

Differential effects of central oxytocin on social versus
cued fear in male rodents



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vorgelegt von Iulia Toth
aus Temeschburg, Rumänien
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Abstract

Fear is an adaptive emotional response to threatening situations that is crucial for the survival of the individual. In human anxiety disorders, such as social anxiety disorder (SAD) and post-traumatic stress disorder (PTSD), fear is unreasonable and excessive and becomes, therefore, maladaptive. SAD and PTSD are both characterized by debilitating fear and avoidance of fearful situations. These patients show similar dysfunctions in the activity of the brain regions implicated in the fear circuitry and are treated with similar psycho- and pharmacotherapy. However, the medication for SAD and PTSD is rather unspecific and, despite considerable efforts, their efficacy is unsatisfactory. The neuropeptide oxytocin (OT) has been suggested as a possible therapeutic agent for SAD and PTSD, as it facilitates a wide variety of social behaviors and decreases stress and anxiety in both humans and rodents.

As the fear responses, the fear circuitry, and the behavioral and physiological effects of OT are highly conserved among mammalian species, findings from animal models of SAD and PTSD might provide important information for clinical trials using OT. The cued fear conditioning paradigm has proven to be a powerful model to study the normal and pathological processes involved in fear learning and extinction (the gradual decrease in the fear response as result of repeated exposure to the feared situation), and models some symptoms of PTSD. This paradigm implies exposing animals to a well-defined cue, usually a tone or light, which co-terminates with a foot shock, and results in specific fear of this certain cue. Although several paradigms have been shown to induce social avoidance and fear in addition to other behavioral deficits, there are no appropriate and specific animal models available to study SAD.

Therefore, the three main aims of this thesis were (1) to develop and validate an animal model that mimics the main behavioral symptom of SAD, namely social fear and avoidance, and (2)

to use this paradigm, which I termed “social fear conditioning”, to assess the therapeutic efficacy of OT in reversing social fear. Finally, in order to compare OT effects on social and non-social fear, I aimed (3) to assess the effects of OT in cued fear in an animal model of PTSD, i.e. the cued fear conditioning paradigm.

I could show that specific social fear can be induced in naïve mice by administering electric foot shocks when they approach and actively investigate a con-specific, i.e. a social stimulus. Social fear was expressed as reduced investigation of unfamiliar social stimuli and aversive responses towards them, such as freezing, stretched approaches, and defensive burying. Furthermore, the social fear was expressed for at least two weeks and sensitized over time; it was only triggered by social stimuli and did not lead to other behavioral deficits, such as fear of novelty, general anxiety, depression, or impaired locomotion. Moreover, I could demonstrate the predictive validity of the social fear conditioning model by showing that social fear is reversed by acute diazepam and chronic paroxetine treatment, pharmacotherapy currently used in SAD patients.

Having achieved the first aim, I could then show that central infusion of synthetic OT before the extinction procedure, which would be the comparable time-point for psychotherapy in SAD and PTSD patients, reversed social fear, but impaired extinction of cued fear, indicating that OT might represent a promising therapeutic approach for SAD, but not PTSD patients when administered at this time-point. Both the reversing effect on social fear and the impairing effect on cued fear extinction were mediated by the OT receptors (OTR), as central administration of an OT receptor antagonist (OTR-A) prior to OT blocked the observed effects. Furthermore, blockade of OT neurotransmission with OTR-A before the extinction procedure impaired social investigation in unconditioned animals, indicating that the endogenous OT system is needed for naturally-occurring social investigation. In contrast,

blockade of OT neurotransmission at this same time-point did not affect cued fear extinction, indicating that the endogenous OT system is not involved in this process.

Administration of OT before cued fear conditioning, however, facilitated, whereas OTR-A impaired cued fear extinction 24 h later, indicating that an activated endogenous OT system during traumatic events is likely to attenuate formation of fear memories. As both the conditioning and extinction procedure involve learning, these results suggest that exogenous OT impairs learning processes that occur during non-social fearful situations.

In an attempt to identify the neurocircuitry of social fear, I could show that social fear was accompanied by alterations in the brain OT system at the level of the limbic system, namely by an increased OTR binding in the bilateral dorso-lateral septum (DLS), right central amygdala, and right hippocampus (dentate gyrus, cornu ammonis 1). Importantly, these alterations normalized after extinction of social fear, suggesting that OTR expression within these brain regions might be involved in the development and/or neural support of social fear. Based on these OTR alterations, I could localize the effects of OT on social fear within the DLS by bilateral administration.

Taken together, I could show that OT has a differential effect on social versus cued fear in rodents, at least when administered before the extinction procedure. This result raises attention to the importance of the feared situation when recommending OT for the treatment of SAD and PTSD. Thus, OT represents a promising therapeutic approach for disorders associated with social deficits, such as SAD and possibly PTSD due to social trauma, but caution is needed before recommending OT for the treatment of PTSD due to non-social trauma. Furthermore, I could identify the DLS as a part of the brain network involved in mediating OT effects on social fear.

Chapter 1

General introduction

Fear and anxiety are crucial emotional behaviors that are expressed to some degree in all chordates. Although many definitions exist, most of these agree that fear is a normal emotional response to a real external threat or danger, whereas anxiety is an emotional response to a potential threat or danger (McNaughton and Zangrossi, 2008). From an ethological perspective, both fear and anxiety are highly adaptive responses, whereas in modern psychiatry fear is generally regarded as adaptive, while anxiety is generally regarded as maladaptive. For the purposes of this thesis, the term “fear” will refer to a response to threat in both humans and animals. The term “anxiety” will refer to a pathological condition in humans, and to an unconditioned fear response (see Table 1) in animals.

The behavioral patterns, functions, and mechanisms of fear and anxiety received intense scientific attention, mainly fueled by the desire to understand and ameliorate human anxiety disorders. In this chapter, I will shortly address the behavioral and physiological fear responses, as well as their function and underlying neural circuitry. I will describe the symptoms, current treatment, and some animal models used to study two highly prevalent anxiety disorders, namely social anxiety disorder (SAD) and post-traumatic stress disorder (PTSD). This description will highlight the need for more specific models to assess the etiology and treatment of these disorders in preclinical research and the rationale for using oxytocin (OT) in the treatment of SAD- and PTSD-like symptoms.

1.1. Fear and the fear response

The current belief is that fears can be both innate and learned throughout life. From an evolutionary perspective, innate fears have been selected for across generations as they help an organism to adapt better to the environment and, therefore, survive. Startle responses to loud noises and eye blinking when objects rapidly approach the eye are examples of innate fears. Fear can also be learned by direct experience, by observing the experiences of others, or

by verbal instruction. In humans and monkeys, for example, although fear of snakes is not innate, it can develop by getting attacked, observing a con-specific being attacked, or, at least in humans, by being told that snakes are dangerous.

Activation of the fear system leads to a series of behavioral and physiological responses which are highly conserved among mammals, highlighting their evolutionary importance. These responses function to remove the organism from the threat, so called escape behaviors, or to prevent the organism from entering or re-entering a dangerous situation, so called avoidant behaviors. The behavioral responses have been characterized in a variety of species and include flight, avoidance, freezing, defensive threat, defensive attack, and risk assessment (Edmunds, 1974; Blanchard et al., 2003). Burying novel, aversive or potentially dangerous objects (Treit et al., 1981), alarm cries (Litvin et al., 2007), and cessation of ongoing behavior (Estes and Skinner, 1941; Brady and Hunt, 1951) have also been described. The physiological responses to threat include activation of the autonomic nervous system (Schneidermann et al., 1974; Cohen and Randall, 1984), release of stress hormones (Mason et al., 1961; Korte et al., 1992), pain suppression (Watkins and Mayer, 1982), and potentiation of somatic reflexes such as startle (Davis, 1986) and eyeblink (Weisz and McInerney, 1990). All these responses are innate and have evolved to increase the chance of survival and to reduce harm to the threatened individual. The freezing response, for example, reduces the likelihood of attack for two reasons. First, if the predator has not yet spotted the prey, it is more difficult to detect when motionless, and second, even if the prey is detected, it is less likely to be attacked if motionless (Fanselow and Lester, 1988). Defensive threat and attack reduce predation by signaling the intention to attack or by actually hurting the predator (Cowlshaw, 1994). Risk assessment enables the individual to assess the characteristics of a threatening stimulus or to determine whether a threat is present or not (Pinel et al., 1989), whereas defensive burying may reduce the risk of accidental contact with potentially harmful stimuli (Pinel et al., 1989).

