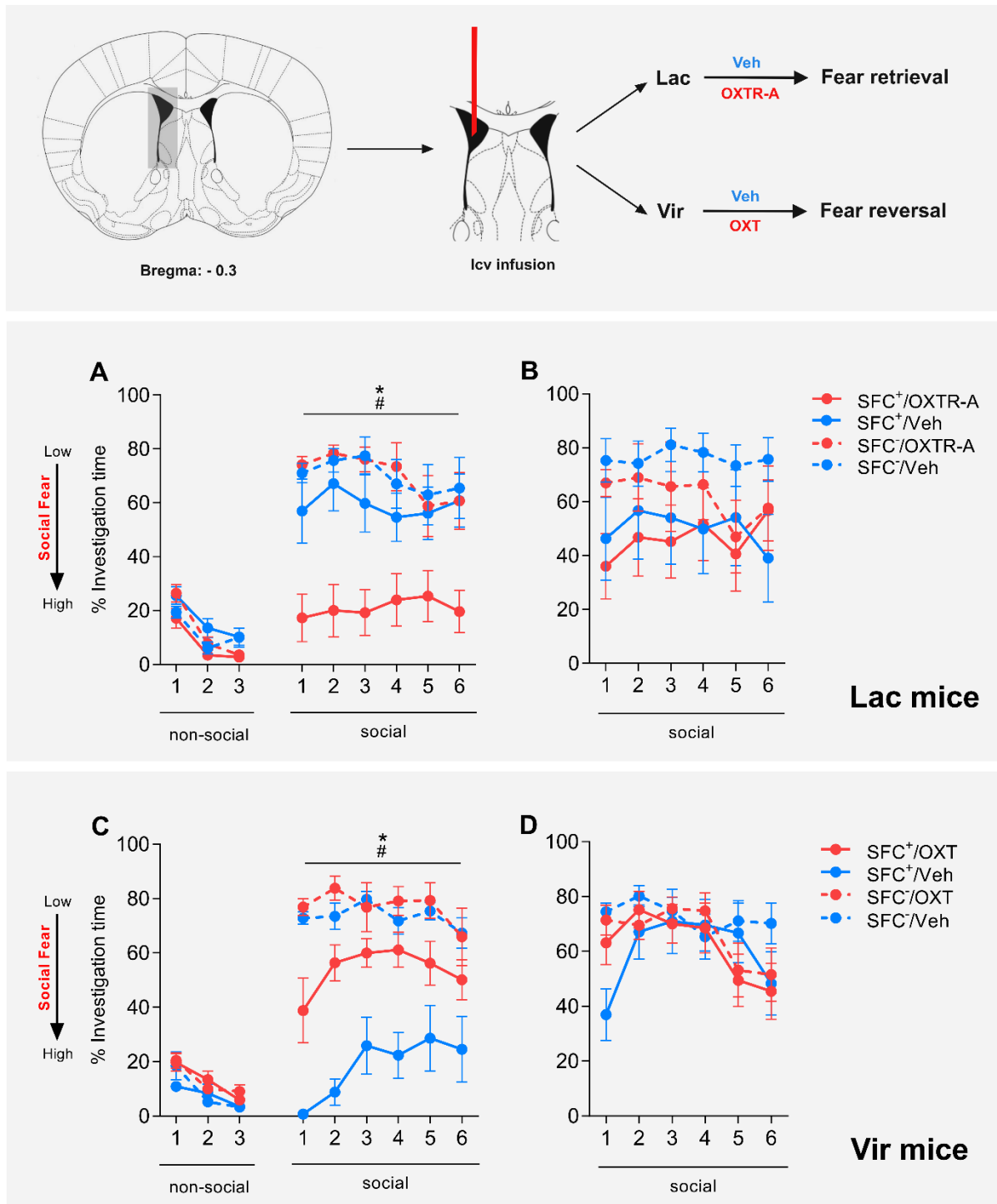
Fig 9. *Icv blockade of the oxytocin receptor (OXTR) signaling with a specific antagonist (OXTR-A) in lactating mice rescues whereas icv activation of the OXTR signaling with synthetic oxytocin (OXT) in virgin mice reverses SFC-induced social fear. Virgin (Vir) and lactating (Lac) mice (n = 6 – 11/ group) were fear conditioned (SFC$^+$) or left unconditioned (SFC$^-$) on day 1; the percentage of investigation of 3 non-social (empty cage) and 6 social (cage with con-specific) stimuli after icv infusion with either vehicle (Veh; blue) or OXTR-A (2µg/2µl; red; lac mice) 30 min before social fear extinction (A; day 2) and veh (blue) or OXT (10 ng/2µl; red; vir mice) 10 min before social fear extinction (C; day 2) was measured; social fear extinction recall (B: lac mice; C: vir mice; day 3) was also monitored. Data represents mean ± SEM. * p<0.05 vs SFC$^+$/Veh (A, C); # p<0.05 vs all SFC$^-$ mice (A, C). Fig 9C and 9D were obtained from experiments performed by Iulia Zoicas.*

3.1.4 Brain regions are differentially activated lactating and virgin mice in response to SFC

To establish the neuronal correlates of reduced social fear in lactating mice using an unbiased approach, I continued to compare the neuronal activation in the whole brain in response to the social fear acquisition and extinction between lactating and virgin mice using cFos immunohistochemistry. This would also help me to localize the OXT-mediated effect on SFC to a specific brain region. I decided to proceed with cFos immunohistochemistry as an immediate early gene, as cFos is a robust marker for neuronal activation. The brain regions analyzed for cFos immunoreactivity were as follows:

1. LS (Fig 10A)

2. BLA (Fig 10B)

3. PVN (Fig 10C)

4. Primary somatosensory cortex (SCX1; Fig 10D)

Virgin mice showed pronounced (6-fold) neuronal activation of the LS in comparison to lactating mice which was indicated by the higher number of cFos-immunopositive neurons at every time point studied within the SFC paradigm with major differences at 90 min after social fear acquisition ($p<0.05$ vs. Vir SFC$^-$, $p<0.05$ vs. Lac SFC$^+$; Fig 10A). The observed activation of the LS in Vir SFC$^+$ mice returned to baseline levels 90 min after social fear extinction ($p<0.05$ vs. Vir SFC$^+$/Ext; Fig 10A).

SFC-induced neuronal activation was also observed in the BLA of Vir SFC$^+$ mice 90 min after social fear acquisition ($p<0.05$ vs. Vir SFC$^-$; Fig 10B), while such an effect was not seen in lactating mice. Within the PVN an overall significance for factor time (indicating a dynamic regulation of cFos-positive neurons) was observed ($p = 0.0161$; Fig 10C). However, multiple comparisons using Bonferroni's *post-hoc* analysis did not reveal any difference between specific groups. No significant differences were observed in the PVN of lactating mice in

comparison to virgin mice at any time point during SFC even after separate statistics. Analysis of the SCX1 showed no significant differential activation between lactating and virgin mice at all assessed time points during SFC indicating similar levels of pain perception (please check for follow-up experiment in section 7).

Statistics:

SFC (Status: Vir vs. Lac mice; 2way ANOVA)	*Group effect (Group: status x SFC)*	*Group x stimulus effect*
Social fear extinction (Fig 10F)	$F(3, 16) = 2.835$, $p < 0.05$*	$F(24, 128) = 3.91$, $p < 0.05$*
cFos immunohistochemistry (Vir vs. Lac; 2way ANOVA)	*Group effect (Status: Vir vs. Lac mice)*	*Group x Conditioning effect (SFC⁻ vs SFC⁺ vs SFC⁺/Ext)*
cFos positive cells in LS (Fig 10A)	$F(1, 28) = 24.1$, $p < 0.05$*	$F(2, 28) = 4.204$, $p < 0.05$*
cFos positive cells in BLA (Fig 10B)	$F(1, 28) = 7.864$, $p < 0.05$*	$F(2, 28) = 0.3731$, $p = 0.692$
cFos positive cells in PVN (Fig 10C)	$F(1, 28) = 3.106$, $p = 0.0889$	$F(2, 28) = 0.1129$, $p = 0.8937$
cFos positive cells in SCX1 (Fig 10D)	$F(1, 26) = 2.441$, $p = 0.6254$	$F(2, 26) = 0.1451$, $p = 0.8657$
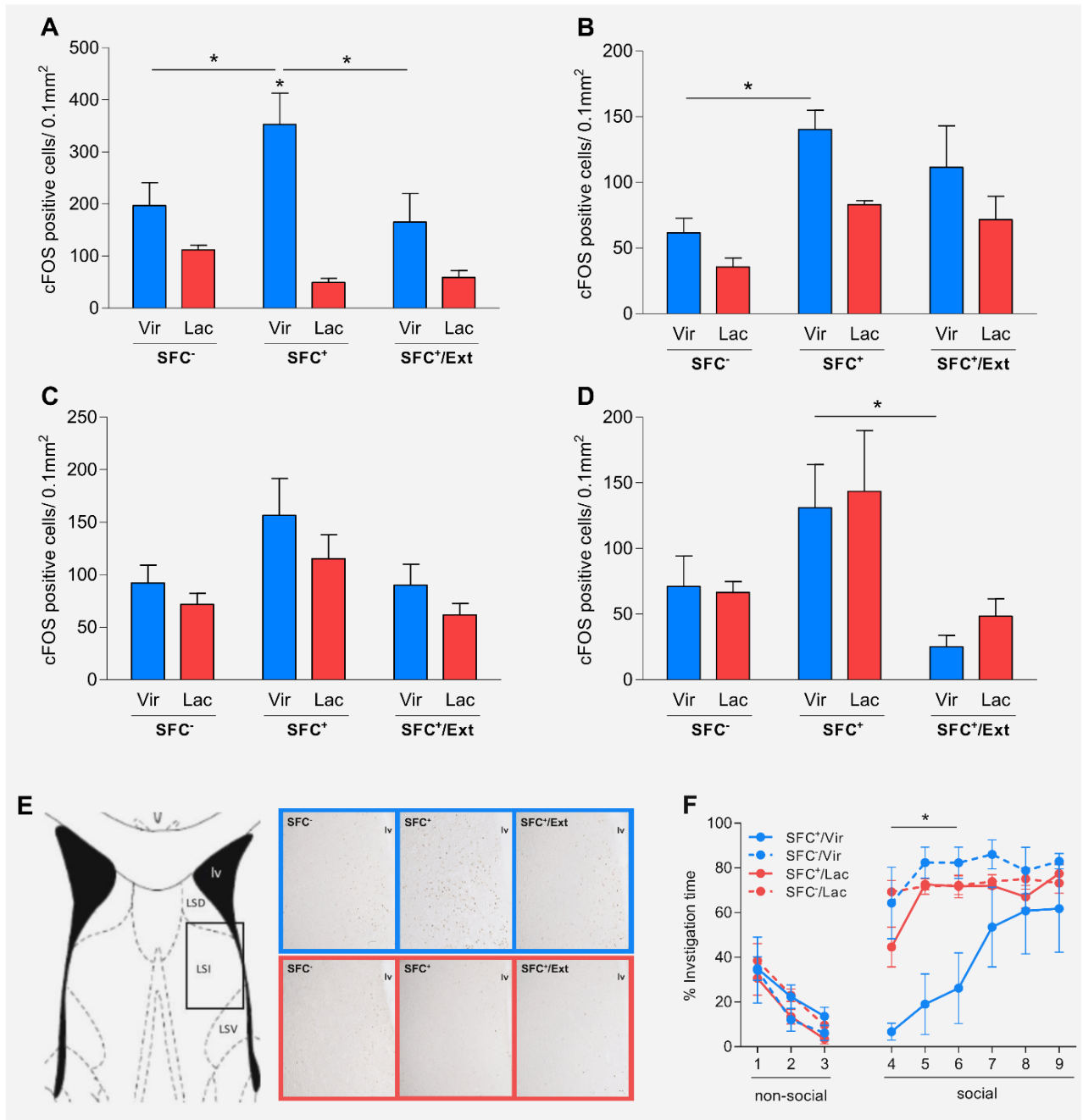
Fig 10. *Number of cFos-immunopositive neurons indicating neuronal activation for virgin (Vir, blue) and lactating (Lac, red) mice within the lateral septum (LS; Bregma 0.86, E). Vir and Lac mice were sacrificed 90 min after either social fear acquisition (SFC- and SFC+; day 1) or after social fear extinction (SFC+/Ext; day 2). The percentage investigation of 3 non-social (empty cage) and 6 social (cage with a con-specific) stimuli was monitored during social fear extinction (day 2; F) for Vir (SFC-/Vir and SFC+/Vir, blue) and Lac (SFC-/lac and SFC+/lac, red) mice. Data represents mean number of cFos-immunopositive neurons/0.1mm^2 ± SEM (A-D) or percentage investigation time ± SEM (F). *p<0.05 (A, B, and D); *p<0.05 for Vir SFC+ vs Lac SFC+, Lac SFC-, and Vir SFC- (F).*

3.1.5 Bidirectional manipulation of OXTR-mediated signaling within the LS causes bidirectionally alters SFC-induced fear in lactating and virgin mice

As mentioned before, the LS-OXT system has been implicated in the regulation of social memory and SFC-induced social fear in male mice (Guzman, Tronson et al. 2013, Zoicas, Slattery et al. 2014). These results combined with my data from the cFos experiment (see Fig 10) led me to the hypothesis that high levels of endogenous OXT within the LS of lactating mice might be the root cause of their apparent immunity against the robust fear-inducing effect of SFC. To clarify this, I endeavored to modulate the LS-OXT system of lactating and virgin mice before social fear extinction using local infusions of the OXTR-A and synthetic OXT, respectively.

Lactating mice: During social fear extinction SFC$^+$/OXTR-A mice expressed higher SFC-induced social fear demonstrated by the highly reduced time they spent investigating the social stimulus in comparison to SFC$^+$/Veh mice and all SFC$^-$ control mice (p<0.05; Fig 11A). As for the SFC$^+$/Veh mice, the lack of fear of a conspecific they exhibited confirmed our previous finding that the upregulation of OXT during lactation reduces the impact of SFC (Fig 11A). The success of the social fear extinction was demonstrated during fear recall on day three wherein all groups of mice spent a high percentage of time investigating the social stimulus (Fig 11B).

Virgin mice: SFC$^+$/OXT mice showed increased investigation of the social stimulus during extinction in comparison the SFC$^+$/Veh mice (p<0.05). However, during the last two social stimuli, there was no significant difference between the two groups showing successful fear extinction in SFC$^+$/Veh mice (Fig 11C). Their amount of social investigation was at par with that of the SFC$^-$ controls which was indicative of the fact that lateral septal OXT treatment reversed SFC-induced social fear (Fig 11C). All groups of mice showed high amount of social investigation during social fear recall which meant that the extinction was successful (Fig 11D).

Statistics:

SFC (Lac mice: Veh vs. OXTR-A; 2way ANOVA)	Group effect (Group: Treatment vs. SFC)	Group x stimulus effect
Social fear extinction (Fig 11A)	$F(3, 24) = 10.70, p < 0.05^*$	$F(24, 192) = 5.014, p < 0.05^*$
Social fear recall (Fig 11B)	$F(3, 24) = 4.09, p = 0.071$	$F(15, 120) = 1.17, p = 0.302$
SFC (Vir mice: Veh vs. OXT; 2way ANOVA)	Group effect (Group: Treatment vs. SFC)	Group x stimulus effect
Social fear extinction (Fig 11C)	$F(3, 20) = 5.47, p < 0.05^*$	$F(24, 160) = 2.6, p < 0.05^*$
Social fear recall (Fig 11D)	$F(3, 20) = 1.55, p = 0.2313$	$F(15, 100) = 0.474, p = 0.94$

Fig 11. *Local lateral septum (LS) blockade of the oxytocin receptor (OXTR) with a specific antagonist (OXTR-A) in lactating mice rescues whereas LS activation of the OXTR with synthetic oxytocin (OXT) in virgin mice reverses SFC-induced social fear. Virgin (Vir) and lactating (Lac) mice (n = 7 – 9/ group) were fear conditioned (SFC+) or left unconditioned (SFC-) on day 1; the percentage of investigation of 3 non-social (empty cage) and 6 social (cage with con-specific) stimuli after bilateral LS infusion with either vehicle (Veh; blue) or OXTR-A (20 ng/0.2 µl/ side; red; lac mice) 30 min before social fear extinction (A; day 2) and Veh (blue) or OXT (0.5 ng/0.2 µl/ side; red; vir mice) 10 min before social fear extinction (C; day 2) was measured; social fear extinction recall (B: lac mice; C: vir mice; day 3) was also monitored. Data represents mean percentage of investigation time ± SEM. * p<0.05 vs. SFC+/Veh (A, C); # p<0.05 vs. all SFC- mice (A, C).*

3.1.6 Lactation prevents CFC-induced cued fear in mice

Based on my results that the state of lactation prevents SFC-induced social fear, I tested the effect of an upregulated endogenous OXT system on the non-social form of conditioned fear, i.e., cued fear induced by CFC. To this end virgin and lactating (LD 5-7) mice were cued fear conditioned using the CFC paradigm. Lactating mice showed impaired acquisition of cued fear in comparison to their virgin counterparts (p<0.05, Fig 12A). Expectedly, on the following day during cued fear extinction (Fig 12B) lactating mice displayed no freezing on CS-presentations reflecting that they had no fear of the tone. This was in stark contrast to the behavior of virgin mice, who showed significantly higher percentage of freezing on CS-presentation (p<0.05, Fig 12B). During cued fear recall all mice showed a low amount of freezing on CS-presentation indicating successful extinction.