Fear responses are therefore adaptive, as they protect the individual from life-threatening situations and improve the evolutionary fitness of a species.

In the context of human pathology, however, fear responses become maladaptive as they are inappropriately activated by innocuous stimuli, or are activated in an excessive, recurring, or prolonged way. Excessive and debilitating fear is a prominent symptom in many anxiety disorders, such as generalized anxiety disorder, SAD, specific phobia, obsessive-compulsive disorder, panic disorder, and PTSD. As SAD and PTSD are the most relevant anxiety disorders for my thesis, I will describe them in more detail.

1.2. Social anxiety disorder

SAD has been defined as a "marked and persistent fear of one or more social or performance situations in which the person is exposed to unfamiliar people or possible scrutiny by others" in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; American Psychiatric Association, 1994). SAD patients often avoid the feared social situations or else endure them with intense anxiety and distress. Epidemiologically, SAD is the second most common anxiety disorder after specific phobia, with a 12-month and lifetime prevalence of 6.8% and 12.1%, respectively (Kessler et al., 2005a,b), and is more prevalent in women than in men (Schneier et al., 1992; Talepasand and Nokani, 2010). Two subtypes of SAD can currently be diagnosed according to DSM-IV criteria, namely specific and generalized SAD. Specific SAD involves fear and avoidance of a particular social situation and includes performance anxiety (e.g. fear of public speaking, eating or drinking in public), interaction anxiety (fear of social interaction and observation situations), and fear of showing anxiety symptoms (Bögels et al., 2010). Patients with generalized SAD are more impaired as they fear and avoid a wide range of social situations (den Boer, 1997; Kessler et al., 1998; Ruipérez et al., 2002). The avoidant behavior is often the greatest impairment in SAD and

ranges from subtle safety behavior, such as avoidance of eye contact, to avoidance of all interpersonal contact outside the family. Moreover, the avoidant behavior plays a critical role in the maintenance of SAD and prevents the reversal of fear in social situations (American Psychiatric Association, 1994; Stangier et al., 2006). The majority of SAD patients report at least one other psychiatric disorder, such as agoraphobia (Magee et al., 1996), depression (Schneier et al., 1992; Regier et al., 1998), or substance abuse (Schneier et al., 2010; Regier et al., 1998). However, SAD generally precedes all these disorders, indicating that SAD is a major risk factor for developing additional psychiatric disorders and that the comorbid disorders are secondary to the main SAD symptoms.

1.3. Post-traumatic stress disorder

PTSD is a severe anxiety disorder that can develop after exposure to an event that results in psychological trauma and is characterized by persistent re-experiencing of the trauma through flashbacks or nightmares, avoidance of stimuli associated with the trauma, and hyper-arousal. Although the consequences of severe trauma have been described since ancient times and several war-related syndromes were reported after the World Wars (Jones, 1995), PTSD has only been officially categorized as an anxiety disorder in DSM-III (American Psychiatric Association, 1980). Epidemiologically, PTSD is the third most common anxiety disorder, with a 12-month and lifetime prevalence of 3.5% and 6.8%, respectively (Kessler et al., 2005a,b), and, like SAD, is more prevalent in women than in men (Kessler et al., 1995; Breslau et al., 1998). The type of trauma is an important predictor of PTSD, as individuals who experienced assaultive violence, especially rape and physical assault, are more likely to develop PTSD than individuals who experienced other types of trauma, such as natural disasters (Kilpatrick et al., 1989; Rothbaum et al., 1992; Breslau et al., 1998). PTSD patients who experienced assaultive violence often show impaired social interaction and emotional reward in addition to the main symptoms (Olf et al., 2010). Like SAD, PTSD is a highly

comorbid anxiety disorder, with depression, substance abuse, and specific phobia being the most common comorbid diagnoses (Kessler et al., 1995). However, unlike SAD, it is not clear which diagnosis occurred first. Estimates provided by Kessler et al., 1995 suggest that while PTSD often precedes other comorbid diagnoses, it usually succeeds at least one diagnosis.

1.4. Neurocircuitry of SAD and PTSD

Given that excessive fear and avoidance of fearful situations are key components of both SAD and PTSD, it is not surprising that the neurocircuitry of these disorders show considerable overlap. A series of neuroimaging studies identified the amygdala, medial prefrontal cortex (mPFC), and hippocampus as the most important brain regions mediating learned fear responses in healthy humans (see Figure 1). These same brain regions are often dysfunctional in SAD and PTSD, as demonstrated by symptom provocation and cognitive-emotional studies. Symptom provocation studies are designed to elicit fear responses by public speaking anticipation and/or performance (SAD) or by specifically recalling a traumatic event (PTSD), whereas cognitive-emotional studies involve exposure to emotional stimuli, such as fearful or angry faces, to study generalization of fear responses. These studies indicate that the amygdala, an almond-shaped group of nuclei involved in the perception, learning, and expression of fear (Fanselow and LeDoux, 1999), is hyperactive in both SAD (Tillfors et al., 2001; Lorberbaum et al., 2004; Phan et al., 2006; Guyer et al., 2008) and PTSD (Shin et al., 1997; Liberzon et al., 1999; Rauch et al., 2000; Pissiota et al., 2002) patients, which might account for exaggerated fear responses and persistence of traumatic memories.

The mPFC, a brain region involved in decision making, goal-directed behavior, and working memory (Vertes, 2004), regulates amygdala output and, thereby, the fear response. More specifically, a region in the ventral part of the mPFC (vmPFC), the infralimbic cortex (IL), inhibits amygdala output and fear expression, whereas a region in the dorsal part of the mPFC

(dmPFC), the prelimbic cortex (PL), increases amygdala output and promotes fear expression (Sotres-Bayon and Quirk, 2010). Accordingly, symptom provocation and cognitive-emotional studies indicate that the vmPFC is hypoactive in PTSD patients (Bremner et al., 1999; Lindauer et al., 2004; Shin et al., 2004a; Yang et al., 2004; Hou et al., 2007) and fails to inhibit the amygdala, whereas the dmPFC shows either normal activity or hyperactivity (Bryant et al., 2005; Felmingham et al., 2009). It is not clear which of these dysfunctions is responsible for the overall outcome in PTSD patients, but a hyperactive amygdala coupled with a hypoactive vmPFC may lead to deficits in fear extinction and emotion regulation in these patients (Liberzon and Sripada, 2008). The involvement of vmPFC in SAD is less clear, with studies showing either hyperactivity (Goyer et al., 2008; Blair et al., 2010), or hypoactivity (Van Ameringen et al., 2004; Evans et al., 2009) of the vmPFC. Additionally, both SAD and PTSD patients show reduced functional connectivity between the amygdala and mPFC during resting-states (Hahn et al., 2011; Sripada et al., 2012), which may contribute to the pathological fear responses observed in these patients.

The hippocampus, a brain region involved in declarative memory (Squire, 1992), processes information about the context, i.e. environment of a fearful situation (Kim et al., 1993). Its involvement in SAD and PTSD is less clear, with studies showing either hypoactivity of this region in SAD (Kilts et al., 2006) and PTSD (Bremner et al., 2003; Shin et al., 2004b) patients, or hyperactivity in SAD (Schneider et al., 1999) and PTSD (Thomaes et al., 2009; Werner et al., 2009) patients. The differences in brain activity between studies may relate to the type of task employed and the selection of patients, as both SAD and PTSD are highly heterogeneous anxiety disorders.

Preclinical research using SAD- and PTSD-relevant animal models (see section 1.7. for more details) has identified that the same neurocircuitry mediates learned fear responses in animals (especially rodents and primates; Figure 1), highlighting again the evolutionary importance of

the fear responses. Although human studies tend to focus on the amygdala, mPFC, and hippocampus, animal studies revealed additional brain regions, such as the striatum, lateral septum (LS), bed nucleus of the stria terminalis (BNST), dorsal raphe nucleus, and periaqueductal gray (Maren, 2001; Calandreau et al., 2007), which are critical either for recognition of a dangerous situation, fear learning, or for appropriate fear expression. Considering the highly conserved fear circuitry among mammals, these regions are likely to play an important role in humans as well.

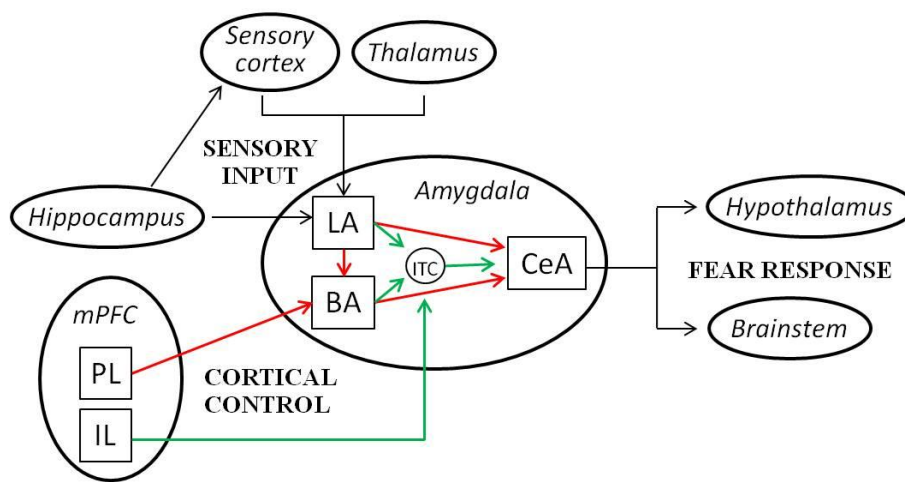


Figure 1. Simplified diagram depicting the major components of the fear circuitry. The amygdala is the central component of the fear circuitry. The lateral amygdala (LA) receives sensory information from the thalamus and sensory cortex. The tha-

lamo-amygdala pathway sends rapid and crude information about the fear-eliciting stimulus without filtering by conscious control. The cortico-amygdala pathway provides slower, but more detailed sensory information. The LA also receives information about the context of the fear-eliciting stimulus from the hippocampus either directly or indirectly through the perirhinal, entorhinal, and parahippocampal cortices. The LA projects to the central amygdala (CeA) either directly or indirectly through the basal amygdala (BA). The LA and BA also project to the intercalated (ITC) cell masses, a GABAergic neuronal network that inhibits the CeA when activated. The CeA mediates fear responses through projections to the hypothalamus and brainstem. The expression of fear is regulated by the medial prefrontal cortex (mPFC) via projections to the LA, BA, and ITC. A region in the dorsal part of the mPFC, the prelimbic cortex (PL), projects to the BA, which excites the CeA, thereby promoting fear expression, whereas a region in the ventral part of the mPFC, the infralimbic cortex (IL), projects to ITC, which inhibits the CeA, and, thereby, fear expression. Red lines indicate pathways that promote CeA output, green lines indicate pathways that dampen CeA output, and black lines provide input to or output from the amygdala.

1.5. Treatment of SAD and PTSD

The best treatment outcomes in both SAD and PTSD patients are obtained with cognitive-behavioral therapy (Gould et al., 1997; Fedoroff and Taylor, 2001), a psychotherapeutic approach where the maladaptive thoughts that produce and maintain anxiety are identified and replaced with more realistic and positive thoughts, thereby reducing emotional distress. One of the techniques used in cognitive-behavioral therapy is exposure therapy, which leads to a gradual fear extinction, i.e. a decline in the fear response as a result of repeated exposure to the feared situation. This psychotherapy is often combined with a rather unspecific pharmacotherapy, originally designed for generalized anxiety or depression, such as benzodiazepines and antidepressants, with selective serotonin reuptake inhibitors (SSRIs) providing the best response rates in both SAD (Liebowitz et al., 1992; Baldwin et al., 1999; Van Ameringen et al., 2001) and PTSD (Marshall and Pierce, 2000; Stein et al., 2006, 2009) patients. Probably due to the insufficient understanding of these disorders and the unspecific medication, many SAD and PTSD patients fail to respond to the available treatment options, achieve only partial remission of symptoms, or show a high relapse rate after treatment discontinuation (Blanco et al., 2002; Davidson et al., 2004; Bisson et al., 2007; Brunello et al., 2001; Ipser et al., 2006). Novel therapeutic strategies make use of cognitive enhancers, which are administered either before the psychotherapy session to increase learning, or immediately after the psychotherapy session to facilitate the consolidation of the “safety” memory trained during the session. For example, augmentation of exposure therapy with D-cycloserine, a glutamatergic N-methyl-d-aspartate receptor agonist, was shown to improve some anxiety symptoms in both SAD (Hofmann et al., 2006; Guastella et al., 2008) and PTSD (Heresco-Levy et al., 2002; de Kleine et al., 2012) patients. Brain neuropeptide systems, such as OT, arginine vasopressin (AVP), neuropeptide Y, neuropeptide S (NPS), and substance P (Fendt et al., 2010; Viero et al., 2010; Meyer-Lindenberg et al., 2011; Bowers et al., 2012; Dunlop et

al., 2012), characterized by discrete neuropeptide synthesis and release sites, distinct receptor distribution, and multiple behavioral functions, represent other interesting research candidates with respect to both pathophysiology and treatment of SAD and PTSD. In this thesis, I investigated the effects of OT in two animal models of SAD and PTSD.

1.6. Oxytocin

OT is a nine amino acid neuropeptide that has been recently proposed as a potential therapeutic agent for SAD (Heinrichs et al., 2003; Kirsch et al., 2005; Labuschagne et al., 2010) and PTSD (Olf et al., 2010) due to its pro-social, anxiolytic, and stress-attenuating effects. OT is mainly synthesized in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, which project to the posterior pituitary and release OT into the bloodstream (Swaab et al., 1975; Vandesande et al., 1975; Brownstein et al., 1980). OT is also synthesized in parvocellular neurons of the PVN, which project to the brainstem, spinal cord, and the limbic system (Swanson and McKellar, 1979; de Vries and Buijs, 1983; Wagner and Clemens, 1991). Centrally, OT is released within the PVN and SON (Moos et al., 1989; Russell et al., 1992; Neumann et al., 1993; Bosch et al., 2005) from dendrites or perikarya of magnocellular neurons (Ludwig and Leng, 2006), and within limbic regions, such as the septum, hippocampus, and central amygdala (CeA; Landgraf et al., 1988; Neumann and Landgraf, 1989; Ebner et al., 2000, 2005; Bosch et al., 2005) from axonal or collateral projections of parvocellular and magnocellular neurons.

The effects of OT are mediated via one single type of OT receptor (OTR), which is a polypeptide with 7 transmembrane domains belonging to the G protein-coupled receptor family. OTR are widespread throughout the central nervous system, in regions such as the cortex, the olfactory system, the basal ganglia, limbic areas such as the BNST, septum, hippocampus, CeA, and ventral subiculum, the thalamus, hypothalamus, the brain stem, and

the spinal cord (Brinton et al., 1984; de Kloet et al., 1985; Elands et al., 1988; Tribollet et al., 1988). Therefore, OT can exert its effects in all brain regions implicated in the fear circuitry so far (see section 1.4. and Figure 1), providing an important reason for studying its involvement in SAD and PTSD, especially in fear learning and extinction (see section 1.7.2. and Table 1).