Statistics:

CFC (Vir vs. Lac)	Group effect (Status: Vir vs. Lac mice)	Group x stimulus effect
2way ANOVA: CFC – acquisition (Fig 12A) CFC – extinction (Fig 12B) *Student's T-test:* CFC – recall (Fig 12C)	$F(1, 14) = 6.59, p < 0.05*$ $F(1, 13) = 8.94, p < 0.05*$ $T(5) = 0.6932, p = 0.5191$	$F(4, 56) = 3.035, p < 0.05*$ $F(9, 117) = 1.608, p = 0.121$
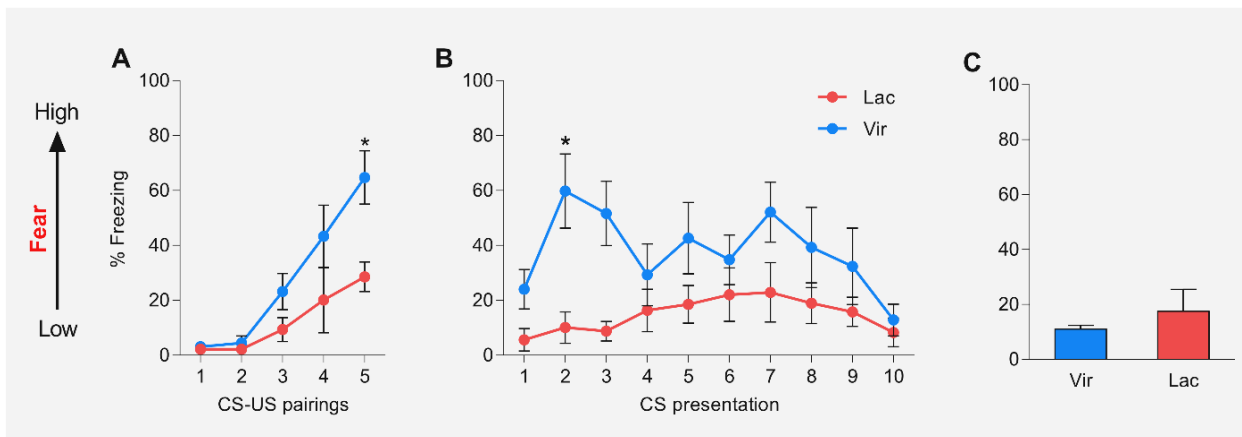
*Fig 12. Lactation results in impaired cued fear learning in mice as reflected by the percentage time of freezing in lactating (Lac, red) and virgin (Vir, blue) mice (n = 8/ group) during cued fear acquisition (A), extinction (B) and extinction retention (C). Data represents mean ± SEM. * p<0.05 vs Lac mice (A and B).*

3.1.7 Icv pharmacological blockade of OXTR does not affect CFC-induced fear in lactating mice irrespective of the time point of infusion.

Applying a similar logic as in experiment 2, I tried to implicate the OXT system in this apparent lack of CFC learning seen in lactating mice. In order to do so, I performed an icv application of OXTR-A in lactating mice 30 min before acquisition and 30 min before extinction in 2 separate experimental cohorts. In both cases, icv OXTR-A application failed to revive the fear to normal levels as all groups of lactating mice whether OXTR-A or Veh infused did not show any freezing during CFC-acquisition (Fig 13A and 13D), extinction (Fig 13B and 13E) and recall (Fig 13C and 13D). This meant that the lack of CFC-induced generalized fear in lactating mice is not due to their overactive OXT system.

Statistics:

CFC (Lac mice: Veh vs. OXTR-A)	Group effect (Treatment: OXTR-A vs. Veh; pre-extinction infusion)	Group x stimulus effect
2way ANOVA: CFC – acquisition (Fig 13A) CFC – extinction (Fig 13B)	 F(1, 12) = 4.207, p = 0.5288 F(1, 14) = 1.88, p = 0.1921	F(4, 48) = 0.6004, p = 0.6641 F(9, 126) = 0.721, p = 0.688
Student's T-test: CFC – recall (Fig 13C)	 T(6) = 0.568, p = 0.5906	
CFC (Lac mice: Veh vs. OXTR-A)	**Group effect (Treatment: OXTR-A vs. Veh; pre-acquisition infusion)**	**Group x stimulus effect**
2way ANOVA: CFC – acquisition (Fig 13D) CFC – extinction (Fig 13E)	F(1, 12) = 4.207, p = 0.5288 F(1, 14) = 1.88, p = 0.1921	F(4, 48) = 0.6004, p = 0.6641 F(9, 126) = 0.721, p = 0.688
Student's T-test: CFC – recall (Fig 13F)	 T(6) = 0.568, p = 0.5906	
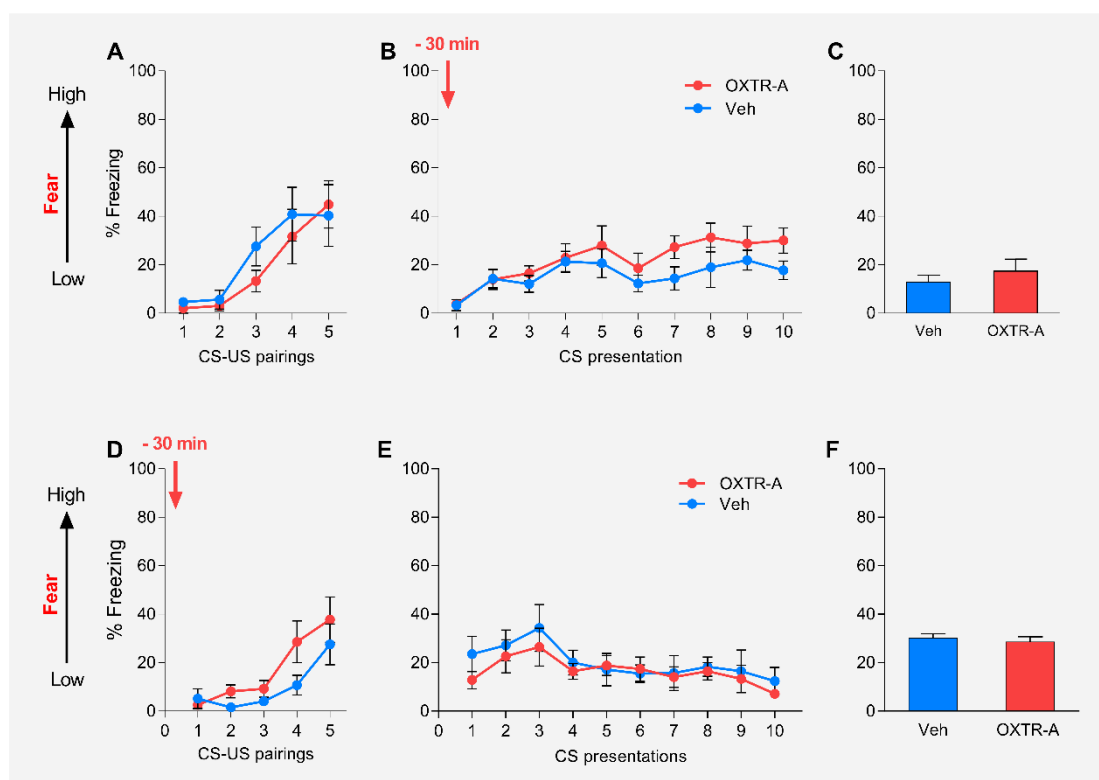
Fig 13. *Blocking the OXT-system in lactating mice does not rescue CFC-induced cued fear as reflected by the percentage time of freezing in lactating (Lac) mice during cued fear acquisition (A), extinction (B) and extinction retention (C) after OXTR-A or veh infusion. Lac mice (n = 8/ group) were either infused with either vehicle (Veh) or OXTR-A (2µg/2µl), 30 min before cued fear acquisition (A and D) or 30 min before cued fear extinction (B and E; day 2). Arrows in the Fig (B and D) indicate the timepoint of infusion in two separate CFC experiments wherein A-C represents experiment 1 and D-F represents experiment 2. Cued fear recall was performed on day 3 (C and F). Data represents mean ± SEM.*

3.1.8 Pain perception remains unaltered in mice during lactation.

OXT released by the magnocellular OXT neurons has been shown to alleviate acute pain (Eliava, Melchior et al. 2016). Thus in addition to showing the ability of lactating mice to repel the robust fear generating effects during fear conditioning paradigms such as SFC and CFC, I had to exclude the possibility that these effects were mediated by the reduced perception of pain in lactating mice. To this end, I performed the Hargreaves' plantar test.

Comparing lactating and virgin mice using the test mentioned above at two different heat intensities revealed no differences in pain perception, which was reflected by the similar latency to withdraw the hind paw on heat stimulation of the plantar region (Fig 14). This result could be seen in congruence to the same number of cFos positive cells in the SCX1 of lactating and virgin mice (Fig 14D) that are activated in response to fear conditioning during SFC. From this, we inferred that although lactating mice appear to feel the same pain, they merely lack the normal CS-US association (in the case of CFC) and do not express social fear despite normal social fear acquisition.

Statistics:

Hargreaves plantar test (Vir vs. Lac; 2way ANOVA)	Group effect (Status: Vir vs. Lac mice)	Group x intensity effect
Pain perception (Fig 14)	$F_{(1, 32)} = 0.3916$, $p = 0.5359$	$F_{(1, 32)} = 0.0289$, $p = 0.87$
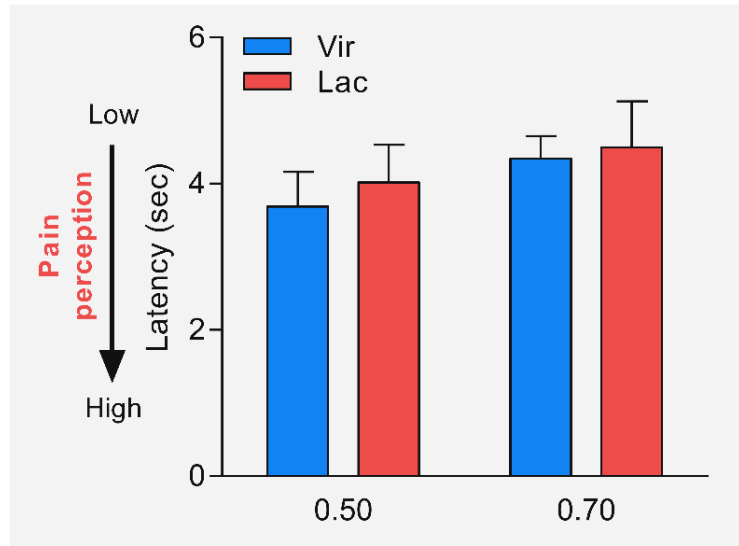
Fig 14. Pain sensitivity was tested using the Hargreaves' plantar test and is reflected by the latency paw withdrawal upon heat stimulation, which did not differ between lactating (Lac, red) and virgin (Vir, blue) mice. Data represents mean ± SEM.

3.1.9 Lactation induces an increase in OXT-positive fibers in the mouse LS

Immunohistochemical characterization of the LS revealed a 2-fold increase in the number of OXT-positive neuronal fibers in lactating (LD 5) mice ($p < 0.05$ vs. virgin; Fig 15A, B, and E). Furthermore, a proximity between branching OXT-positive axons with multiple varicosities and OXTR-GFP neurons was seen in lactating OXTR reporter mice (Figs 15C and D). We also found that the majority of the OXTR-expressing neurons within the LS are GABAergic neurons, as revealed by the high number of cells immunopositive for both GFP (expressed under the control of the OXTR gene promoter) and calbindin (GABAergic interneuronal marker). ***This experiment was performed in collaboration with Dr. Valery Grinevich's lab.***

Statistics:

OXT-positive fiber immunohistochemistry	*Group effect (Status: Vir vs. Lac mice)*
Fiber count (Fig 15)	$T(28) = 7.247$, $p < 0.05*$
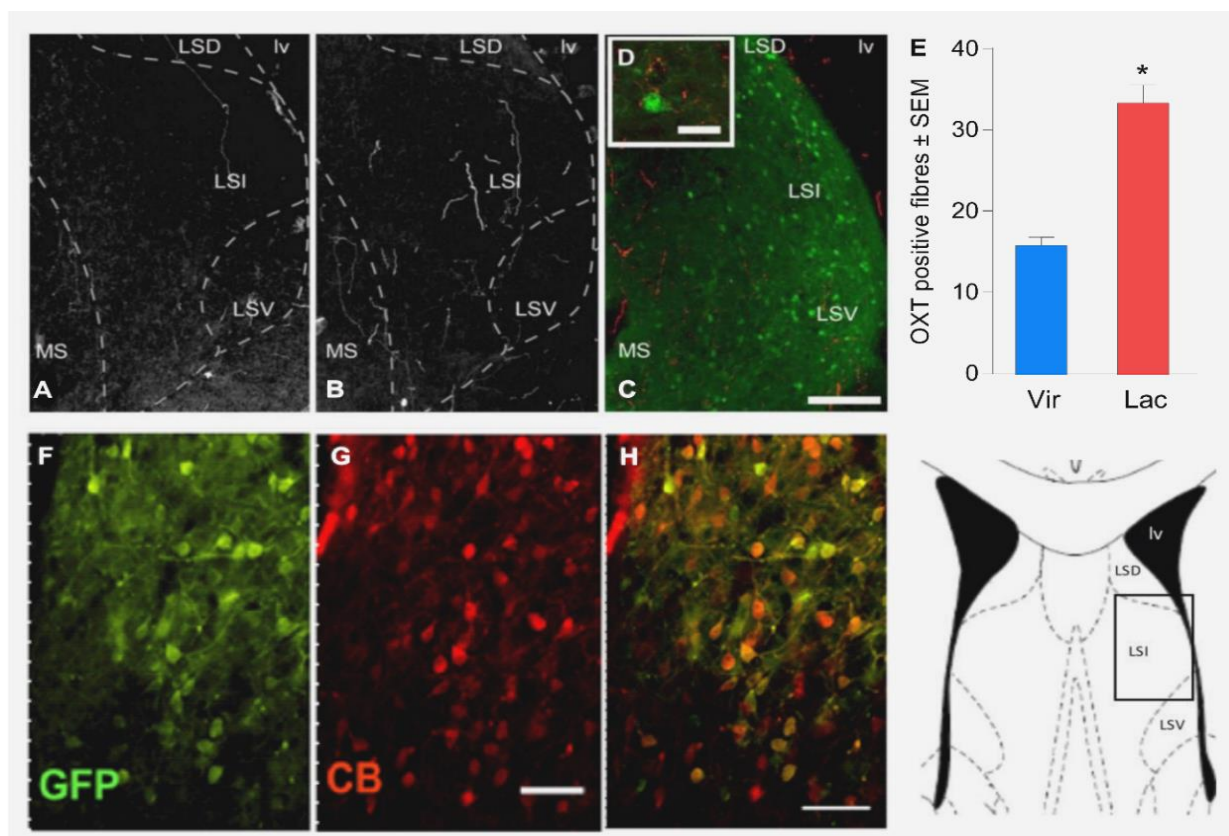
Fig 15. *Lactation induces an increase in the number of OXT-positive neuronal fibers, and a high percentage of the OXTR-positive neurons in the LS are GABAergic. The LS of lactating (Lac; LD 5; B-D) and virgin (Vir; A, F-H) mice were immunohistochemically characterized for the number of OXT-positive fibers and the nature of OXTR-positive neurons. The highest number of OXT-positive fibers were found in LSI of Lac mice (B). In C, the OXT-positive fibers (red) were seen to be localized in the close proximity to the OXTR-positive neuronal cell bodies (green, the staining was performed in OXTR reporter mouse brain). The high magnification of the OXTR Venus-positive neuron body surrounded by the OXT-positive fibers is shown boxed in D. The percentage of green fluoroscent protein (GFP; F) and calbindin (CB) positive (G) positive neurons from the LS of virgin OXTR reporter mice was examined. H is a merged image showin GFP and calbindin positive neurons. Data represents mean number of OXT positive neurons ± SEM. *P<0.05 vs. Vir (E). Scale bars in A, B, C: 500µm, in D: 25µm and G, H: 200µm. Abbreviations: LSD, LSI, LSV – Dorsal, Intermediate and Ventral subdivisions of Lateral Septal (LS) nucleus. MS- medial septal nucleus, lv – lateral ventricle.*

3.1.10 SFC blocks oxytocin release within the female mouse LS

To reveal the functional significance of the increased OXT innervation of the LS in lactation I performed intracerebral microdialysis during social fear extinction. During extinction of social fear, Vir SFC[+] mice expressed fear levels, which were significantly higher than that of Vir SFC[-] mice (p<0.05; Fig 16B). However, Vir SFC[-] mice displayed high social investigation in comparison to the SFC[+] Lac and Vir mice indicating lack of social fear (p<0.05; Fig 16B). I observed a significant increase in the measurable amount of OXT in microdialysates sampled within the LS of SFC[-] virgin mice during the social investigation of the first three conspecifics

compared to basal (p<0.05, Fig 16C and D). A similar rise was observed in SFC[+] lactating mice in response to consecutive exposure to social stimuli (p<0.05; Fig 16C and D), indicative of increased septal OXT release. In contrast, the increase in local OXT release observed in the SFC[+] virgin group did not reach statistical significance.