The reason for the interest in OT in relation with SAD and PTSD includes the fact that OT has been shown to exert pro-social, anxiolytic, and stress-attenuating effects both in rodents and humans. In more detail, OT facilitates a broad variety of social behaviors in rodents, including the onset and maintenance of maternal behavior in lactation (Pedersen and Prange, 1979; Bosch et al., 2005), regulation of male and female sexual behavior (Arletti and Bertolini, 1985; Arletti et al., 1985; Waldherr and Neumann, 2007; Nyuyki et al., 2011), pair bonding in female voles (Williams et al., 1994), social preference (Lukas et al., 2011), and social recognition in male and female rodents (Popik and van Ree, 1991; Ferguson et al., 2000; Choleris et al., 2003; Engelmann et al., 1998; Lukas, Toth et al., in press). In addition to these pro-social effects, both synthetic and endogenous OT exert anxiolytic properties in rodents (McCarthy et al., 1996; Waldherr and Neumann, 2007; Blume et al., 2008; Jurek et al., 2012), and inhibit the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Windle et al., 1997; Neumann et al., 2000).

Comparable effects have also been described in humans, where synthetic OT was shown to exert pro-social effects, such as increased trust and social risk-taking (Kosfeld et al., 2005; Baumgartner et al., 2008; Theodoridou et al., 2009; Mikolajczak et al., 2010a,b), increased attachment (Buchheim et al., 2009), cooperative behavior (Declerck et al., 2010), emotional and cognitive empathy (Bartz et al., 2010; Hurlemann et al., 2010), improved emotion recognition (Domes et al., 2007a; Savaskan et al., 2008) and memory for positive social information (i.e. happy faces, Di Simplicio et al., 2009; Rimmele et al., 2009; Marsh et al.,

2010). Additionally, OT was shown to reduce the autonomic response to stress (Heinrichs et al., 2003; Kirsch et al., 2005; Kubzansky et al., 2009; Quirin et al., 2011), to decrease aversion to angry faces (Evans et al., 2010), and to increase positive communication during couple conflict (Ditzen et al., 2009). Imaging studies revealed that OT reduces the activity of the amygdala to painful stimulation (Singer et al., 2008), threatening faces and scenes (Kirsch et al., 2005; Domes et al., 2007b; Gamer et al., 2010), and conditioned faces (Petrovic et al., 2008a). Although OT did not reduce anxiety symptoms in SAD patients (Kirsch et al., 2005; Domes et al., 2007b), it improved speech performance (Guastella et al., 2009) and dampened the hyperactivity of the amygdala to fearful faces (Labuschagne et al., 2010). In autistic patients, which are characterized by marked social deficits, OT was shown to increase social interaction (Andari et al., 2010), emotion recognition (Guastella et al., 2010), comprehension of affective speech (Hollander et al., 2007), and gaze to the eye region (Andari et al., 2010). More indirect evidence for the anxiolytic and antistress effects of OT in humans comes from nursing mothers who are calmer and less anxious during stressful situations, possibly due to high brain OT activity (Heinrichs et al., 2001; Carter et al., 2001; Slattery and Neumann, 2008).

As fear responses are highly conserved across species, studies on the neural basis of fear in experimental animals made important contribution to understanding the normal processes underlying fear responses. Importantly, the highly conserved fear circuitry combined with the highly conserved effects of OT among mammalian species suggests that findings from experimental animals might be translatable to humans. However, in order to understand the etiology and the underlying neurobiological mechanisms of pathological social and cued fear, which might in turn provide important information for the development of more specific medication for SAD and PTSD, animal models that mimic the main behavioral symptoms of these anxiety disorders are needed.

1.7. Animal models used to study SAD and PTSD

As both SAD and PTSD are complex anxiety disorders with strong psychological components, animal models cannot mimic these disorders in their entire complexity. However, although human performance anxiety and fear of showing anxiety symptoms (see section 1.2.) cannot be reliably modeled in laboratory animals, several paradigms have been shown to induce severe behavioral deficits in rodents, including social avoidance and fear, and can be used to model human interaction anxiety. These paradigms include social defeat (Huhman, 2006; Yan et al., 2010), conditioned defeat (Hammack et al., 2012), foot-shock exposure (Haller and Bakos, 2002; Louvart et al., 2005), maternal separation (Franklin et al., 2011), and restraint stress (Gehlert et al., 2005; Doremus-Fitzwater et al., 2010). Among the animal models used to study PTSD, cued and context fear conditioning (Sanders et al., 2003), active and passive avoidance (Kovács et al., 1979; Ibragimov, 1990), predator and predator scent exposure (Adamec and Shallow, 1993, Takahashi et al., 2008), and fear potentiated startle (Davis, 1986) have proven to be powerful models to study the neural circuitries and mechanisms involved in fear learning and, perhaps more importantly, in fear extinction. As social defeat and cued fear conditioning are the most relevant models for my thesis, I will describe these two paradigms in more detail. For more details about the other models, please see the following reviews: Davis, 1986; Korte, 2001; Sanders et al., 2003; Henn and Vollmayr, 2005; Takahashi et al., 2008; Kikusui and Mori, 2009; Hammack et al., 2012.

1.7.1. Social defeat

The social defeat paradigm was established by Klaus Miczek (1979) and consists of placing a smaller animal in the home cage of a larger aggressive male, which defends its territory and defeats the experimental animal, forming thereby a typical dominant-subordinate relationship. This procedure can be performed once, i.e. acute social defeat, or repeatedly using several

dominant males, i.e. chronic social defeat (Huhman, 2006; Yan et al., 2010). Both acute and chronic social defeat result in persistent fear and avoidance of con-specifics, however, the severity of the induced behavioral alterations depends on the type and length of the defeat. Acute social defeat induces avoidance only of the dominant con-specific that performed the defeat (Lai et al., 2005; Lukas et al., 2011) and is, therefore, less relevant as an animal model of SAD. Chronic social defeat, on the other hand, induces a general avoidance of con-specifics (Avgustinovich et al., 2005; Berton et al., 2006), which makes this model a powerful tool to study the neural circuitries underlying social fear. However, chronic social defeat also induces a large variety of behavioral and physiological alterations, such as increased general anxiety (Keeney and Hogg, 1999; Avgustinovich et al., 2005; Denmark et al., 2010), decreased locomotor activity (Koolhaas et al., 1997; Rygula et al., 2005), depressive-like behavior (Avgustinovich et al., 2005; Rygula et al., 2005; Hollis et al., 2010), anhedonia (Von Frijtag et al., 2000; Rygula et al., 2005), changes in circadian rhythms (Tornatzky and Miczek, 1993; Meerlo et al., 1997), sleep patterns (Kinn et al., 2008), feeding and body weight (Foster et al., 2006), increased heart rate and blood pressure (Fokkema et al., 1986; Sgoifo et al., 1999; Costoli et al., 2004), increased body temperature (Keeney et al., 2001; Hayashida et al., 2010), and suppression of immune responses (Stefanski, 1998; Avitsur et al., 2009; Chester et al., 2010). Given that social fear might either represent the major symptom of the anxiety disorder, as seen in SAD, or a comorbid condition to other psychiatric disorders, such as depression and schizophrenia, animal models that induce specific social fear without any confounding behavioral alterations are required to study the etiology of social fear, which might lead to more specific and efficient medications for these patients.

1.7.2. Cued fear conditioning

The cued fear conditioning paradigm was originally developed in humans (Watson and Rayner, 1920) and later translated to animals (Estes and Skinner, 1941). With their famous

experiment on “little Albert”, an eleven-month-old child, Watson and Rayner could demonstrate for the first time that fear responses can be conditioned in humans. They exposed the child to a rat, to which he initially showed no fear response, and made a loud noise by hitting a metal pipe with a hammer, causing Albert to startle and fall forward. After repeatedly pairing the rat with the loud noise, Albert started crying at the sight of the rat and crawled away from it (Watson and Rayner, 1920).

Although fear conditioning studies employing association of innocuous stimuli, such as pictures of faces or landscapes, tones, and words with mild aversive stimuli, such as electric shocks, painful pressure, heat pain, air puffs, and aversive pictures, tones, tastes or odors (Mechias et al., 2010) are largely being used in humans nowadays, most of the indepth knowledge we have about the pathways and neural mechanisms involved in conditioned fear (see section 1.4.) comes from studies in rodents. A typical cued fear conditioning experiment in rodents consists of three experimental phases, namely fear conditioning, fear extinction, and extinction recall (see Table 1 for terminology used in fear conditioning experiments and throughout this thesis). During fear conditioning, rodents are presented with an innocuous stimulus, usually a tone or light (conditioned stimulus, CS), which co-terminates with an aversive stimulus, such as an electrical foot shock (unconditioned stimulus, US). Through repeated CS-US associations (typically 2 - 5), animals learn that the CS predicts the US, and the CS will elicit a fear response even when presented in the absence of the US. The conditioned fear responses elicited by the CS are highly conserved among mammals and are similar to those elicited by natural threats, such as predators (see section 1.1.). During fear extinction, rodents are repeatedly presented with the CS without the US, which leads to a gradual decrease in the fear responses, and is similar to the repeated exposure to the feared situation during cognitive-behavioral therapy in humans (see section 1.5.). There is a growing body of evidence suggesting that fear extinction is different from forgetting and does not

eliminate the original fear memory, but generates new “safety” memory that inhibits the expression of fear in response to the CS (Bouton, 2004; Quirk et al., 2010). During extinction recall, rodents are presented with the CS without the US to determine whether the “safety” memory trained during fear extinction was consolidated. Although the cued fear conditioning paradigm does not fully model PTSD due to the complexity of the disorder, it allows the study of the neural circuitries and mechanisms involved in fear learning and fear extinction.

| | |
|-----------------------------|--|
| Fear conditioning | A process by which fear of an innocuous stimulus is learned through repeated associations of this stimulus with a noxious stimulus |
| Unconditioned stimulus (US) | A noxious stimulus (i.e. electric foot shock) that triggers an automatic, unlearned fear response |
| Unconditioned response (UR) | An automatic, unlearned fear response (i.e. freezing, startle) to an US |
| Conditioned stimulus (CS) | An innocuous stimulus (i.e. tone, light, context) that, after fear conditioning, triggers a learned fear response |
| Conditioned response (CR) | A learned fear response (i.e. freezing) to a CS |
| Fear acquisition | A process by which the association between the CS and US is encoded; it represents the initial stage of fear conditioning |
| Fear consolidation | A process by which the association between the CS and US is stabilized and maintained after fear acquisition |
| Fear expression | A process by which CR are triggered by the CS |
| Fear extinction | A process by which CR gradually decrease as a result of repeated presentations of the CS without the US |
| Extinction recall | A process by which is determined whether CR are triggered by the CS after consolidation of fear extinction |

Table 1. Terminology used in fear conditioning experiments

1.8. Aim of the present thesis

Given the lack of animal models that induce general social fear without confounding deficits, the high prevalence and unsatisfactory treatment options for both SAD and PTSD, and the

potential therapeutic role of OT in SAD and PTSD, I aimed to:

1. Develop and validate an animal model that mimics the main behavioral symptom of SAD, namely social fear and avoidance.
2. Use this novel animal model, i.e. the social fear conditioning paradigm, to verify the therapeutic efficacy of OT in reversing social fear.
3. Verify the therapeutic efficacy of OT in reversing cued fear in an animal model of PTSD, the cued fear conditioning paradigm.

1.9. Outline of the present thesis

Chapter 2 describes a novel and specific animal model to study SAD, the social fear conditioning paradigm, and its effects on social investigation, general anxiety, depressive-like behavior, and locomotion. It also describes the effects of typical SAD medication, i.e. the benzodiazepine diazepam and the antidepressant paroxetine, on the induced social fear.

Chapter 3 describes step by step how to perform the social fear conditioning paradigm. It also describes theoretical and practical considerations for rats and mice, and for the analysis and interpretation of the obtained data.

Chapter 4 describes the central and local effects of synthetic OT on social fear induced by social fear conditioning. It also describes the alterations that occur at the level of the brain OT system after social fear conditioning and social fear extinction.

Chapter 5 describes the central effects of synthetic OT on cued fear induced by cued fear conditioning, and emphasizes the importance of the time-point of OT administration during the conditioning procedure.

Chapter 6 summarizes and discusses the most relevant findings of this thesis.

Chapter 2

Social fear conditioning: a novel and specific animal model to study social anxiety disorder

Author's contribution:

Toth: designed research, performed research, analyzed data, wrote the manuscript

Neumann: designed research, revised the manuscript

Slaterry: designed research, revised the manuscript

[adapted from: Toth I, Neumann ID, Slaterry DA (2012) Social fear conditioning: a novel and specific animal model to study social anxiety disorder. *Neuropsychopharmacology* 37(6):1433-1443]

ABSTRACT

SAD is a major health concern with high lifetime prevalence. The current medication is rather unspecific and, despite considerable efforts, its efficacy is still unsatisfactory. However, there are no appropriate and specific animal models available to study the underlying etiology of the disorder. Therefore, we aimed to establish a model of specific social fear in mice and use this social fear conditioning paradigm to assess the therapeutic efficacy of the benzodiazepine diazepam and of the antidepressant paroxetine, treatments currently used for SAD patients. We show that by administering electric foot shocks (2 - 5, 1 s, 0.7 mA, pulsed current) during the investigation of a con-specific, the investigation of unfamiliar con-specifics was reduced for both the short- and long-term, indicating lasting social fear. The induced fear was specific to social stimuli and did not lead to other behavioral alterations, such as fear of novelty, general anxiety, depression, and impaired locomotion. We show that social fear was dose-dependently reversed by acute diazepam, at doses that were not anxiolytic in a non-social context, such as the elevated plus-maze (EPM). Finally, we show that chronic paroxetine treatment reversed social fear. All in all, we demonstrated robust social fear after exposure to social fear conditioning in mice, which was reversed with both acute benzodiazepine and chronic antidepressant treatment. We propose the social fear conditioning model as an appropriate animal model to identify the underlying etiology of SAD and possible novel treatment approaches.

INTRODUCTION

SAD, often referred to as social phobia, is characterized by persistent fear and avoidance of social situations. Epidemiologically, SAD is the third most common psychiatric disorder, with a 12-month and lifetime prevalence of 6.8% and 12.1%, respectively (Kessler et al., 2005a,b). For diagnostic purposes, SAD has been divided in two subtypes: specific and generalized SAD. The specific form refers to the fear and avoidance of a particular social situation and includes performance anxiety (e.g. fear of giving a public speech), interaction anxiety (fear of social interaction and observation situations), and fear of showing anxiety symptoms (Bögels et al., 2010). Patients with generalized SAD are more impaired as they fear and avoid a wide range of social situations (den Boer., 1997; Kessler et al., 1998; Ruipérez et al., 2002). This avoidant behavior has an important role in the maintenance of SAD and prevents the reversal of fear in social situations (American Psychiatric Association, 1994; Stangier et al., 2006).