Statistics:

SFC (Status: Vir vs. Lac mice; 2way ANOVA)	*Group effect (Group: Status vs. SFC)*	*Group x stimulus effect*
Social fear extinction (Fig 16B)	$F(2, 16) = 12.8$, $p < 0.05*$	$F(16, 128) = 9.12$, $p < 0.05*$
Microdialysis (Status: Vir vs. Lac mice)	*Group effect (Group: Status vs. SFC)*	*Group x stimulus effect*
1way ANOVA: OXT release (% baseline) (Fig 16C)	$F(1, 16) = 3.23$, $p = 0.06$	$F(8, 64) = 4.74$, $p < 0.05*$
2way ANOVA: OXT release (absolute content) (Fig 16D)	$F(1, 28) = 29.5$, $p < 0.05*$	$F(2, 228) = 0.878$, $p = 0.427$
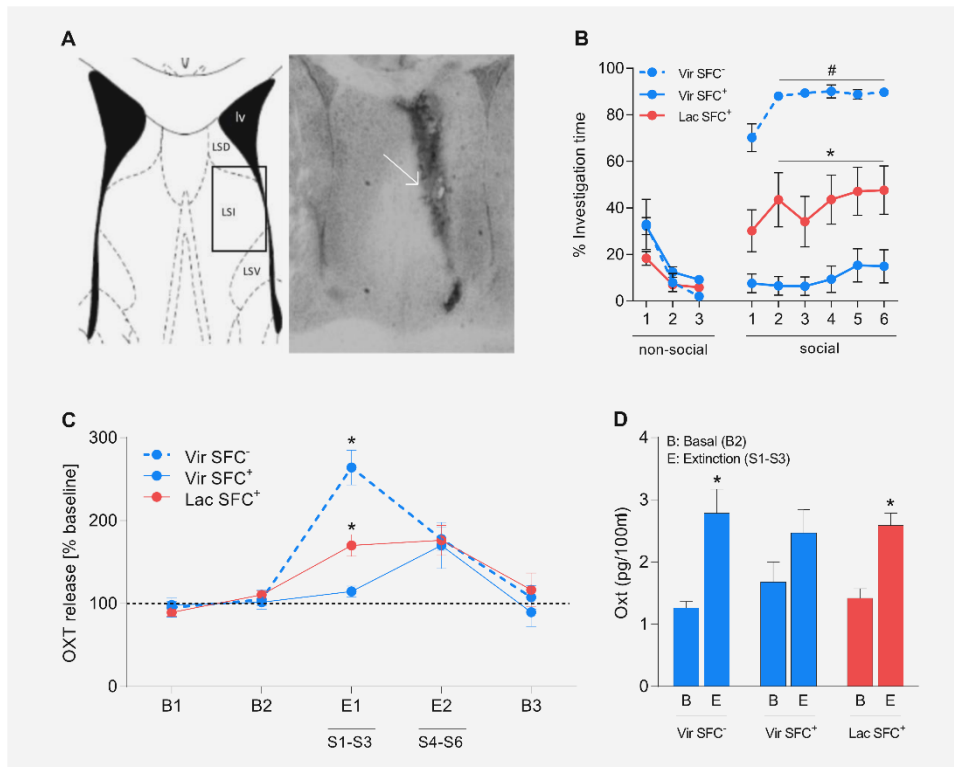
Fig 16. *OXT release within the LS in response to social fear extinction monitored by intracerebral microdialysis. Virgin (vir, blue) and lactating (lac, LD 5, red) mice were exposed to SFC on day 1 (Vir SFC+: n = 6, Vir SFC-: n = 6 and Lac SFC+: n = 6) and to social fear extinction training 24 hrs later (B). Microdialysates were collected before (dialysates B1 and B2; basal conditions), during (dialysate E1 during exposure to social stimuli 1-3, dialysate E2 during exposure to social stimuli 4-6), and after (dialysate B5; basal conditions) the extinction training. Data represent: mean percentage of investigation time ± SEM (B), percentage of mean basal values ± SEM (C), and mean absolute OXT concentrations in 100 ul microdialysates ± SEM. * p<0.05 vs. Vir SFC+ and # p<0.05 vs. Vir SFC+ and Lac SFC+ using 2-way ANOVA followed by Bonferroni's posthoc analysis (B)* p<0.05 vs. Vir SFC+ using 1-way ANOVA with repeated measures followed by Bonferroni's posthoc analysis (C); * p<0.05 using 2-way ANOVA followed by Bonferroni's posthoc analysis (D).*

3.1.11 Genetic manipulation of the OXTR-expressing neurons alters social fear expression

OXT is known to be a partial agonist for receptors of its sister peptide vasopressin (AVP) (Jurek and Neumann 2018). Although pre-extinction icv infusion of AVP does not affect social fear expression in male mice (Zoicas, Slattery et al. 2014), I aimed to provide more specific evidence for an OXTR-mediated effect of OXT within the LS to reverse social fear.

Firstly, I used a recombinant adeno-associated virus (AAV) -mediated transfection of the LS with an EF1-OXTRpr-IRES:GFP (IRES) construct resulting in constitutive enhancement of translation within the transfected OXTR-positive neurons (Johnson, Grosely et al. 2017). Virgin mice were bilaterally infused with IRES (70 nl/ infusion site) at four different, adjacent loci

(350 µm from ventral to dorsal) within each LS or equal volumes of the vehicle. AAV-mediated translational activation of OXTR-positive neurons resulted in partial reversal of social fear expression. This is demonstrated by the fact that SFC$^+$ IRES-infused mice showed increased social investigation during exposure to the first conspecific (Fig 17E), although the level of social investigation was still lower than that observed in SFC$^-$ controls ($p<0.05$; Fig 17E). However, the social investigation exhibited by IRES-infused mice matched that of the SFC$^-$ control mice by the second social stimulus (Fig 17E). Such differences were present despite regular social fear acquisition: all conditioned mice received a similar number of CS-US pairings (Fig 17D) indicating that modulating OXTR-signaling did not affect acquisition of social fear. Again, during social fear extinction recall, all animals displayed a similar level of social investigation.

In this context, we identified OXTR-positive neurons as GABA-ergic neurons within the LS using co-staining for GFP (Fig 17A and B), and calbindin, a GABAergic interneuronal marker. More than 97% of OXTR-positive neurons within the LS could be characterized to be GABAergic (Fig 17C).

Following this, I inquired the effect of constitutive genetic loss of function of OXTR signaling on SFC behavior using OXTR knockout (KO) mice (Sala, Braida et al. 2011). For this, we fear-conditioned KO and OXTR wildtype (Wt) mice. During social fear acquisition, both groups of SFC$^+$ mice received the same number of CS-US pairings (Fig 17G). During extinction of social fear, all groups of female mice showed a similar investigation of the non-social stimulus indicating a similar level of non-social anxiety. Also, both KO and Wt SFC$^+$ mice showed reduced social investigation of the first conspecific in comparison to their SFC$^-$ counterparts, indicating similar social fear acquisition and expression. However, KO exhibited impaired social fear extinction, as reflected by reduced social exploration of conspecifics 2 to 4 (Fig 17H). The impaired fear extinction of KO mice was still visible during fear extinction recall, as

SFC[+]/ KO mice showed reduced social investigation in comparison to SFC[+]/ Wt mice ($p<0.05$; Fig 17I) providing further evidence for the importance of OXTR signaling in reversing SFC-induced social fear.

Interestingly, abovementioned genetic manipulations, including IRES-mediated enhancement of translation within OXTR-expressing cells within the LS, and constitutive knockout of OXTR did not affect the behavioral performance and expression of fear in the cued fear conditioning paradigm (data not shown). Thus, our data indicate that the LS-OXT system of female mice including local GABAergic OXTR-positive neurons is specifically involved in the extinction of traumatic events in a social context.

This dataset was generated in collaboration with Thomas Grund and Dr. Iulia Zoicas.

Statistics:

SFC (Vir mice: Veh vs. IRES)	*Group effect (Group: Treatment vs. SFC)*	*Group x stimulus effect*
Student's T-test:		
Social fear acquisition (Fig 17D)	$T(21) = 0.159$, $p = 8754$	
2way ANOVA:		
Social fear extinction (Fig 17E)	$F(3, 29) = 18.1$, $p < 0.05*$	$F(24, 232) = 5.56$, $p < 0.05*$
Social fear recall (Fig 17F)	$F(3, 14) = 0.6244$, $p = 0.611$	$F(15, 70) = 0.776$, $p = 0.69$
SFC (Vir mice: Wt vs. KO)	*Group effect (Group: genotype vs. SFC)*	*Group x stimulus effect*
Student's T-test:		
Social fear acquisition (Fig 17G)	$T(16) = 9.177$, $p = 0.3724$	
2way ANOVA:		
Social fear extinction (Fig 17H)	$F(3, 288) = 72.22$, $p < 0.05*$	$F(24, 288) = 2.6$, $p < 0.05*$
Social fear recall (Fig 17I)	$F(3, 192) = 34.43$, $p < 0.05*$	$F(15, 192) = 0.511$, $p = 0.9328$
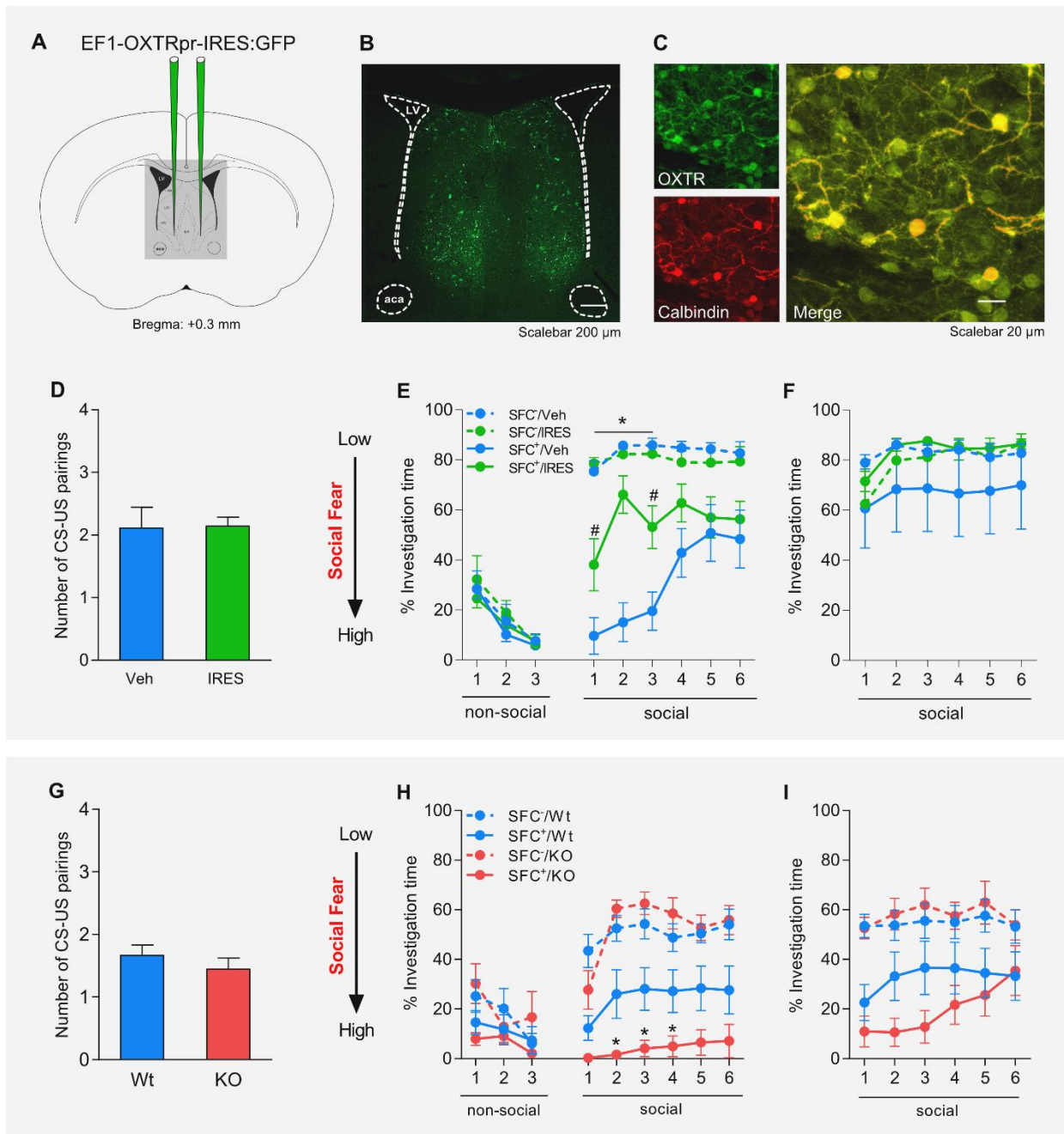
Fig 17. *Enhancement of translational activity of oxytocin receptor (OXTR)-expressing GABAergic neurons within the LS of virgin mice by local infusion of an AAV-containing EF1-OXTRpr-IRES:GFP (IRES; green) construct or vehicle (Veh; blue) (scheme of infusion site, A-B). Co-staining for OXTR-GFP (green) with calbindin (red) (C). IRES and Veh-treated mice were either social fear conditioned (SFC+) or left unconditioned (SFC-) (Day 1; D; n = 7-13/ group). Percentage investigation of 3 non-social (empty cage) and six social (cage with a conspecific) stimuli during social fear extinction (Day 2; E) and social fear extinction recall (Day 3; F) were measured. Data represent mean ± SEM. * p<0.05: SFC+/IRES vs. SFC+/Veh; # p<0.05: SFC+/IRES vs. all SFC- groups (E). OXTR knockout (KO; red) mice show impaired social fear extinction. Wildtype (Wt; blue) and KO mice were social fear conditioned (SFC+) or left unconditioned (SFC-) on (Day 1; G; n = 9-10/ group). Percentage investigation of 3 non-social (empty cage) and six social (cage with a conspecific) stimuli during social fear extinction (Day 2; H) and social fear extinction recall (Day 3; I) were assessed. Data represent mean ± SEM. *p<0.05: SFC+/KO vs. SFC+/Wt (H).*

3.1.12 Magnocellular OXTergic SON-LS projections are involved in reduced social fear in lactating mice

To provide final evidence for the functional involvement of OXT in the blunted social fear in lactation, I aimed to specifically target those OXTergic projections from the hypothalamus to the LS, which are activated during lactation. For this purpose, we used a dual virus approach, wherein we injected CAV2-Cre (70nl/ infusion; 4 infusion sites/ hemisphere), a retrogradely transducing virus (Schwarz, Miyamichi et al. 2015), bilaterally into the LS in combination with a floxed rAAV bilaterally into the hypothalamic PVN and SON (70nl/ infusion) expressing an inhibitory DREADD specifically in OXT neurons following Cre activity (OXTpr-DIO-hM4Di:mCherry). After allowing a two week period for AAV expression, we performed SFC during which lactating mice received an intraperitoneal (i.p.) infusion of vehicle or clozapine N-oxide (CNO; 15mg/kg) 30 min before social fear extinction. This strategy allowed us to precisely silence the subpopulation of LS-projecting hypothalamic OXT neurons.

On the first day of the SFC paradigm, all lactating animals underwent regular social fear acquisition, characterized by the same number of CS-US pairings (Fig 18B) for all SFC[+] mice. During social fear extinction, vehicle-injected SFC[+] mice displayed a naturally high level of social investigation from the first social stimulus (Fig 18C). In contrast, i.p. infusion of CNO and consequent silencing of OXTergic hypothalamic projections to the LS completely blocked any social investigation in all mice (Fig 18C). In confirmation, CNO-injected mice also expressed high social fear throughout social fear extinction recall in comparison to vehicle-injected SFC[+] mice (Fig 18C). Lactating mice injected with CNO (with prior infusion of the vehicle instead of virus) behaved exactly like vehicle-infused lactating mice (Fig 18C) showing that CNO infusion alone does not affect the low social fear expressed by lactating mice.

There were no differences in the investigation of the non-social stimuli between vehicle- and CNO- treated mice indicating unaltered general anxiety. Two days after the SFC paradigm, i.e.,

on LD 9, we analyzed their maternal behavior after another i.p. injection of either CNO or vehicle, wherein we were unable to ascertain any differences between both groups (data not provided). One day after the maternal behavior recordings, mice were injected i.p. with the retrograde tracer fluorogold (FG), which is exclusively taken up by neuronal fiber in regions lacking a blood-brain barrier such as the neurohypophysis.

Coronal brain slices obtained from these mice were co-stained for OXT, mCherry, and FG. I found that a high number (25 ± 4) of the LS-projecting hypothalamic OXT neurons originated in the SON and to be solely magnocellular (Fig 18E). In contrast, only very few (4 ± 2) OXT neurons which project to the LS were found in the PVN. These data show that magnocellular OXTergic afferents to the LS primarily originate in the SON and are specifically responsible for reduced social fear in lactating mice.

This dataset was generated in collaboration with Thomas Grund, Dr. Iulia Zoicas and Dr. Valery Grinevich's lab.

Statistics:

SFC (Vir mice: Veh/Veh vs Veh/CNO vs. DIO/CNO)	Group effect (Group: Treatment vs. SFC)	Group x stimulus effect
1way ANOVA: Social fear acquisition (Fig 18B)	$F_{(2, 24)} = 0.4624$, $p = 0.635$	
2way ANOVA: Social fear extinction (Fig 18C) Social fear recall (Fig 18D)	$F_{(3, 28)} = 43.82$, $p < 0.05*$ $F_{(3, 16)} = 11.37$, $p < 0.05*$	$F_{(24, 224)} = 20.27$, $p < 0.05*$ $F_{(15, 80)} = 1.571$, $p = 0.101$
Immunohistochemistry (PVN vs. SON)	**Group effect (Group: genotype vs. SFC)**	
Student's T-test: mCherry labeled cells (Fig 18G)	$T_{(12)} = 5.78$, $p < 0.05*$	
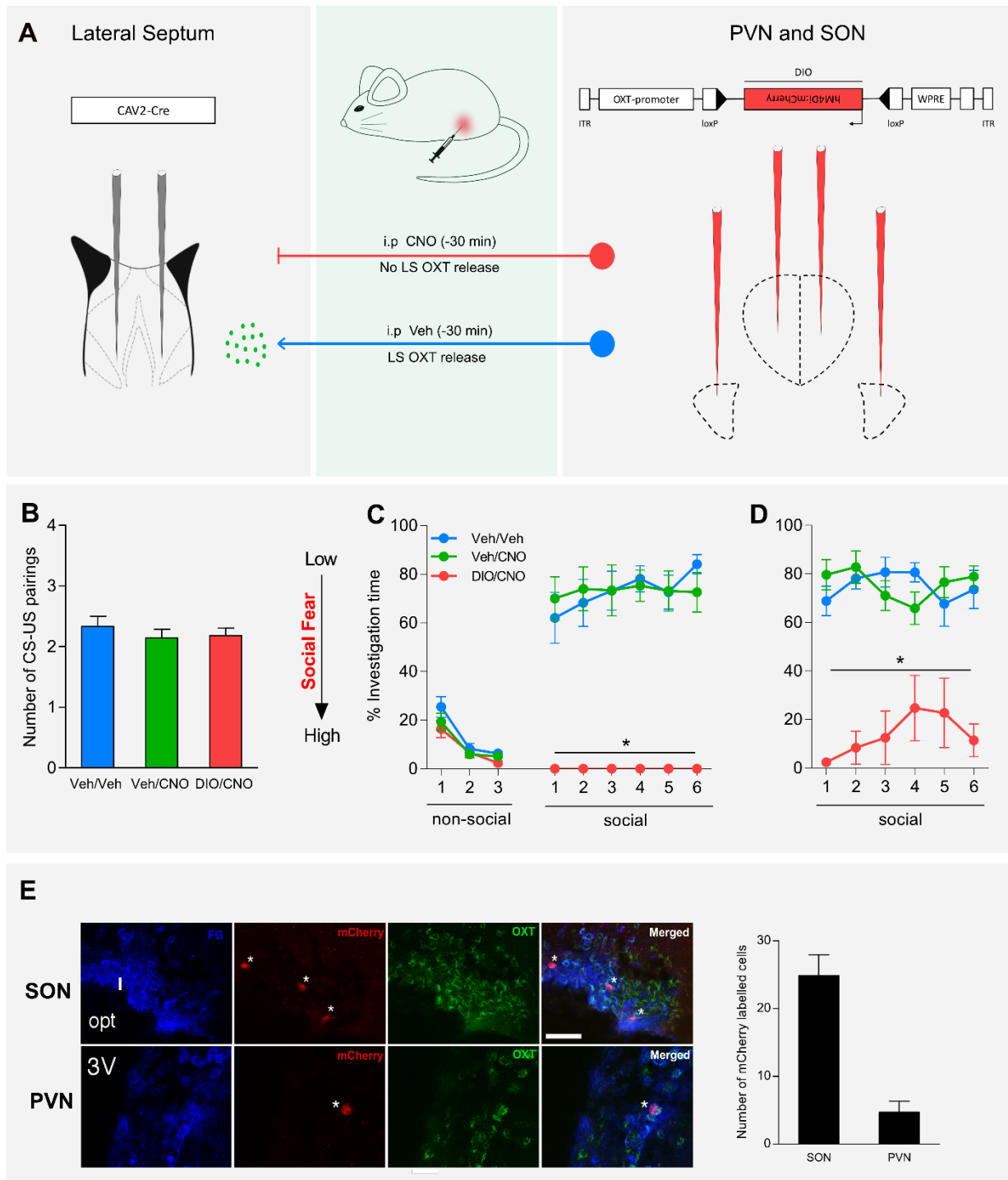
Fig 18. Schematic representation of viral (DIO) or vehicle (Veh) infusions into the lateral septum (LS), supraoptic (SON) and paraventricular (PVN) hypothalamic nuclei and intraperitoneal (i.p) administration of vehicle (Veh) or clozapine-N-oxide (CNO). (B) Chemogenetic blockade of social investigation in lactation (Lac) mice. Lac mice (preinfused with Veh or DIO) were social fear conditioned (SFC⁺) (Day 1; B; n = 6-9/ group). Percentage investigation of 3 non-social (empty cage) and six social (cage with a conspecific) stimuli during social fear extinction (Day 2; C) and social fear extinction recall (Day 3; D) was measured. SFC⁺ mice were i.p infused with Veh or CNO (15mg/kg) 30 min before extinction training. Data represent mean ± SEM. *p<0.05: DIO/CNO vs. Veh/Veh and Veh/CNO (C) using 2way ANOVA followed by Bonferroni's posthoc analysis . Immunohistochemical staining for fluorogold (FG; blue), mCherry (red) and oxytocin (OXT; green) within the SON and PVN and the average number of labelled neurons indicating OXTergic afferrents to the lateral septum (E) of virus infused lac mice. Data represent mean number of neurons ± SEM. *p<0.05 using Student's T-test (G).

3.2 Septal HDAC1 regulates SFC-induced social fear in male mice.

3.2.1 Summary

In this part of my thesis, I endeavored to reveal the brain region-specific role of epigenetic factors, such as HDACs, involved in the social fear acquisition, memory and extinction. Following cFos immunohistochemistry, I was able to ascertain that LS is specifically activated in response to acquisition of social fear also in male mice. Analysis of the mRNA levels of all class I HDACs (selected based on their extensive implication in cognition and memory formation: see section (1.9.1). In line with this, pharmacological blockade of HDAC1 using MS275 (a specific HDAC1 inhibitor) only facilitated extinction of social fear, but not that of cued fear. Following this up with a microarray I was able to identify genes, which were differentially regulated in response to SFC within the LS. Cross referencing this gene list against published HDAC1 regulated genes (Rouillard, Gundersen et al. 2016), I found a set of genes that could possibly be involved regulation of SFC-induced social fear in mice**.** Overall, in this study using molecular and pharmacological techniques I provide compelling evidence for a specific HDAC1-mediated social fear regulatory mechanism within the mice LS.

3.2.2 SFC and CFC lead to differential activation of LS and CA2 in male mice.

First, I compared the neuronal activation patterns in the brain in response to SFC and CFC. For SFC, male mice were sacrificed on day 1 of the SFC paradigm either with or without social fear acquisition (SFC$^+$/Ext$^-$ and SFC$^-$/Ext$^-$) or on day two after social fear extinction training (SFC$^+$/Ext$^+$). All animals were sacrificed 90 min post-treatment. For CFC, brains were collected at analogous time points with the corresponding groups being: CFC$^+$/Ext$^-$, CFC$^-$/Ext$^-$, and CFC$^+$/Ext$^+$. These brains were immunohistochemically stained for cFos, and immunopositive cells were counted in the BLA, LS, PVN and the hippocampal CA2 nucleus.

Behavior: The success of fear conditioning against both a social stimulus (for SFC) and a tone (non-social stimulus; CFC) could be assessed during fear extinction on day 2 during which all SFC$^+$ mice spent significantly low amount of time investigating the social stimulus (reflecting increased social fear) ($p<0.05$ vs. SFC$^-$ mice; Fig 19A) and the CFC$^+$ mice exhibited increased freezing on CS-presentation ($p<0.05$ vs. CFC$^-$ mice; Fig 19F).

LS: A significant increase in the number of cFos-positive neurons was seen within the LS (Fig 19B) of SFC$^+$/Ext$^-$ mice 90 min after social fear acquisition ($p<0.05$ vs. SFC$^-$/Ext$^-$), which returned to baseline levels after extinction ($p<0.05$ vs. SFC$^+$/Ext$^+$). Such dynamic response to fear acquisition or fear extinction was not observed within the LS (Fig 19G) at any time point during CFC. This suggested that LS is specifically activated by the acquisition of social fear.

CA2: As with the LS, I observed a robust increase in cFos-positive neurons within the CA2 (Fig 19E) 90 min after the social fear acquisition in the SFC$^+$/Ext$^-$ mice ($p<0.05$ vs. SFC$^+$/Ext$^+$). Such an increase was absent when the mice were cued fear conditioned (Fig 19J).

BLA and PVN: Neuronal activation within the BLA and PVN was measured as a positive control, as the BLA is a well-known neuronal correlate of fear expression in mice (Felix-Ortiz, Beyeler et al. 2013, Tovote, Fadok et al. 2015), and the PVN is known to be involved in stress response (Tan and Nagata 2002). Expectedly, increased activation could also be seen within the

BLA (Fig 19C) and PVN (Fig 19D) of SFC^+/Ext^- mice 90 min after social fear acquisition ($p<0.05$ vs. SFC^-/Ext^-). However, in contrast to the LS and CA2, both BLA (Fig 19H) and PVN (Fig 19I) were also activated 90 min after cued fear acquisition ($p<0.05$ vs. CFC^-/Ext^-) indicating the success of the cued fear acquisition process and the specificity of LS and CA2 neural correlates of social fear acquisition.

Statistics:

SFC (1way ANOVA)	*Group effect (SFC)*	*Group x stimulus effect*
Social fear extinction (Fig 19A)	$F(1, 10) = 11.25$, $p < 0.05^*$	$F(8, 80) = 7.22$, $p < 0.05^*$
	Group effect (SFC)	
cFos immunohistochemistry (1way ANOVA)		
	$F(3, 15) = 0.254$, $p < 0.05^*$	
cFos positive cells in LS (Fig 19B)	$F(3, 13) = 0.8004$, $p < 0.05^*$	
cFos positive cells in BLA (Fig 19C)	$F(3, 14) = 2.05$, $p < 0.05^*$	
cFos positive cells in PVN (Fig 19D)	$F(3, 16) = 2.036$, $p < 0.05^*$	
cFos positive cells in CA2 (Fig 19E)		
	Group effect (CFC)	*Group x stimulus effect*
CFC (1way ANOVA)	$F(1, 100) = 172.4$, $p < 0.05^*$	$F(9, 100) = 22.78$, $p < 0.05^*$
CFC – extinction (Fig 19F)	*Group effect (CFC)*	
cFos immunohistochemistry (1way ANOVA)		
	$F(1, 17) = 0.546$, $p = 0.8245$	
cFos positive cells in LS (Fig 19G)	$F(3, 15) = 0.8004$, $p < 0.05^*$	
cFos positive cells in BLA (Fig 19H)	$F(3, 14) = 1.303$, $p < 0.05^*$	
cFos positive cells in PVN (Fig 19I)	$F(3, 16) = 0.511$, $p = 0.7133$	
cFos positive cells in CA2 (Fig 19J)		

Fig 19. *Social fear conditioning (SFC) leads to specific activation of the lateral septum (LS). Number of cFos-immunopositive neurons (indicating neuronal activation) were counted from mice after SFC (A-E) and cued fear conditioning (CFC; F-J) within the LS (B and G), basolateral amygdala (BLA; C and H), paraventricular nucleus (PVN; D and I) and hippocampal CA2 (E and J). Mice were sacrificed 90 min after, either social fear acquisition (SFC-/Ext- and SFC+/Ext-; n =6) and after cued fear acquisition (CFC-/Ext- and CFC+/Ext-; n =6) on day 1 or after social fear extinction (A; SFC+/Ext+ and SFC+/Ext+; n =6) and after cued fear acquisition (D; CFC-/Ext+ and CFC+/Ext+; n =6) on day 2. Data represents mean number of cFos-immunopositive neurons/0.1mm^2 ± SEM (B-E and G-J), mean percentage of investigation time ± SEM (A) and mean percentage of CS-elicited freezing ± SEM (F). *p<0.05 vs SFC- (A), CFC (F) and respectively marked groups (B, C, D, E, H and I).*

3.2.3 Hdac1 mRNA is dynamically regulated within the LS of male mice during SFC.

In order to reveal the involvement of epigenetic mechanisms, I proceeded to analyze the expression of class I *Hdacs (Hdac1, Hdac2, Hdac3, Hdac8)* within the LS and CA2. Using qRT-PCR I quantified the mRNA levels of all class I *Hdacs* within the LS and CA2 at different time points during SFC. No differential expression of any class I *Hdacs* could be seen within the CA2 hippocampal nuclei (data not shown). Only *Hdac1*, but none of the other class I *Hdacs*, showed significant changes in response to the social fear acquisition and social fear extinction. Specifically, *Hdac1* mRNA levels were elevated in the SFC$^+$/Ext$^-$ mice, i.e., 120 min after social fear acquisition on day 1 of the SFC paradigm (p<0.05 vs. SFC$^-$/Ext$^-$; Fig 20A), and returned to baseline 120 min after social fear extinction on day 2. This result pointed towards the possible involvement of *Hdac1*, specifically within the LS in regulating SFC-induced social fear.

Statistics:

| qRT-PCR (mRNA levels; 1way ANOVA)	Group effect (Group: Treatment vs. SFC)
Hdac1 mRNA (Fig 20A)	$F(3, 39) = 1.904$, $p < 0.05$*
Hdac2 mRNA (Fig 20B)	$F(3, 13) = 0.6430$, $p = 0.572$
Hdac3 mRNA (Fig 20C)	$F(3, 13) = 0.7107$, $p = 0.587$
Hdac8 mRNA (Fig 20D)	$F(3, 14) = 1.543$, $p = 0.1487$
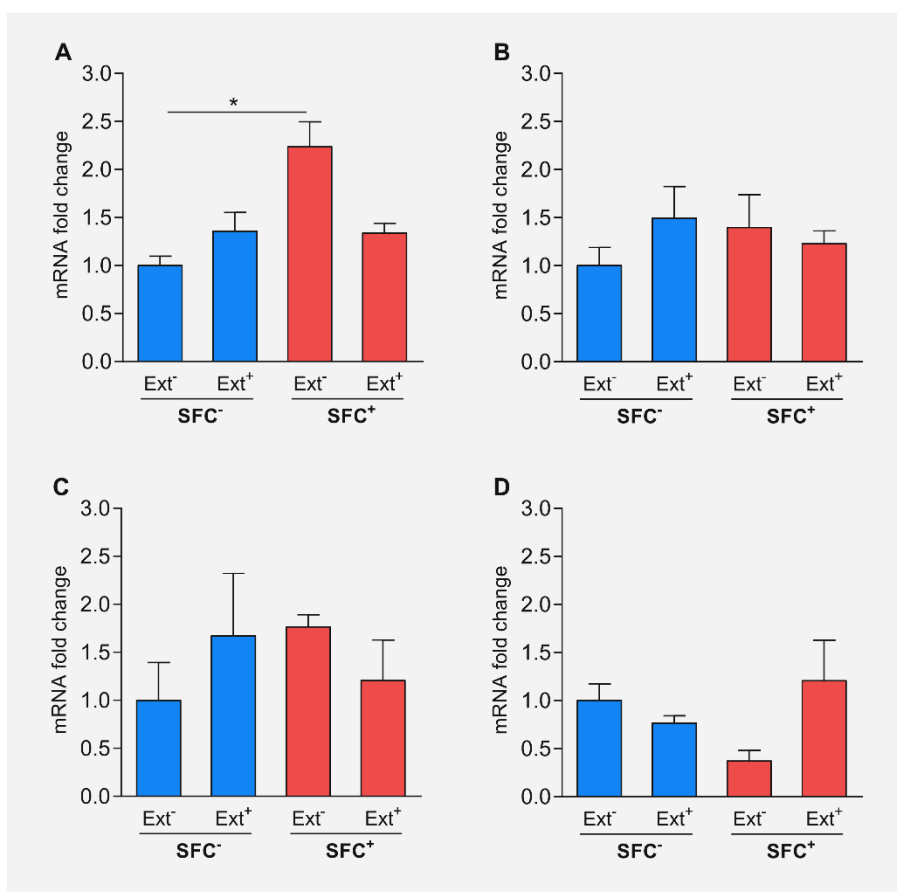
Fig 20. *Hdac1 mRNA is specifically upregulated after social fear conditioning within the male mouse LS. mRNA was isolated from mice LS micropunches 120 min after social fear conditioning (SFC⁻/Ext⁻ and SFC⁺/Ext⁻; n = 6; Day 1) or after extinction (SFC⁻/Ext⁺ and SFC⁺/Ext⁺; n = 6; Day 2) and gene expression for Hdac1 (A), Hdac2 (B), Hdac3 (C), and Hdac8 (D) was measured. Data represents mean of normalized fold change ± SEM. *p<0.05.*

3.2.4 Pre-extinction pharmacological inhibition of HDAC1 within the LS of male mice facilitates extinction of SFC-induced social fear.