At present, SAD treatment consists of cognitive-behavioral therapy (Gould et al., 1997; Fedoroff and Taylor, 2001), which leads to gradual fear extinction, i.e. a decline in the fear response as a result of repeated exposure to the feared situation, and is often combined with medication originally designed for depression or generalized anxiety, such as antidepressants, β -blockers, and benzodiazepines. However, a high percentage of SAD patients fail to respond to the available treatment options, or achieve only partial remission of symptoms, with SSRIs providing the best response rates (Liebowitz et al., 1992; Baldwin et al., 1999; Van Ameringen et al., 2001). Given the high prevalence and unsatisfactory treatment options for SAD, a better understanding of the etiology and underlying neurobiological mechanisms of social fear, particularly extinction of social fear, is urgently needed. This, in turn, might provide important information for the development of more specific medication and an improved treatment outcome for SAD patients.

However, there are currently no appropriate animal models available to study the disorder. Social fear and avoidance is presently induced using a number of paradigms, including the social defeat paradigm, which can be used both acutely and chronically (Huhman, 2006; Yan et al., 2010), and foot-shock exposure (Haller and Bakos, 2002; Louvart et al., 2005; Mikics et al., 2008a). However, these paradigms are rather unspecific with respect to the behavioral alterations they induce, as increased general anxiety, depression, and impaired locomotion were found to accompany the social avoidance (Denmark et al., 2010; Hollis et al., 2010).

Therefore, we aimed to establish a novel and specific animal model of SAD using the social fear conditioning paradigm, and use this model to assess the therapeutic efficacy of diazepam and paroxetine, currently used for SAD patients. The SAD-like phenotype was induced in naïve mice by punishing them when investigating an unfamiliar conspecific. Mice were conditioned to associate a shock-induced pain with the investigation of a social stimulus and, therefore, avoid social stimuli. The conditioned social fear is specific to several social stimuli, long-lasting and not accompanied by changes in general anxiety, depressive-like behavior, and locomotion. The social fearful phenotype was dose-dependently reversed by acute diazepam, at a dose that was not anxiolytic in a non-social context, i.e. the EPM. Furthermore, chronic antidepressant treatment also reversed social fear, thus validating the social fear conditioning model.

MATERIALS AND METHODS

Animals

Male CD1 mice (Charles River, Sulzfeld, Germany) weighing 30 - 35 g were individually housed in polycarbonate cages (16 × 22 × 14 cm) for 1 week before experiments started, and

remained isolated throughout. Isolation was shown to increase social motivation (Niesink and Van Ree, 1982) and prevent the attenuation of behavioral effects of stressors observed in group-housed mice (Ruis et al., 1999; Cherng et al., 2010). Mice were transferred to observation cages (30 × 23 × 36 cm) 3 days before experiments started. Age- and weight-matched male CD1 mice were used as social stimuli.

Mice were maintained under standard laboratory conditions (12:12 light/dark cycle, lights on at 06:00, 22°C, 60% humidity, food and water ad libitum). Experiments were performed during the light phase, between 08:00 and 12:00, in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the National Institutes of Health.

Social fear conditioning paradigm

On day 1, mice were conditioned for social fear, whereas on days 2 and 3 or 15 and 16, social investigation was assessed as readout of short- and long-term social fear and fear extinction.

Social fear conditioning was performed with a computerized fear conditioning system (TSE System GmbH, Bad Homburg, Germany). The conditioning chamber consisted of a transparent Perspex box (45 × 22 × 40 cm) enclosed in a wooden chamber to reduce external noise and visual stimulation. The floor consisted of a removable stainless steel grid connected to a shock delivery unit used for manual application of foot shocks. A video camera at the top of the chamber enabled video recording.

Social fear conditioning (day 1). Mice were placed in the conditioning chamber and, after a 30-s adaptation period, an empty wire mesh cage (7 × 7 × 6 cm) was placed as a non-social stimulus near one of the short walls. Mice were allowed to investigate the non-social stimulus for 3 min, before it was replaced by an identical cage containing an unfamiliar male mouse. Unconditioned (UC) mice were allowed to investigate the social stimulus for 3 min.

Conditioned (C) mice were given a 1-s electric foot shock (0.7 mA, pulsed current) each time they investigated the social stimulus, defined by direct contact with the mouse. Mice received between 2 and 5 foot shocks, with a variable inter-shock interval, depending on when direct social contact was made. The first social contact and, therefore, foot shock occurred within 15 - 30 s. Mice were returned to their home cage when no further social contact was made for 2 min, meaning that conditioned mice spent between 3 and 6 min in the conditioning chamber while the social stimulus was present. The time mice spent investigating the non-social stimulus, as a pre-conditioning measure of non-social anxiety, was analyzed using the JWatcher program (V 1.0, Macquarie University and UCLA).

Social fear extinction (day 2 or 15). To investigate whether conditioned mice displayed social fear and whether this fear could be extinguished, social investigation was assessed in the home cage 1 or 15 days after social fear conditioning for short-term and long-term social fear, respectively. In detail, social fear extinction consisted of exposing the mice to 3 empty cages identical to the cage used during day 1 (non-social stimuli) to assess non-social investigation. Mice were then exposed to 6 unfamiliar male mice enclosed in wire mesh cages (social stimuli) to assess social investigation. Each stimulus was placed near a short wall of the home cage and presented for 3 min, with a 3-min inter-exposure interval. Reduced social investigation and aversive responses toward the social stimuli, such as freezing, stretched approaches, and defensive burying indicated social fear and successful social fear conditioning. As the non-social stimulus elicited a fear response in conditioned mice, an empty cage was placed over night in the home cage to extinguish the fear of the cage.

Extinction recall (day 3 or 16). To investigate whether repeated exposure to social stimuli during social fear extinction leads to a complete reversal of social fear, social investigation was assessed in the home cage 1 day after social fear extinction. Extinction recall consisted of

exposing the mice to 6 unfamiliar social stimuli for 3 min, with a 3-min inter-exposure interval.

Elevated plus-maze

The EPM (Pellow et al., 1985; Lister, 1987) was performed 1 day after social fear conditioning to assess general anxiety-related behavior as previously described (Reber et al., 2007). Briefly, the maze consisted of two open ($6 \times 30 \times 0.2$ cm, 110 lx) and two closed ($6 \times 30 \times 16$ cm, 25 lx) arms radiating from a central platform (6×6 cm) elevated 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 was considered as anxiety-related parameter. The number of entries into the closed arms was considered as indicator of locomotor activity. The experiment was analyzed using the Plus-maze program (version 2.0; E. Fricke).

Forced swim test

The forced swim test (FST; Porsolt et al., 1977) was performed 1 day after social fear conditioning to assess depressive-like behavior. Mice were individually placed into a plexiglass cylinder (13 cm diameter, 25 cm height) filled with 25°C water to a depth of 14 cm for 6 min. Immobility was scored during the last 4 min using the JWatcher program, and was defined as the animal floating in water without swimming and making only movements necessary to maintain its head above the water (Slattery et al., 2005).

Locomotor activity

Home cage locomotion was assessed 1 day after social fear conditioning. Mice were taken out of the cage for 1 min to achieve similar arousal levels in all mice and returned to the home

cage thereafter. Locomotion was measured for 1 h using the Noldus Ethovision XT 5.1 program (Noldus Information Technology, Wageningen, The Netherlands).

Novel object investigation

Novel object and social investigation were assessed in the home cage 1 day after social fear conditioning to differentiate between fear of novelty and social fear. Mice were exposed to three non-social stimuli, three cages containing a white ball (novel object stimuli, size and color matched to the social stimuli), and three unfamiliar social stimuli. Each stimulus was presented for 3 min, with a 3-min inter-exposure interval.

Electric foot-shock exposure

To assess the effects of foot-shock exposure in the absence of a social stimulus on social fear, mice were placed in the empty conditioning chamber and, after a 30-s adaptation period, received five electric foot shocks (1 s, 0.7 mA, pulsed current, i.e. the maximum number received during social fear conditioning), with a 2-min inter-shock interval. Mice were returned to their home cage 2 min after the last foot shock. An empty cage was placed in their home cage over night to allow for comparable behavioral effects with the social fear conditioning experiments. One day later, social investigation was assessed during social fear extinction.