Based on the elevated *Hdac1* mRNA levels within the LS revealed by qRT-PCR, I endeavored to study the consequences of the local blockade of HDAC1 before fear extinction on SFC-induced social fear expression. All groups of SFC⁺ mice required the same number CS-US pairings before they displayed social avoidance during social fear acquisition (Fig 21A). During social fear extinction, mice infused with selective HDAC1 inhibitor MS275 displayed similar low levels of social investigation during the first social presentation, but social fear extinction was significantly accelerated in these animals, as seen by the steeper increase in social

investigation in the MS275/SFC$^+$ group (p<0.05 vs. Veh/SFC$^+$; Fig 21A) especially of the 2nd and 3rd social stimulus. Investigation times of the 4th to 6th social stimuli still tended to be elevated in the MS275/SFC$^+$ group, but these differences did not reach statistical significance. Treatment with MS275 in SFC$^-$ mice did not affect social investigation times. All groups showed similar high level of social investigation during social fear recall on day 3 of the SFC paradigm (Fig 21C) indicating the success of extinction procedure.

Statistics:

SFC (Veh vs. MS275)	Group effect (Group: Treatment vs. SFC)	Group x stimulus effect
Student's T-test: Social fear acquisition (Fig 21A)	T(16) = 0.9177, p = 0.3724	
2way ANOVA: Social fear extinction (Fig 21B) Social fear recall (Fig 21C)	F(3, 234) = 38.17, p < 0.05* F(3, 156) = 4.77, p = 0.333	F(24, 234) = 3.06, p < 0.05* F(15, 156) = 0.776, p > 0.99
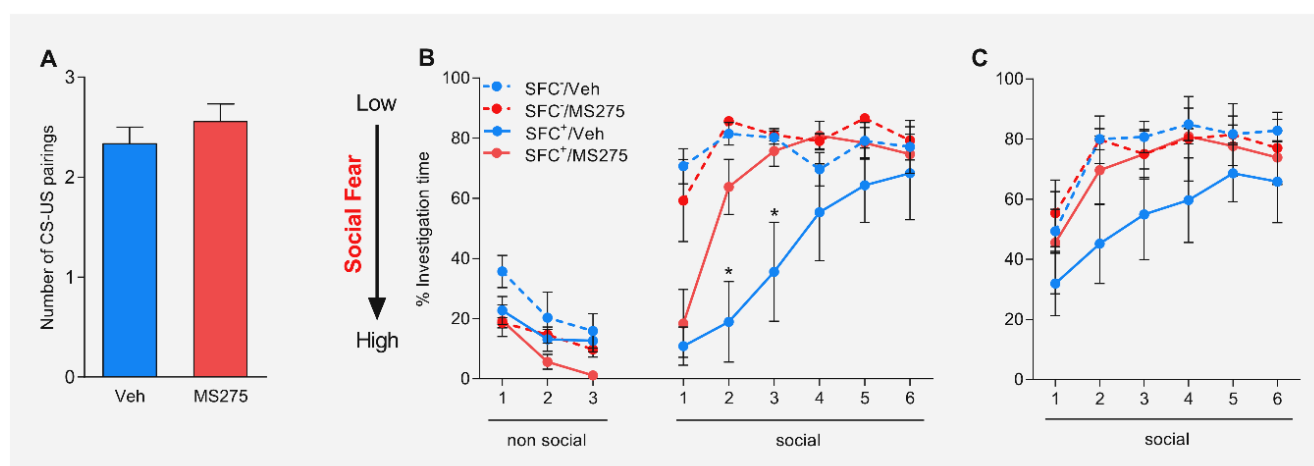
*Fig 21. Extinction of SFC-induced social fear could be facilitated by blocking HDAC1 in the lateral septum. Percentage investigation of 3 non-social (empty cage) and 6 social (cage with a conspecific) stimuli during social fear extinction (day 2; B) and social fear extinction recall (day 3; C) was measured. During social fear acqusition (day 1; A) mice were left unconditioned (SFC$^-$) and conditioned (SFC$^+$) following which they were bilaterally infused with vehicle (Veh; 0.5% DMSO in Ringer) or MS275 (375 ng/0.2 µl/side) into the LS 90 min before extinction training (n = 7-9/ group). Data represents mean percentage of ± SEM. *p<0.05 vs. SFC$^+$/MS275, SFC$^-$/Veh, and SFC$^-$/MS275 (B).*

3.2.5 Pre-extinction pharmacological inhibition of HDAC1 within the LS of male mice has no effect on CFC-induced fear.

To test for the role of LS HDAC1 in non-social fear induced by CFC, I blocked HDAC1 by bilateral infusion of MS275 into the LS of SFC[+] mice with Veh as a control group. All mice achieved similar levels of tone – foot shock association independent of subsequent treatment reflected by CS-elicited freezing at the final CS-presentation during fear acquisition on day 1 (Fig 22A). Importantly, no significant difference in CS-elicited freezing was observed during cued fear extinction training or extinction retention between mice treated with either vehicle or MS275 90 min before cued fear extinction. This demonstrates that the cued fear expression is not regulated by the HDAC1 activity within the LS (Figs 22B and 22C).

Statistics:

| CFC (Veh vs. MS275)	Group effect (Treatment)	Group x stimulus effect
2way ANOVA: CFC – acquisition (Fig 22A) CFC – extinction (Fig 22B)	$F_{(1, 14)} = 6.59$, $p < 0.05$* $F_{(1, 7)} = 0.048$ $p = 0.8322$ $T_{(14)} = 0.4648$, $p = 0.6492$	$F_{(4, 56)} = 3.035$, $p < 0.05$* $F_{(9, 63)} = 1.078$, $p = 0.3912$
Student's T-test: CFC – recall (Fig 22C)		
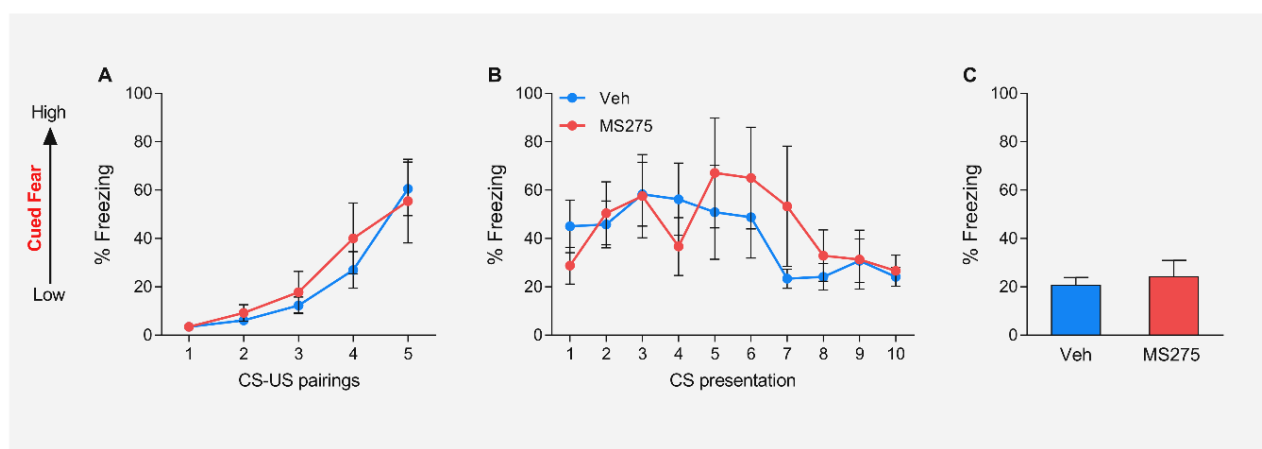
Fig 22. Blocking HDAC1 within the mouse LS does not affect CFC-induced fear expression. Mice were fear conditioned on day 1 (A), infused with Veh (0.5% DMSO in Ringer; n = 8) or MS275 (375 ng/0.2 µl/side; n = 8) 90 min before fear extinction on day 2 (B) and extinction retention was measured on day 3 (C). Data represents mean percentage of CS-elicited freezing ± SEM.

3.2.6 HDAC1 is ubiquitously expressed in neurons and astrocytes within the adult mouse brain

Although traditionally HDAC1 was thought to be expressed exclusively in astrocytes within the adult mouse CNS (MacDonald and Roskams 2008), recent studies have suggested that HDAC1 is also expressed in neurons of the adult mouse brain (Zhu, Vidaurre et al. 2017). Thus in order to find out, whether it is the neuronal or the astrocytic HDAC1 which regulates social fear within the LS I co-stained brain slices of SFC$^+$ and SFC$^-$ mice for HDAC1 with either NeuN as a neuronal marker (Duan, Zhang et al. 2016) or with glial fibrillary acidic protein (GFAP) as an astrocytic marker (Baba, Nakahira et al. 1997). Confocal imaging revealed the HDAC1 co-localized with both NeuN (Fig 23 B and C) indicative of expression in neurons and GFAP (Fig 24 B and C) indicative of expression in astrocytes. In both cases, HDAC1 signals also colocalized with that of DAPI (Fig 23 B and C; Fig 24 B and C). Colocalization with DAPI suggested that HDAC1 was expressed within the nucleus of neurons and astrocytes. Also, there was no observable difference in HDAC1 expression between the SFC$^+$ and SFC$^-$ mice.
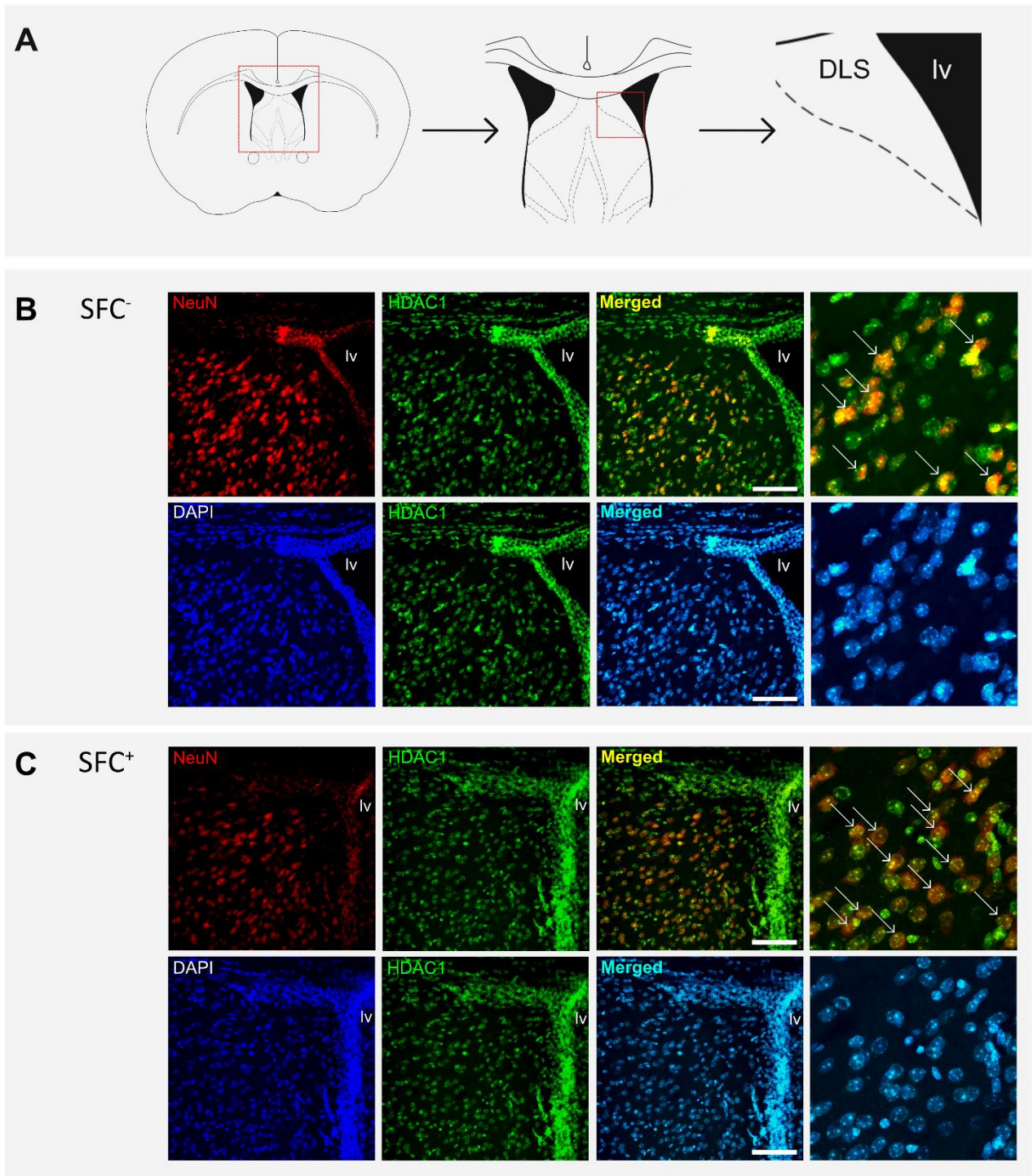
Fig 23. Schematic representation of the analysed region (DLS: dorsal lateral septum; lv: lateral ventricle; A). Immunofluorescence staining followed by confocal imaging was performed 60 min after social fear acquisition on day 1 with unconditioned (SFC⁻; B) and conditioned (SFC⁺; C) mice. Panels show costaining of HDAC1 with either NeuN (neuronal marker) or DAPI (nuclear marker). Scalebar = 0.1 mm².
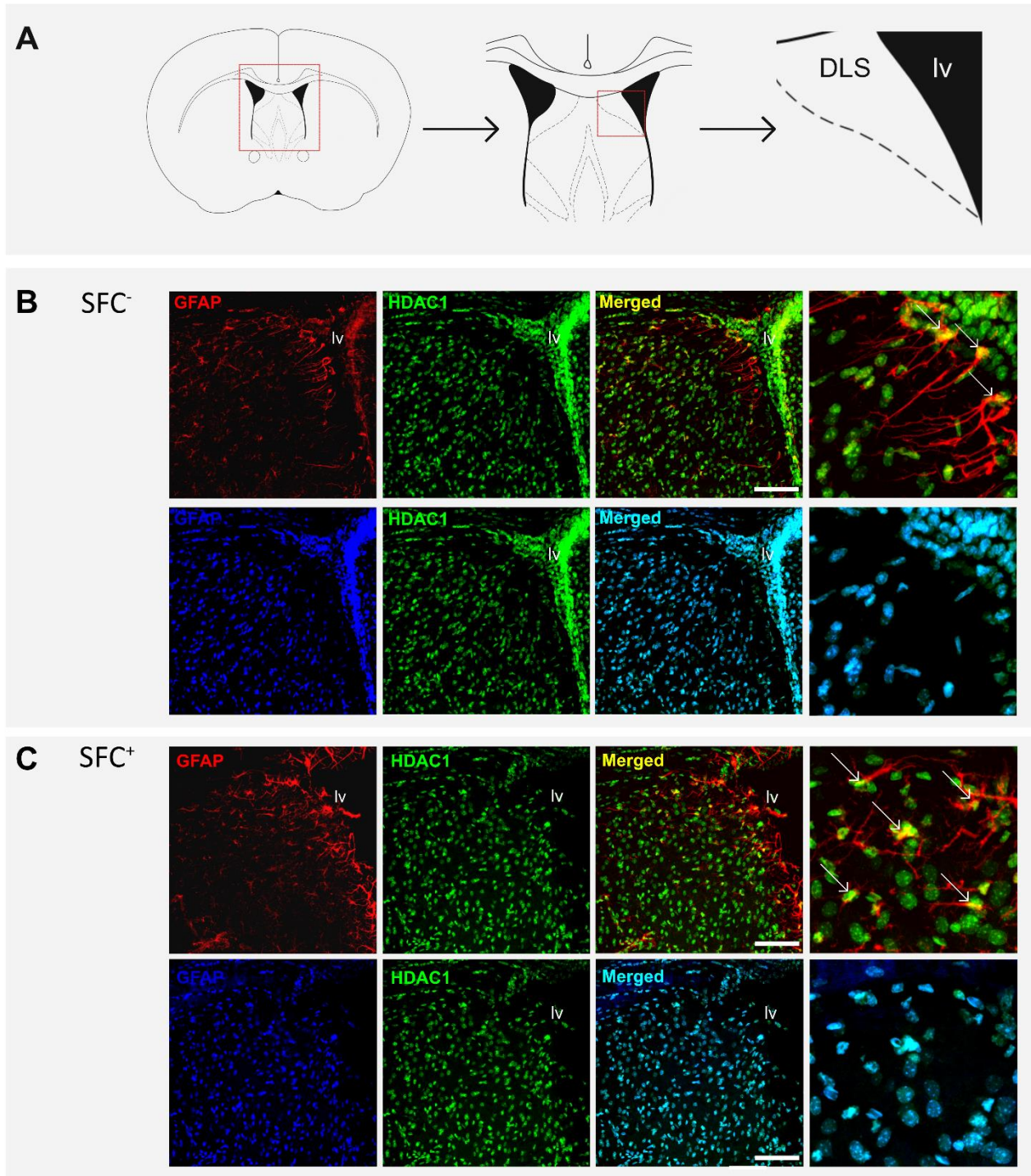
*Fig 24.* : Schematic representation of the analysed region (DLS: dorsal lateral septum; lv: lateral ventricle; A). Immunofluorescence staining followed by confocal imaging was performed 60 min after social fear acquisition on day 1 with unconditioned (SFC⁻; B) and conditioned (SFC⁺; C) mice. Panels show co-staining of HDAC1 with either GFAP (astrocytic marker) or DAPI (nuclear marker). Scalebar = 0.1 mm².

3.2.7 Whole genome mRNA expression analysis revealed possible candidates for HDAC1 targets

To assess the differential gene expression within the LS in response to SFC, I performed microarray using Affymetrix Mouse Gene 2.1 ST array (SFC$^+$ vs. SFC$^-$; n =3/ group). I identified a total of 96 differentially expressed genes (p<0.05; fold change < 1.5) within the LS of SFC$^+$ mice in comparison to SFC$^-$ mice. Cross-referencing these genes with potential HDAC1 binding sites obtained from 'Harmonizome', which is a collection of processed datasets gathered from over 70 major online resources (Rouillard, Gundersen et al. 2016), I identified 16 genes, which (i) are known to have HDAC1 binding sites and (ii) to be regulated by HDAC1 responsive to SFC.

Affymetrix Mouse
Gene 2.1 ST array

Harmonizome
ChIP seq database

96   **16**   13852

**Final list of genes
displayed in table 2**
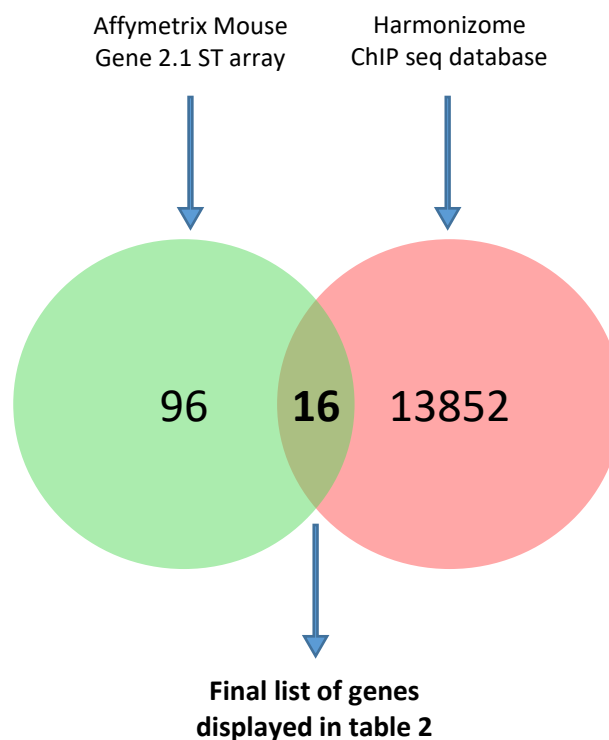
*Fig 25. Schematic representation of genes differentially regulated
by SFC as found using the Affymetrix array and known to possess
HDAC1 binding sites obtained from Harmonizome.*

No	Probe ID	Accession No	Gene Symbol	Fold change	P value	References
1	17515402	AK035548	*Cnn1*	2,23	0,01	(Anastasiadou and Knoll 2016, Hossain, Zhao et al. 2016)
2	17291183	AK010121	*Hist1h3f*	1,90	0,01	(Mirisola, Mora et al. 2011, Datson, van den Oever et al. 2013)
3	17374972	AB195274	*Pla2g4b*	1,75	0,01	(Tao, Yu et al. 2005, Matsunami, Hensel et al. 2014)
4	17547744	AK002451	*Cks2*	1,71	0,01	(Frontini, Kukalev et al. 2012, Lv, Zhang et al. 2013)
5	17464282	AK148179	*Sspn*	1,70	0,00	(Grady, Zhou et al. 2000, Fort, Estrada et al. 2005)
6	17527661	AB024538	*Islr*	1,64	0,01	(Homma, Shimada et al. 2009, Sollner and Wright 2009)
7	17484897	AK012402	*Rplp2*	1,54	0,02	(Shimada, Matsui et al. 2009, Artero-Castro, Castellvi et al. 2011)
8	17458514	AK002982	*Npy*	1,54	0,04	(Reichmann, Wegerer et al. 2016, Schmeltzer, Herman et al. 2016)
9	17312312	AK153712	*Mapk15*	-1,54	0,04	(Rossi, Colecchia et al. 2011, Colecchia, Strambi et al. 2012)
10	17456084	AK051668	*Foxp2*	-1,54	0,03	(Bracha 2006, Chen, Kuo et al. 2016)
11	17442307	BC150919	*Lrrc43*	-1,56	0,02	(Strausberg, Feingold et al. 2002, Kimura, Wakamatsu et al. 2006)
12	17210904	AK039151	*Oprk1*	-1,64	0,01	(Bilkei-Gorzo, ERK et al. 2012, Ji, Wang et al. 2015)
13	17504935	AK006041	*Tsnaxip1*	-1,65	0,03	(Bray, Chennathukuzhi et al. 2002, Yang and Hecht 2004)
14	17320571	AB026807	*Syt10*	-1,77	0,02	(Cao, Maximov et al. 2011, Woitecki, Muller et al. 2016)
15	17282557	AY993933	*Syndig1*	-1,82	0,02	(Diaz 2012, Lovero, Blankenship et al. 2013)
16	17542194	AK079258	*Gabre*	-2,07	0,01	(Fatemi, Folsom et al. 2013, Bersten, Wright et al. 2014)

Table 2. *Final set of differentially regulated (upregulated: green; downregulated: red) genes known to be regulated by HDAC1*

Among these 16 genes (summarized in table 1) were 8 upregulated genes including histone cluster 1 H3F *(Hist1h3f) and* CDC28 protein kinase regulatory subunit 2 *(Cks2),* which are involved in maintaining the integrity of the cell cycle. Moreover, genes like ribosomal protein,

large subunit 2 *(Rplp2) and* phospholipase A2, group 4B *(Pla2G4b),* which regulate cellular metabolism. Also, among the upregulated genes was neuropeptide Y *(Npy),* which is known for its anxiolytic effect within the brain. Amongst the eight downregulated genes were genes involved in maintenance and regulation of synaptic plasticity like synaptogamin 10 *(Syt10),* synapse differentiation inducing 1 like *(Syndig1l),* and forkhead box protein P2 *(Foxp2)* - a master transcription factor known to be involved in speech development and social behavior (ultrasonic vocalization).

Discussion

Although mechanisms underlying general fear and anxiety are fairly well understood, similar knowledge regarding molecular and neuronal mechanisms differentially regulating specific subtypes of fear are seldom studied. During the course of my thesis, I was able to advance the current knowledge regarding the molecular mechanism specifically regulating social fear in mice. More precisely, I could reveal the following aspects regarding the molecular underpinnings of social fear in mice:

a. I was able to show that endogenous OXT signaling within the LS regulates social fear in female mice (section 4.1).

b. I was able to show that HDAC1 within the DLS acts as a key regulator of social fear extinction (section 4.2).

4.1 Septal oxytocin signaling regulates social fear in female mice

4.1.1 Lactating mice exhibit reduced fear expression without confounding alterations in general anxiety, social preference or pain perception

To assess the response of female mice with an activated brain OXT system to traumatic experiences in both a social and non-social context, we first compared the SFC- and CFC-induced fear responses between lactating and virgin mice. Lactating mice, unlike their virgin counterparts, did not express social fear after normal social fear acquisition (Fig 8D). In addition to the abolished social fear expression, lactating mice also did not express any cued fear during cued fear extinction (Fig 12B). However, in contrast to SFC, they already displayed an attenuated cued fear learning during cued fear acquisition (Fig 12A). This differential acquisition of traumatic experiences in a social versus a non-social context could stem from the higher ethological relevance a social stimulus (a conspecific mouse) holds compared to a non-

social stimulus (in my case a tone; 80 dB, 8 kHz, 30 s continuous sound) for all social mammals, which includes mice and humans (Insel 2002, Young 2002). Such differences mean that neurobiological and physiological changes that accompany lactation could have different effects on acquisition of social versus cued fear. Differences in fear expression during extinction could be attributed to impaired acquisition only in the case of CFC. However, for SFC, deficits in social fear expression could not be attributed to the impaired fear acquisition. Thus, for proper interpretation of my results, it was imperative to test for lactation-associated alterations in general anxiety-related behavior, social preference behavior, and pain perception. Testing on the EPM revealed a similar level of general anxiety in virgin and lactating mice (Fig 8A). Therefore, I could exclude a significant contribution of altered general anxiety-related behavior to the observed lack of social (and cued) fear expression seen in conditioned lactating mice. This finding of unaltered anxiety-related behavior in lactating mice is rather surprising, as reduced anxiety levels have been described in lactating rats under some experimental circumstances, which was found to be due to the high activity of the brain OXT system in the peripartum period (Neumann, Torner et al. 2000, Jurek, Slattery et al. 2012). Also, both lactating and virgin female mice showed similar levels of social preference behavior towards an unknown virgin female mouse as tested in the SPT (Fig 8C), which seems to be another prerequisite for the proper interpretation of my data. This result is analogous to the preference of lactating SFC⁻ and virgin SFC⁻ mice for conspecifics (Fig 8D, social stimulus 1-6) compared with the low level of investigation of the empty cages (Fig 8D, non-social stimulus 1-3) during social fear extinction. Moreover, lactation-induced attenuation in nociception could impair associative learning, which forms the experimental basis of the two fear conditioning paradigms used this study and lead to enfeeblement of fear expression during extinction irrespective of the context. This theory is supported by studies corroborating the nociceptive properties of OXT in rats (Knobloch, Charlet et al. 2012), the more as overactivation of the brain OXT system during lactation is a cornerstone of this study. Using Hargreaves' plantar test, I found that pain

perception was not altered in lactating mice (Fig 14). In support, the primary somatosensory cortex, which is a region known to be activated in response to painful stimuli (Vierck, Whitsel et al. 2013), showed similar neuronal activity as indicated by cFos-immunoreactivity in virgin and lactating SFC[+] mice 90 min after fear conditioning. Studies dealing with pain perception in lactation have mainly been performed in rats and yielded contradictory results depending on the species, the phase of lactation at which the animals were tested or duration of separation from pups at the time of testing (Gintzler 1980, Rushen, Foxcroft et al. 1993, Martinez-Gomez, Cruz et al. 1994). Comparable data in lactating mice have not been available so far. Thus, taken together, these results exclude the possibility that the lack of SFC and CFC-induced fear expression in lactating mice is due to altered general anxiety, social preference behavior or pain perception.

4.1.2 Neuroanatomical adaptations of the LS-OXT system during lactation

cFos expression has been considered to be an indication of neuronal activity within the brain induced by various kinds of environmental stimuli (Morgan and Curran 1991, Knapska and Maren 2009, Cruz, Koya et al. 2013). Accordingly, I performed cFos immunohistochemistry to identify differentially activated brain regions during SFC between lactating and virgin mice. In virgin SFC[+] mice, I found an activation of the LS and BLA 90 min after social fear acquisition on day 1 in comparison with virgin SFC[-], although this reverted to baseline levels 90 min after social fear extinction (day 2; Fig 10A and B). Interestingly, the LS and BLA showed significantly reduced activity in SFC[+] lactating mice at each time point during SFC in comparison to their corresponding virgin controls. This difference was prominent at 90 min after social fear acquisition (day 1) in the LS, where virgin SFC[+] mice showed an approximately 6-fold increase in cFos-positive cells compared to lactating SFC[+] mice (Fig 10A). The BLA – as amply described in the introduction – is critical in assembling information regarding the fearful stimuli and its activation is essential to produce fear responses. Thus its dampened

activity could be an indication of reduced fear memory formation in lactating mice. The LS is a region known to be activated in response to aversive and stressful stimuli such as exposure to 5-min forced swimming, fear conditioning and immobilization stress (Pezzone, Lee et al. 1992, Beck and Fibiger 1995, Singewald, Salchner et al. 2003). It also has a well-established role in regulating social behavior and is directly connected to regions such as the hippocampal CA2 region, which controls social memory (Hitti and Siegelbaum 2014), or to the anterior hypothalamus to control stress responses (Anthony, Dee et al. 2014). So it is possible that the LS is specifically involved in the coding of social information within the SFC paradigm, and its dampened activity found in lactation could indeed lead to impaired social information processing leading to lack of social fear expression during extinction.

A major brain factor, which I hypothesized to be involved in the reduced conditioned fear expression, is OXT, which has repeatedly been shown to be highly activated in lactation. This is reflected by elevated expression of OXT and its receptor, by increased OXTR binding and activated local release in limbic and hypothalamic regions triggered by suckling and maternal-offspring interactions (Neumann, Russell et al. 1993, Francis, Champagne et al. 2000, Bosch, Kromer et al. 2004, Slattery and Neumann 2008). To identify the anatomical substrate of OXT within the LS of lactating dams, I compared the number of OXT-immunopositive fibers between virgin and lactating mice and found a 2-fold increase in the number of OXT-positive fibers innervating the LS during lactation (Fig 15). Although axonal OXT projections to the LS have been reported previously (Knobloch, Charlet et al. 2012, Grinevich, Knobloch-Bollmann et al. 2016), here I demonstrate for the first time anatomical signs of OXT axon plasticity, such as an increase of OXT-immunosignal in axons of lactating mice. This might reflect the acceleration of axonal OXT trafficking (and probably local OXT release) during lactation. An increased release of OXT within the LS has been previously reported in response to female reproduction-related stimuli (Neumann and Landgraf 1989, Landgraf, Neumann et al. 1991).

To ascertain the functional nature of the lactation-specific neuroanatomical adaptations of the LS-OXT system, I performed intracerebral microdialysis within the LS of virgin and lactating mice before and during social fear extinction. I was unable to detect a significant potentiation in OXT release in response to exposure to the social stimuli in SFC[+] virgin mice, which is very similar to the previous observations in SFC[+] male mice (Zoicas, Slattery et al. 2014). However, in contrast to SFC[+] virgin mice, I detected a significant increase in OXT release in the LS of lactating SFC[+] (and virgin SFC[-] mice) in response to social stimuli during social fear extinction (Fig 16C and D). Thus, I conclude that the observed adaptations of the LS-OXT system are functional in nature.

An interesting point of discussion is the behavior of SFC[+] lactating mice during social fear extinction and ongoing microdialysis experiments. Lactating mice in this experiment did not show the high levels of the social contact shown by the SFC[+] lactating mice in other SFC experiments (check section 3.1.2 and 3.1.3). Specifically, SFC[+] lactating mice with ongoing microdialysis show an impaired social fear extinction, which was very similar to the previously published behavior shown by male SFC[+] mice (Zoicas, Slattery et al. 2014). A blunted social fear extinction shown by the SFC[+] lactating mice could be caused by the implanted microdialysis probe, which could damage some of the OXTR-positive interneurons or even OXT fibers within the LS. Moreover, a local "wash-out" of endogenous OXT within the extracellular fluid might prevent activation of the OXTR signaling within the LS of these mice to of the LS cannot be excluded.

4.1.3 LS-OXT signaling as a critical regulator of social fear expression in female mice

We have previously shown that compensating for the lack of endogenous OXT release during social fear extinction with icv, or local LS infusion of synthetic OXT reverses SFC-induced social fear in male mice (Zoicas, Slattery et al. 2014). Supporting this, pharmacological inhibition of the central OXT signaling with icv infusion of the OXTR-A 30 min before fear

extinction rescued the expression of SFC-induced social fear in lactating mice (Fig 9A) to a level typical seen in virgin SFC$^+$ mice. In line, icv infusion of OXT 10 min prior to extinction reversed SFC-induced social fear in virgin SFC$^+$ mice (Fig 9C). Similar pharmacological blockade of the OXT system in lactating mice before acquisition did not have any discernible effect on social fear expression (data not shown). Corroborating these data, constitutive genetic knockout of the OXTR impaired extinction of social fear (Fig 17H) without affecting social fear acquisition (Fig 17G). This suggests a significant role for brain OXT signaling in supporting extinction of conditioned social fear in female mice.

Based on the abovementioned pharmacological data and the attenuated neuronal activation within the LS of lactating mice after SFC, I tested the hypothesis of a local inhibitory effect of OXT on SFC-induced social fear. Confirming my line of thought, bilateral infusion of OXTR-A into the LS, 30 min before social fear extinction training resulted in a complete retrieval of social fear expression in lactating mice (Fig 11A). Conversely, bilateral infusion of OXT into the LS of virgin SFC$^+$ mice robustly reversed social fear expression (Fig 11C). The capacity of OXT to locally attenuate social fear expression within the LS has been recently shown in male SFC$^+$ mice, as infusion of OXT into the dorsal part of the LS completely reversed social fear (Zoicas, Slattery et al. 2014). In support, conditional genetic downregulation of OXTR within the LS is known to cause deficits in social memory leading to lack of preference for social novelty (Mesic, Guzman et al. 2015). Thus, it seems that OXTR-expressing neurons within the LS seem to mediate increased preference towards a novel neutral conspecific. This again points towards the importance of LS as a structural correlate of social fear extinction.