Drugs

Diazepam (Ratiopharm GmbH, Germany) was freshly dissolved in saline and administered intraperitoneally (i.p.) at a volume of 5 ml/kg and doses between 0.5 and 1.25 mg/kg. The highest doses were chosen based on previous studies (Corbett et al., 1993; Dalvi and Rodgers, 1996; Stachowicz et al., 2008). Paroxetine (Bayer Schering, Germany) was administered over 14 days via the drinking water at a dose of 10 mg/kg/ day. The paroxetine dose was chosen

based on previous studies (Da-Rocha et al., 1997; Hascoët et al., 2000a,b; Massé et al., 2005; Elizalde et al., 2008; Thoeringer et al., 2010).

Experimental design

Effects of social fear conditioning on short- and long-term social fear

Initial experiments were designed to characterize the effects of social fear conditioning on short- and long-term social fear. Therefore, separate groups of mice were subjected to social fear conditioning and social investigation was assessed 1 or 15 days later during social fear extinction (n = 13 per group for short- and n = 9 per group for long-term effects). Extinction recall was measured 1 day later.

Specificity of the induced social fear

To verify the specificity of the induced social fear, separate groups of mice were subjected to general anxiety (EPM; n = 8 per group), depressive-like behavior (FST; n = 8 per group), home cage locomotion (n = 7 per group), or novel object investigation (n = 8 per group) testing 1 day after social fear conditioning. Another group of mice was exposed to electric foot shocks in the absence of the social stimulus, and social investigation was assessed 1 day later during social fear extinction (n = 8 per group).

Reversal of short- and long-term social fear by acute diazepam and chronic paroxetine treatment, respectively

To determine whether the effects of social fear conditioning on short- and long-term social fear could be reversed by medication used for SAD patients, we assessed the effects of diazepam and paroxetine, respectively. Mice (n = 10 per group) were subjected to social fear conditioning. The following day, 30 min before social fear extinction, mice were injected i.p. either with vehicle (5 ml/kg saline) or diazepam (0.5, 0.75, 1.0, and 1.25 mg/kg). Extinction

recall was measured 1 day later. To determine whether diazepam has anxiolytic effects in a non-social context at doses used to reverse social fear, naïve mice were injected i.p. either with vehicle (5 ml/kg saline) or with diazepam (0.5, 0.75, 1.0, and 1.25 mg/kg) 30 min before EPM testing. In a separate group of mice ($n = 8$ per group), social investigation was assessed 15 days after social fear conditioning. Paroxetine (10 mg/kg/day) was administered chronically via the drinking water over 14 days, starting 1 day after social fear conditioning to prevent possible confounding effects on fear memory consolidation. Extinction recall was measured 1 day later.

Statistical analysis

For statistical analysis PASW/SPSS (Version 17) 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 post-hoc analysis whenever appropriate. Statistical significance was set at $p < 0.05$. Overall statistics are shown in Table 2.

RESULTS

Effects of social fear conditioning on short- and long-term social fear

Short-term social fear

Mice showed similar non-social anxiety during social fear conditioning on day 1 (Figure 2A, Table 2). During social fear extinction on day 2, conditioned mice showed similar non-social investigation, but reduced social investigation ($p < 0.05$; Figure 2B) compared with unconditioned mice, reflecting social fear. No difference between the mice was found during extinction recall on day 3 (Figure 2C).

| Experiment | Group effect | Group \times stimulus effect |
|---|------------------------------|--------------------------------|
| Short-term social fear (Figure 2) | | |
| Social fear conditioning (day 1) | $T_{(24)}=0.89, p=0.38$ | |
| Social fear extinction (day 2) | $F_{(1,24)}=13.70, p<0.01^*$ | $F_{(8,192)}=8.96, p<0.01^*$ |
| Extinction recall (day 3) | $F_{(1,24)}=0.92, p=0.35$ | $F_{(5,120)}=1.64, p=0.16$ |
| Long-term social fear (Figure 3) | | |
| Social fear conditioning (day 1) | $T_{(16)}=0.08, p=0.94$ | |
| Social fear extinction (day 15) | $F_{(1,16)}=10.73, p<0.01^*$ | $F_{(8,128)}=4.41, p<0.01^*$ |
| Extinction recall (day 16) | $F_{(1,16)}=4.60, p=0.05^*$ | $F_{(5,80)}=1.37, p=0.25$ |
| Specificity of the induced social fear (Figure 4) | | |
| General anxiety | $T_{(14)}=0.84, p=0.42$ | |
| Depressive-like behavior | $T_{(14)}=-0.70, p=0.50$ | |
| EPM locomotion | $T_{(14)}=-0.19, p=0.86$ | |
| Home cage locomotion | $F_{(1,12)}<0.01, p=0.99$ | |
| Fear of novelty (Figure 5) | | |
| Social fear conditioning (day 1) | $T_{(14)}=0.43, p=0.67$ | |
| Social fear extinction (day 2) | $F_{(1,14)}=6.54, p=0.02^*$ | $F_{(8,112)}=11.09, p<0.01^*$ |
| Foot-shock exposure (Figure 6) | $F_{(1,14)}=1.11, p=0.31$ | $F_{(8,112)}=0.33, p=0.95$ |
| Short-term social fear by diazepam (Figure 7) | | |
| Social fear conditioning (day 1) | $F_{(3,36)}=0.01, p=0.99$ | |
| Social fear extinction (day 2) | $F_{(3,36)}=7.68, p<0.01^*$ | $F_{(24,288)}=4.14, p<0.01^*$ |
| Extinction recall (day 3) | $F_{(3,36)}=1.07, p=0.37$ | $F_{(15,180)}=1.01, p=0.45$ |
| Long-term social fear by paroxetine (Figure 8) | | |
| Social fear conditioning (day 1) | $F_{(3,28)}=0.32, p=0.81$ | |
| Social fear extinction (day 15) | $F_{(3,28)}=32.02, p<0.01^*$ | $F_{(24,224)}=8.13, p<0.01^*$ |
| Extinction recall (day 16) | $F_{(3,28)}=4.96, p<0.01^*$ | $F_{(15,140)}=2.05, p=0.02^*$ |

Table 2. Overall effects for the social fear conditioning data. Stimulus effect refers to both non-social and social stimuli during social fear extinction, while during extinction recall it refers to social stimuli only. EPM, elevated plus-maze. Student's t-tests, one-way or two-way ANOVA for repeated measures followed by Bonferroni post-hoc test; * $p<0.05$.

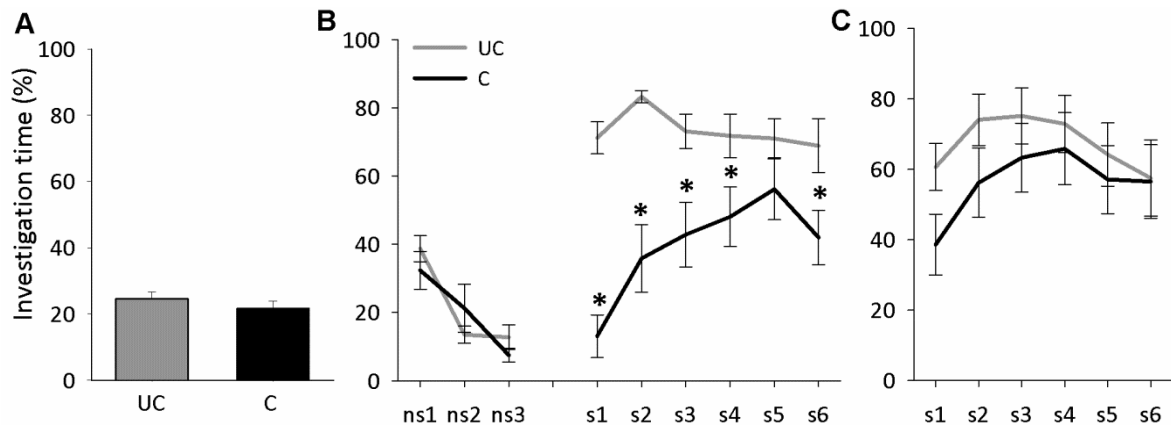


Figure 2. Social fear conditioning induces short-term social fear in mice. (A) Investigation of the non-social stimulus (empty cage) by unconditioned (UC) and conditioned (C) mice during social fear conditioning on day 1 ($n = 13$ per group). (B) Investigation of non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ vs. UC mice.