In acquiescence to the social fear ablative effect of OXT infusion into the LS of virgin and male mice, I found that enhancement of the activity of OXTR-positive neurons within the LS also facilitates extinction of social fear (Fig 17E). Although activation of OXTR-expressing neurons within the LS by IRES is akin to agonizing the OXTR receptors, there are some noteworthy

differences. Firstly, IRES-mediated activation of translation leads to overall upregulation of activity of all those transfected neurons expressing the OXTR in our experimental setting, whereas local synthetic OXT leads to activation of specific OXTR-coupled intraneuronal signaling cascades (Jurek and Neumann, 2018). Thus, IRES-mediated activation might induce fear-enhancing pathways along with fear relieving ones. For example, social defeat-mediated activation of the OXTR-ERK pathway within the LS is correlated with potentiation of contextual fear (Guzman, Tronson et al. 2013). Another recent study has provided compelling evidence for corticotrophin releasing factor receptor 2 (CRFR2) positive GABAergic interneurons within the LS to be mediators of stress-induced anxiety (Anthony, Dee et al. 2014). In my experiments, I also found most (97%) of the OXTR-expressing neurons within the LS to be calbindin-positive (i.e., GABAergic) (Fig 4: A1-A3). Whether a subset of OXTR positive neurons co-express the CRFR2 is a matter of further investigation, but such an occurrence could easily dilute the social fear reversing the effect of the LS-OXT signaling. Secondly, all the studies mentioned above were performed in male mice, whereas this dataset is obtained from female mice. This is important, as sex-specific functions of the OXT system are well known in mice. For example, cortical OXTR-positive interneurons are known to be anxiolytic in male mice and prosocial in females mice (Li, Nakajima et al. 2016, Kim, Yang et al. 2017). Such sexual dimorphism could exist in in the LS-OXT system functionality which potentially uses different mechanisms to negate SFC-induced social fear in males vs. females. Together these results strongly suggest that LS-OXT enhances the positive valence of social-interaction and, thus, helps to counter the effect of SFC-induced social fear with a consequent reduction in social fear expression.

4.1.4 Sufficiency of SON-LS OXTergic projections in countering SFC-induced social fear in female mice

Up until this point, I was able to ascertain that pharmacological and genetic manipulation of the LS-OXT system efficiently regulates social fear expression in a bidirectional fashion. In an attempt to establish the sufficiency of LS-OXT to reduce social fear expression, I also showed that chemogenetic silencing of magnocellular OXTergic afferents to the LS completely blocks social investigation in lactating SFC$^+$ mice (Fig 18). Interestingly, very few mCherry-labelled neurons could be seen in the PVN, which was indicative of the small number of OXTergic projections to the LS originating in the PVN. In contrast, most of these projections arose from the SON and were magnocellular in nature. SON astrocytes are known to undergo extensive plasticity during lactation which helps the magnocellular neuronal population to communicate and fire in bursts leading to milk ejection reflex (Theodosis, Poulain et al. 1981, Wang, Negoro et al. 1996). According to this long-held belief SON, magnocellular neurons have been almost exclusively associated with the peripheral release of OXT. My results in this section surmise the first study showing an adaptive central neuronal circuit involving SON magnocellular OXT neurons. Thus, the release of OXT within the LS by SON magnocellular neurons seems to be the underlying neuronal circuit, which is responsible for the reduction of social fear in lactating mice.

4.1.5 Specificity of LS-OXT signaling in regulation traumatic experiences only in a social context in female mice

This social fear-attenuating effect of LS-OXT is different to what is seen in CFC. I could show that icv OXTR-A infusion 30 min before acquisition or extinction of cued fear and consequent inhibition of endogenous OXT signaling failed to rescue cued fear expression in lactating mice (Fig 13). Corroborating this constitutive knockout of the OXTR in female mice did not affect acquisition or extinction of cued fear (data not shown). Also, IRES-mediated genetic

upregulation of translation within OXTR-expressing neurons failed to affect both acquisition and extinction of cued fear (data not shown). Such differential effects of OXT on the extinction of social vs. non-social fear is not new as icv infusion of OXT before cued fear extinction increases fear expression in male mice (Toth, Neumann et al. 2012). Supporting my point of view, OXT is known to reduce attention towards facial expression with a negative valence, but not towards neutral facial expressions or non-social images in rhesus monkeys (Parr, Modi et al. 2013). My data are indicative of the differential processing of social vs. non-social forms of fear by brain OXT circuits and in this regard specific involvement of the LS-OXT system in the regulation of fear only in a social context. Although this needs more research, one could hypothesize that the lack of cued fear expression in lactating mice is due to other neurophysiological or neuroendocrine adaptations that accompany lactation including reduction in HPA-axis activity (Slattery and Neumann 2008) and increase in prolactin levels in the brain (Torner and Neumann 2002, Torner, Toschi et al. 2002).

As mentioned before SON magnocellular neurons play a crucial role in parturition, milk ejection and peripheral OXT release in response to various physiological stressors. Since many of these functions are essential for maternal behavior, I checked whether LS-OXT signaling played any role in maternal behavior and found no effect of the social fear acquisition on maternal behavior (data not shown). Importantly, the presence of pups did not affect fear expression during social fear extinction (Fig 8F), which indicates that the nature of the social stimuli (adult same-sex conspecific versus juvenile pups) matters in the SFC paradigm. Also, in contrast to what has been described in lactating rats (Neumann et al. 1989) OXT release could not be detected in response suckling in the mouse LS, and chemogenetic silencing of magnocellular SON-LS afferents did not alter maternal behavior (data not shown). Together these data conclusively show that LS-OXT system explicitly regulates extinction of social fear, but does not seem to essential for maternal behavior.

4.1.6 Oxytocin mediates its effects via increasing GABA signaling within the LS

Neuroanatomical assessment of the LS using calbindin-staining of brain slices from the OXTR reporter mice showed that most of the OXTR-expressing neurons within the LS are GABAergic. In many cases, OXT axons carrying varicosities formed closed appositions to somas and dendrites of GABAergic neurons (Fig 15C and D). The latter suggests the existence of precise OXT release sites in proximity to OXTR-expressing GABAergic neurons, whose electrophysiological activation by OXT has been reported for other brain regions, such as the central amygdala (Knobloch, Charlet et al. 2012), and the dorsal (Owen, Tuncdemir et al. 2013) and ventral (Eliava and Grinevich, unpublished) hippocampus. In analogy to these reports, one could speculate that OXT excites GABAergic interneurons in the LS. However, evidence from rats shows that GABA neurons in the LS are reciprocally connected (Jakab and Leranth 1990), and, hence, their overall excitability might be suppressed in the situation of massive OXT release during lactation. This may be reflected by the low cFos signal found under basal and SFC-induced conditions in lactating mice. Indeed, an increase in GABAergic signaling along with reduced cFos expression within the LS has been described during lactation (Lee and Gammie 2007, Lee and Gammie 2009, Zhao, Bao et al. 2012). Thus, I conclude that GABAergic neurons of the LS project, both directly and indirectly, e.g., via the medial septum and diagonal band of Broca, to numerous brain regions, including structures that orchestrate processing and expression of fear such as the amygdala or ventral hippocampus. The lower SFC-induced activation of the BLA in lactating mice (Fig 10B) suggests that this could be a potential target region for the OXTR-expressing GABAergic projections originating in the LS. However, axonal projections from OXTR septal neurons and their central targets, which likely form various neuroanatomical and functional circuits regulating social fear, require further exploration.

4.1.7 Outlook

SFC-induced social fear is in contrast to the innate motivation of a mouse to investigate a novel conspecific. Thus, when confronted with a social stimulus during extinction, the test animal is faced with the dilemma of whether to approach a "novel conspecific" or stay away from a "potential threat". In this thesis, I propose that an increased activity of the brain OXT system within the LS is essential and sufficient to increase social motivation and thus tilt this balance towards approach based behavior (for schematic representation see Fig 26). Such a mechanism, and consequently the brain circuit I describe in this thesis, might be the neuronal substrate, which underlies OXT's ability to overcome the SFC-induced inhibition of social approach and to investigate a novel and neutral conspecific.
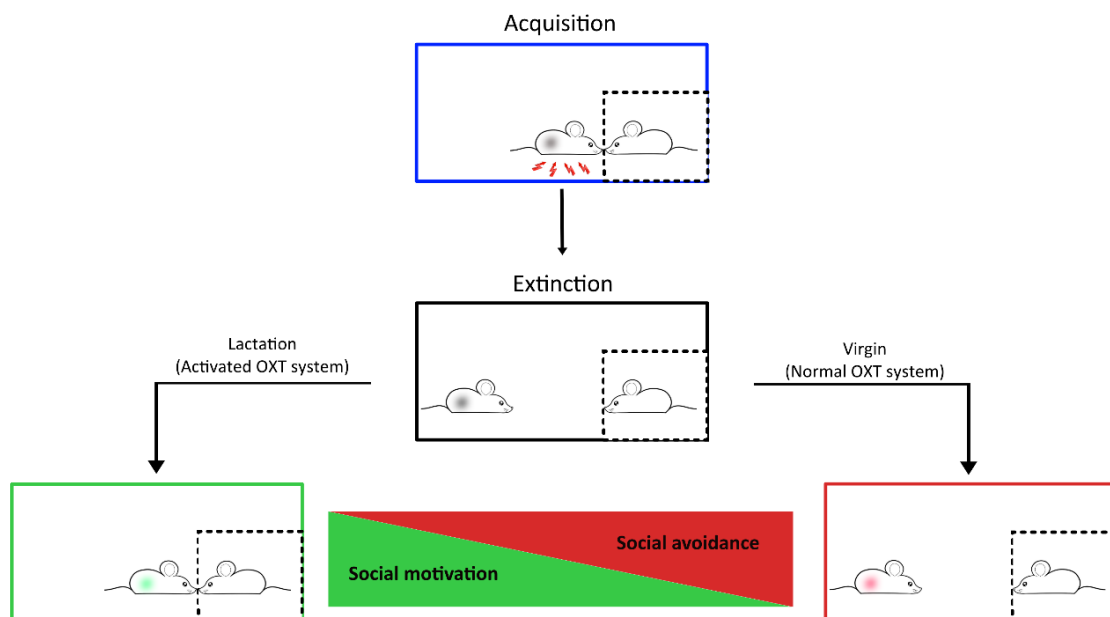

Fig 25. Schematic representation of the hypothesis.

Taken together, my results demonstrate that the lack of SFC-induced social fear in lactating mice is due to the heightened activity of the brain OXT system causing the specific release of OXT in the LS by a population of magnocellular SON neurons, which probably mediate

GABAergic inhibition of brain regions controlling the expression of conditioned social fear. These results are highly relevant for human SAD and other psychopathologies with high comorbidity to SAD such as autism, schizophrenia, and anorexia, which are largely unstudied during the period of breastfeeding.

4.2 Septal HDAC1 regulates social fear in male mice

4.2.1 SFC leads to specific activation of the LS

Neuronal gene expression changes dramatically in response to environmental stimuli and IEG's like cFos, which are specifically upregulated upon neuronal activation in response to environmental stimuli, have long been used as molecular markers of neuronal activity (Sheng and Greenberg 1990). This principle has been applied to fear conditioning paradigms as well (Ranjan, Singh et al. 2017, Sillivan, Joseph et al. 2017). cFos is one of the downstream targets of CREB (Lonze and Ginty 2002) and its activation within the BLA is necessary for the successful formation of auditory fear memory (Han, Kushner et al. 2007). For example, the BLA, which is a critical region for fear learning (Tovote, Fadok et al. 2015), was shown to have increased cFos labeling post contextual fear conditioning (Butler, Ehling et al. 2017). Increased cFos expression within the dorsal hippocampus has also been linked to contextual fear conditioning

(Kwapis, Alaghband et al. 2017). Thus IEG's like cFos are a reliable target for assessing neuronal activation within the brain in response to fear learning.

In my study, I found activation of BLA after both social (Fig 19C) and cued (Fig 19H) fear acquisition which implied that molecular activation of the BLA was essential for learning of both social and cued fear. Similar activation was also seen in the PVN post social (Fig 19D) and cued (Fig 19I) acquisition. This was expected as PVN being involved in stress responsiveness, and both SFC and CFC are also stress-inducing processes. In contrast to the regions mentioned above, LS (Fig 19B) and hippocampal CA2 (Fig 19E) were activated only after the social fear acquisition. LS, as explained abundantly in this thesis, is a brain region heavily implicated in social memory in male mice (Mesic, Guzman et al. 2015) and rats (Lukas, Toth et al. 2013). Although these studies specifically address the role of OXT system within the LS, one cannot rule out more general mechanisms including epigenetic pathways within the LS to be involved in formation and regulation of social memory. Similarly, pyramidal neurons

of the hippocampal CA2 nuclei are known to code for social memory. Genetic silencing of pyramidal neurons within the CA2 region causes loss of social memory in adult male mice (Hitti and Siegelbaum 2014). Such studies are complemented by data from humans which report a reduction in the number of inhibitory neurons within the CA2 of patients suffering from schizophrenia and bipolar disorders (Benes, Kwok et al. 1998). Thus, LS and CA2 seem to specifically code for the social component of fear learning involved in the acquisition of social fear. Thus, they could be considered as key components of the neuronal ensemble of social fear learning.

IEG expression has been linked to changes in histone acetylation. In fact, HDAC1 has previously been shown to reduce cFos expression, with increased HDAC1 levels leading to deacetylation of H3K9 and a corresponding decrease in cFos expression within the hippocampus (Bahari-Javan, Maddalena et al. 2012). In this case, I found both a high number of cFos positive neurons (in both LS and CA2) and an elevation in *Hdac1* mRNA levels post-acquisition of social fear only within the LS. Such discrepancy could be explained by the hypothesis that the increase in *Hdac1* mRNA levels is a response to decreased HDAC1 protein activity within the LS neurons. Therefore, I performed an ELISA to quantify HDAC1 activity with SFC[+] mouse LS protein lysates, and the results showed a decrease in HDAC1 activity post social fear acquisition. However, these results are preliminary and were, hence, not presented in this thesis. Such a decrease in the enzymatic activity of HDAC1 after SFC acquisition could explain the corresponding increase in cFos levels and a compensatory increase in *Hdac1* mRNA levels within the LS of SFC[+] mice.

4.2.2 Inhibition of HDAC1 within the LS facilitates the extinction of SFC-induced social fear

We observed that cFos protein levels within the LS reverted to baseline after the extinction of social fear. At the same time (i.e. 60 min after social fear extinction) we found increase activity

of HDAC1 in SFC$^+$ mice (preliminary results from ELISA; data not shown). Reduction in an activator of transcription like cFos combined with the increased activity of HDAC1 which is an integral unit of various transcription repressor complexes, must promote an overall dampening of gene expression. Considering the plethora of studies which have shown that active gene transcription and translation are a pre-requisites for successful memory formation, the dampening of gene expression caused by abovementioned molecular events could impair extinction memory formation. Such idea is supported by studies, which employ pharmacological or genetic approaches for inhibition of HDACs to enhance cognitive and learning abilities in mice (Guan, Haggarty et al. 2009, Singewald, Schmuckermair et al. 2015, Whittle, Maurer et al. 2016). Within the SFC paradigm, I observed a facilitation of extinction (Fig 21B) after pre-extinction infusion of MS275 (an ortho-amino anilide inhibitor of class I HDACs with high affinity for HDAC1) into the mouse LS. This effect was expectedly absent when the drug was infused before cued fear extinction (Fig 22B) into the LS again evidencing the specificity of LS in regulation social fear. Such a specific effect of HDAC1 inhibition within the LS in enhancing the learning of extinction only in case of social fear goes on to show the importance of LS in social learning.

4.2.3 HDAC1 mediated regulation of gene expression

Checking for differential gene expression in response to SFC would give us an idea about molecular pathways which could be involved in fear memory formation. Thus, I performed a microarray to check for whole genome mRNA expression changes within the LS using tissue micropunches obtained from SFC$^+$ and SFC$^-$ male mice (see section 3.2.7). Genes, which fulfilled the criteria (i) to be upregulated in SFC$^+$ mice and (ii) to be regulated by one of the HDAC1-mediated repressor complexes can be considered as potential targets for fear memory formation. Although fear memory formation and extinction memory formation are two entirely different processes, there are overlapping components (Orsini and Maren 2012), and one could

use this information to identify the elusive molecular mechanism by which HDAC1 might repress formation of extinction memory. Out of the 16 potential gene targets, which differentially regulated by social fear conditioning and have been previously shown to be regulated by HDAC1 interesting candidates include *Npy (*which is known for its anxiolytic effect within the brain), *Syt10* (known for its neuroprotective effect) and *Syndig1l* (known to be involved in synapse maturation). These genes are currently being validated and assessed for their role in the regulation of SFC-induced social fear within the male mouse LS.

4.2.4 HDAC1 is ubiquitously expressed across different cell types within the adult mouse brain

HDAC1 and its mouse brain HDAC1 is known to be expressed only in astrocytes and HDAC2 is expressed in neurons (MacDonald and Roskams 2008). This original finding has been countered by many studies over the last 5 years, which have suggested neuronal HDAC1 to be a critical regulator of neuropsychiatric disorders such as schizophrenia (Bahari-Javan, Varbanov et al. 2017), anxiety disorder (Bahari-Javan, Maddalena et al. 2012, Bowers, Xia et al. 2015) and substance use disorder (Wang, Zhang et al. 2010) amongst others. Considering these abovementioned contradictory results, it was important for me to find out if it was neuronal HDAC1 that regulates social fear extinction in the SFC paradigm. I found ubiquitous expression of HDAC1 in neurons and astrocytes in both SFC$^-$ and SFC$^+$ male mice. Such ubiquitous expression of HDAC1 was also seen in both SFC$^-$ and SFC$^+$ male mice after extinction.

A recent study, which deals with HDAC1 nuclear export under neurotoxic conditions, found HDAC expression within the CA1 hippocampal neurons in adult mice (Zhu, Vidaurre et al. 2017). Therefore, despite the fact that downregulation of HDAC1 during development is more pronounced within the neurons, one has to take into consideration the fact that HDAC1 is still expressed in moderate levels within these cells. Through this thesis, I can suggest that a

harmony in brain functionality, emotionality and social behaviors depend on the delicate balance of HDAC1 expression within the neurons and astrocytes LS and consequently, any disruption of HDAC1 function in limbic circuitries, could lead to psychopathological manifestations.

4.2.5 Outlook

The focus of this chapter was on the role of LS HDAC1 in the regulation of gene expression and consequently in regulation fear memory formation and extinction memory formation within the SFC paradigm. However, one needs to consider the histone code (which includes all the different histone PTM's) along with DNA methylation and ncRNA mediated regulation of transcription together in order to understand the environment's influence on behavior. Considering this, future studies should focus on delineating the following mechanisms:

- The contribution of DNA methylation towards regulation of SFC-generated social fear phenotype.

- The contribution of ncRNA mediated regulation of SFC-induced fear.

Other than this, it is well known that HDAC1 activity is known to be regulated by phosphorylation at its ser431 residue (Segre and Chiocca 2011) and I would like to investigate the dynamics of HDAC1 PTM in response to SFC and correlate this with HDAC1 activity. Also, HDAC1 has high sequence similarity (85%) to its class I comrade, i.e., HDAC2 (de Ruijter, van Gennip et al. 2003). Although *Hdac2* mRNA level did not change in response to social fear acquisition or extinction, it would be interesting to analyze HDAC2 protein and activity dynamics across different time points in the SFC paradigm.

Although a plethora of studies in the recent past has demonstrated dynamic epigenetic regulation, it is important to consider the fact that these changes do not fit into the traditional definition of epigenetics, which require these modifications to be heritable. Thus a slight change of the traditional concept of epigenetics has led to the emergence of the field of 'cognitive

epigenetics' which states that neurons, in essence, seem to have chosen the epigenetic mechanisms in order to adapt to environmental stimuli while other cells use it to perpetuate their phenotype. In the case of SFC, HDAC1 inhibition seems to prime the cells for better learning as seen during the extinction of social fear. Thus, targeting factors regulating epigenetic mechanisms like HDACs seems to be a fruitful strategy to enhance cognition and is probably the future of treatment for neuropsychiatric disorders.

4.3 Future studies

In this short section, I am discussing data from two studies which extremely pertinent to the present thesis but are still in their infancy and hence not presented as complete chapters within this thesis.

4.3.1 Mating before extinction reduces SFC-induced social fear in male mice.

The study on the role high endogenous OXT-system activity during lactation in a reversal of social fear focused on female mice. Mating-induced OXT release within the PVN is known to induce anxiolysis in male rats (Waldherr and Neumann 2007). Considering these data, in the follow-up project, we try to assess the role of endogenous OXT system in fear regulation in both social and non-social context in male mice. For this purpose, we used mating in male mice as a model for activated brain OXT system. Male mice were divided into separate groups on the basis of the way they mated as shown in figure 12 below. All groups of SFC- mice were put together in 1 group named: SFC$^-$/Mating irrespective of their mating pattern and animals that were not given a sexual stimulus were categorized as nonmating.
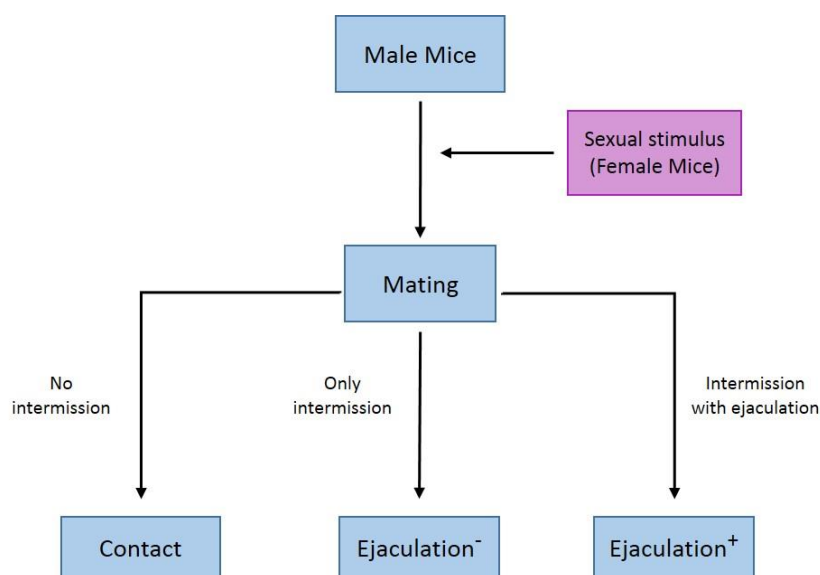

Fig 26. Schematic overview of the groups within mating male mice

Preliminary results:

We found that pre-extinction exposure to a primed female reduces expression of SFC-induced social fear in male CD1 mice only if they ejaculated in the process (Fig 27):
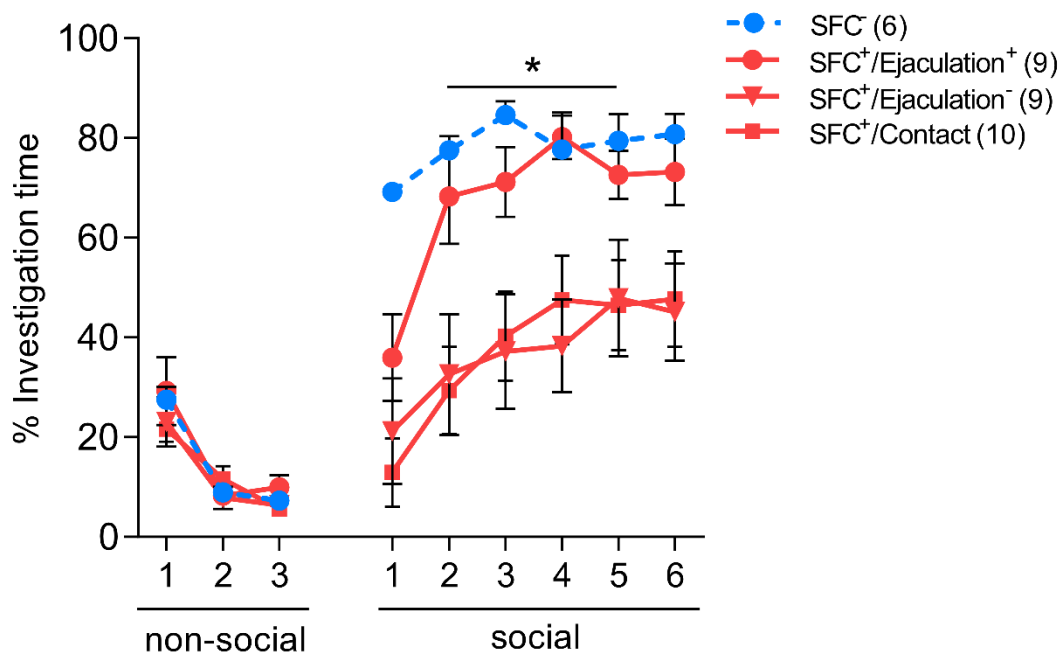

*Fig 27. Mating induced ejaculation reduces expression of SFC-induced social fear (social fear extinction). Male mice (n = 6 – 10/ group) were social fear conditioned (SFC+; red) or left unconditioned (SFC-; blue) during social fear acquisition (day 1), and the percentage investigation of 3 non-social (empty cage) and 6 social (cage with a con-specific) stimuli was monitored during social fear extinction (day 2; D, F) and social fear extinction recall (day 3; E). Data show social fear extinction and represents mean of percentage investigation time ± SEM. * p<0.05 SFC+/Contact vs. SFC+/Ejaculation+, SFC-.*

Further studies in this project will inspect specific molecular pathways and brain regions which mediate social fear reducing the effect of ejaculation in male mice.

4.3.2 SFC leads to an increase in the *Oxtr* mRNA levels within the LS possibly by altering the methylation of specific CpG's at its promoter.

Following points were considered as a background for the following study:

1. OXTR binding is elevated post-acquisition of social fear in the LS of SFC[+] male mice (Zoicas, Slattery et al. 2014).

2. Methylation of the *Oxtr* promoter is known to effect its expression in male mice (Mamrut, Harony et al. 2013, Harony-Nicolas, Mamrut et al. 2014).

3. Decreased methylation at the *Oxtr* promoter (possibly leading to increased *Oxtr* mRNA levels) has been seen in the blood of SAD patients (Ziegler, Dannlowski et al. 2015).

Considering the abovementioned points, I endeavored to check for DNA methylation changes at the *Oxtr* promoter in response to SFC. *Oxtr* mRNA levels were measured using qRT-PCR at different time points during SFC. This was followed by targeted bisulfite sequencing at two specific loci selected based on previously published data (Mamrut, Harony et al. 2013).
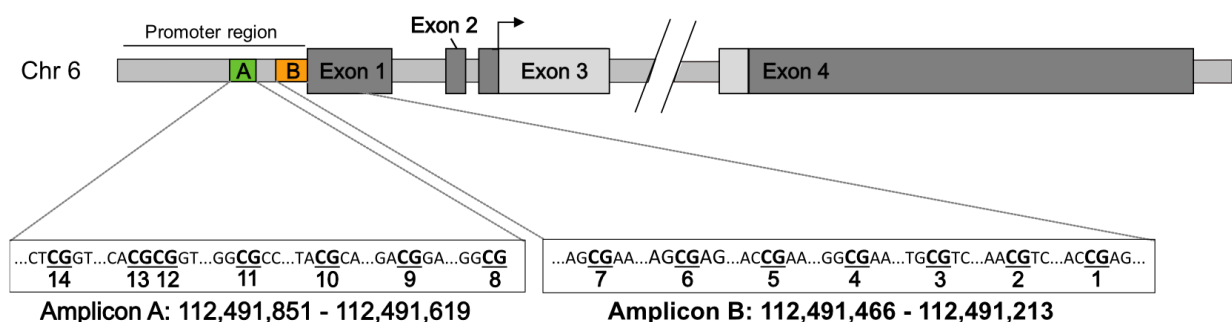

Fig 28. *Schematic overview of the regions within mouse Oxtr promoter which were selected for targeted bisulfite sequencing*

Preliminary results:

Oxtr mRNA levels were elevated in the LS of SFC$^+$ mice post social fear acquisition. This went back to baseline levels 120 min after social fear extinction.
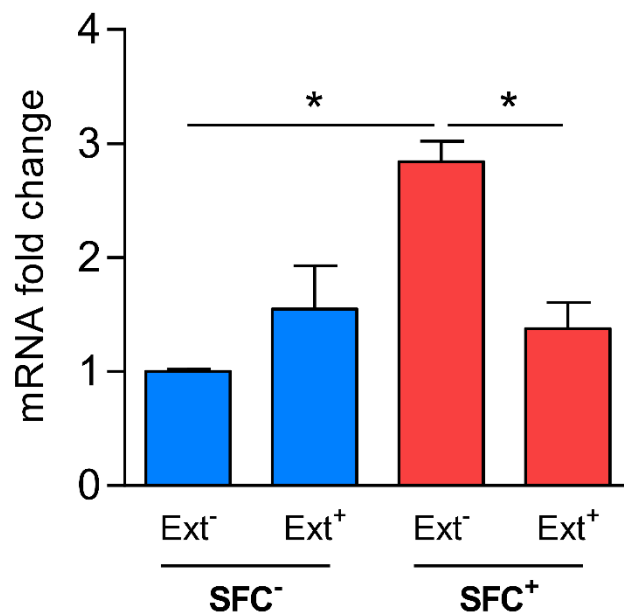

Fig 28. Oxtr mRNA is specifically upregulated after social fear conditioning within the male mouse LS. mRNA was isolated from mice LS micropunches 120 min after social fear conditioning (SFC⁻/Ext⁻ and SFC⁺/Ext⁻; n = 6; Day 1) or after extinction (SFC⁻/Ext⁺ and SFC⁺/Ext⁺; n = 6; Day 2) and gene expression Oxtr was measured. Data represents mean of normalized fold change ± SEM. *p<0.05.

Targeted bisulfite sequencing revealed an increase in methylation at multiple CpG sites (CpG 5, CpG 12 and CpG 14) after extinction in SFC$^-$ mice. This does not explain the slight increase in *Oxtr* mRNA levels at the same timepoint (Fig 28). Having said that, an interesting observation in this regard is that exposure to multiple social stimuli during extinction of social fear leads to release of OXT within the LS (Zoicas, Slattery et al. 2014). Thus the increase in methylation at *Oxtr* promoter might be a response of the system to increase in the availability

of the ligand. Nevertheless, further studies are required to ascertain the precise mechanisms by which the OXTR expression within the LS is regulated in response to SFC.

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Abbreviations

AAV	Adeno-associated virus
ANOVA	Analysis of Variance
AVP	Arginine vasopressin
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminals
CA1	Cornu Ammunis 1
CA2	Cornu Ammunis 2
CA3	Cornu ammunis 3
CBP	CREB binding protein
CBT	Cognitive-behavioral therapy
CeA	Central amygdala
CFC	Cued Fear Conditioning
CNO	Clozapine N-oxide
CRE	CREB response element
CRF	Corticotropin-releasing factor
CS	Conditioned stimulus
DG	Dentate gyrus
DMEM	Dulbecco's modified Eagle's medium
DSM	Diagnostic and Statistical Manual of Mental Disorders
EPM	Elevated plus-maze
ERK	Extracellular regulated kinase
FBS	Foetal bovine serum
GABA	Gamma-aminobutyric acid
GAD	General anxiety disorder
GFAP	Glial fibrillary acidic protein
HAT	Histone acetylaetransferase

Abbreviations

HDAC	Histone deacetylase
HPA	Hypothalamic-pitiutary-adrenal
i.e.	Latin *id est,* meaning "that is"
i.p.	intraperitoneal
icv	intracerebroventricular
ICPE	International Consortium of Psychiatric Epidemiology
IEG	Immediate early genes
ITC	Intercalated cell masses
LA	Lateral amygdala
LDB	Light-dark box
LS	Lateral septum
MeA	Medial amygdala
MEK	MAPK/ERK kinase
mPFC	Medial prefrontal cortex
MPOA	Medial preoptic area
MS	Medial septum
mRNA	Messenger RNA
NAcc	Nucleus accumbens
NMDAR	N-methyl-d-aspartate receptors
OFT	Open field test
OXT	Oxytocin
OXTR	Oxytocin receptor
PAG	Periaqueductal grey
PBS	Phosphate buffered saline
PD	Panic disorder
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RT	Room temperature

SAD	Social anxiety disorder
SFC	Social Fear Conditioning
SNRI	Serotonin-norepinephrine reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
SON	Supraoptic nucleus
SPT	Social Preference Test
SSRI	Selective serotonin reuptake inhibitors
UDG	Uracil DNA glycosylase

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Curriculum Vitae

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Research Interest:

The extent of the environment's influence on an individual's emotional disposition has only been truly appreciated in the last decade. It is the epigenome, with DNA methylation, histone modifications, histone variants, non-coding RNA and other types of epigenetic markings which, in concert, define the localized chromatin structures and provide a molecular bridge between genes and "the environment". My main research interest is in studying as to how these mechanisms orchestrate fear processing in the mice brain. I am specifically interested in the molecular and neuronal mechanisms regulating social fear in mice. In my research I focus specifically on the oxytocinergic system in this context.

Education:

2013 to date: PhD student in Molecular Neurobiology at Prof. Dr. Inga D. Neumann's laboratory, Department of Molecular and Behavioral Neurobiology, University of Regensburg, Regensburg, Germany.

2010 – 2013: Masters of Science in Biology with specialisation in Cell and Molecular Biology, Uppsala University, Sweden.

2008 – 2010: Post Graduate Diploma in Biotechniques, with focus on basic molecular biology and biochemical techniques at the Institute of Bioinformatics and Biotechnology, Bangalore, India.

2004 – 2008:   Bachelors studies in Biotechnology, with focus on Biotechnology, molecular Biology, and biochemistry at Rai Foundation Colleges, Mumbai, India.

Research Experience:

University of Regensburg, Regensburg, Germany

Ph.D. student with Prof. Dr. Inga D. Neumann, 2013 to present

Molecular and neuronal mechanisms of social fear in mice.

- Analysed the epigenetic adaptation of oxytocin system during social fear in mice.

- Examined social fear in lactating mice versus virgins and males.

- Currently, analyzing the molecular and neuronal differences between social and cued fear conditioning.

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Master's thesis and research assistantship with Prof. Dr. Helgi B. Schiöth, 2011 to 2013

Epigenetics of obesity and functional characterization of a novel motif through analysis of locomotor behavior in mice.

- Analysed the link between 3 SNPs namely rs7206629, rs7202116, and rs7202296 within the intron 1 FTO gene and the methylation status of its first intron.

- Studied locomotion and conditioned place preference in SLCZ1 knockout mice along with pharmacological manipulation of the dopaminergic D2 receptors

Awards / Scholarship / Fellowships:

March 2015: Travel Award, ECNP workshop for Junior Scientist, Nice, France

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Publications

- **Menon R**, Grund T, Zoicas I, Fiedler D, Althammer F, Biermeier V, Eliava M, Hiraoka Y, Nishimori K, Grinevich V, Neumann ID. Enhanced oxytocin signaling in the lateral septum prevents social fear expression during lactation in mice. 2017 (in prep).

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