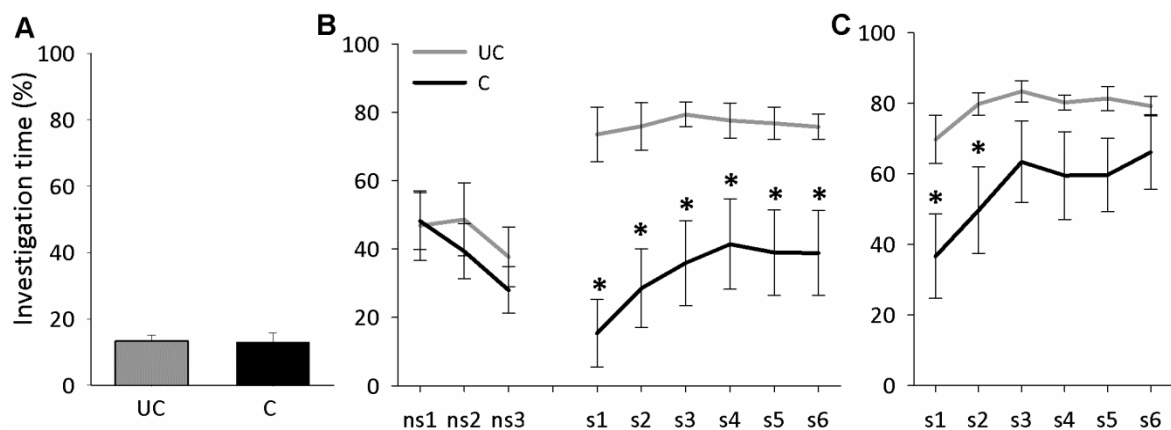


Figure 3. Social fear conditioning induces long-term social fear in mice. (A) Investigation of the non-social stimulus (empty cage) by unconditioned (UC) and conditioned (C) mice during social fear conditioning on day 1 ($n = 9$ per group). (B) Investigation of non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 15 (3 min exposure to stimulus, 3 min inter-exposure interval). (C) Investigation of social stimuli (s1-s6) during extinction recall on day 16. Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ vs. UC mice.

Long-term social fear

Mice showed similar non-social anxiety during social fear conditioning on day 1 (Figure 3A, Table 2). During social fear extinction on day 15, conditioned mice showed similar non-

social investigation, but reduced social investigation ($p < 0.05$; Figure 3B) compared with unconditioned mice, reflecting social fear. In contrast to short-term social fear, conditioned mice still showed reduced social investigation during extinction recall on day 16 ($p < 0.05$; Figure 3C).

Specificity of the induced social fear

No effect of social fear conditioning on general anxiety, depressive-like behavior, and home cage locomotion

Conditioned mice showed no changes in general anxiety (percentage of time on open arms; Figure 4A, Table 2) or locomotion on the EPM (number of closed arm entries; Figure 4C), in depressive-like behavior in the FST (percentage immobility; Figure 4B), or in home cage locomotion (distance moved; Figure 4D) compared with unconditioned mice 1 day after social fear conditioning.

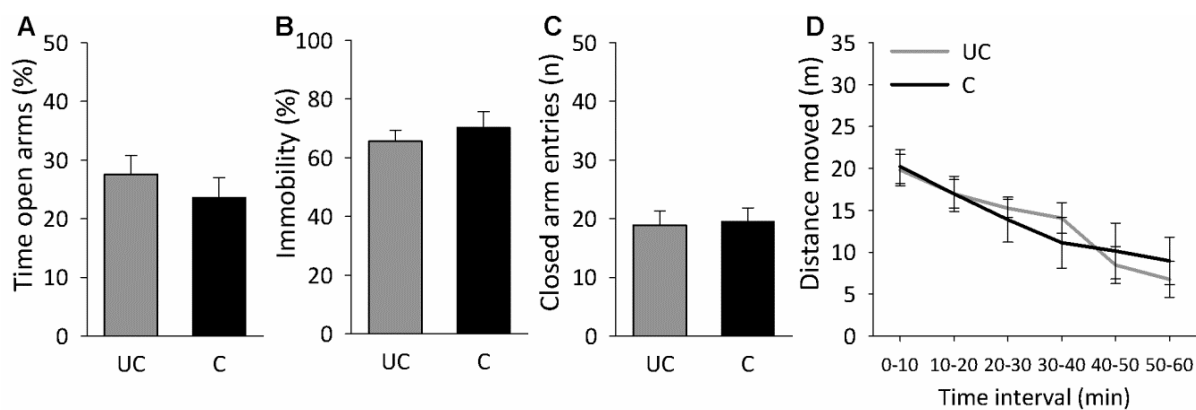


Figure 4. No effect of social fear conditioning on general anxiety on the elevated plus-maze (EPM) (A), depressive-like behavior in the forced swim test (B), and locomotor activity on the EPM (C) and in the home cage (D). Data represent means \pm SEM for $n = 7$ to 8 mice (separate groups). C, conditioned mice; UC, unconditioned mice.

No effect of social fear conditioning on fear of novelty

Mice showed similar non-social anxiety during social fear conditioning on day 1 (Figure 5A, Table 2). One day after social fear conditioning, conditioned mice showed similar non-social and novel object investigation, but reduced social investigation ($p < 0.01$; Figure 5B) compared with unconditioned mice.

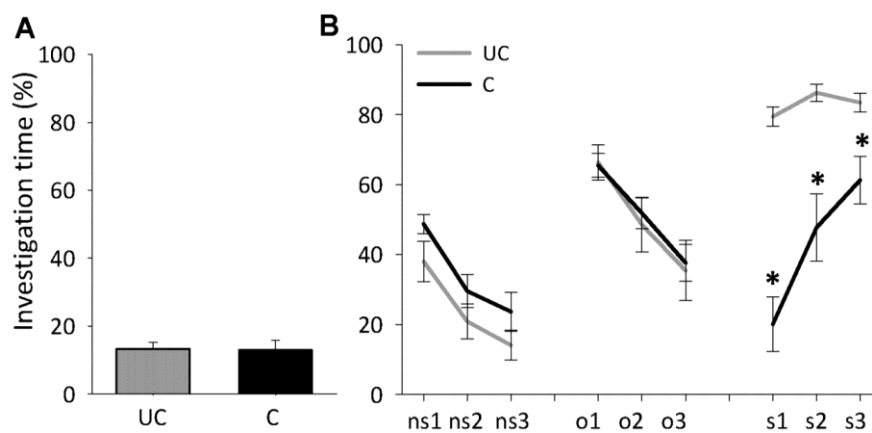


Figure 5. Social fear conditioning does not induce fear of novelty.

(A) Investigation of the non-social stimulus (empty cage) by unconditioned (UC) and conditioned (C) mice during social fear conditioning on day 1

($n = 8$ per group). (B) Investigation of non-social stimuli (ns1-ns3), novel object stimuli (cages with objects; no1-no3), and social stimuli (cages with mice; s1-s6) during novel object investigation on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ vs. UC mice.

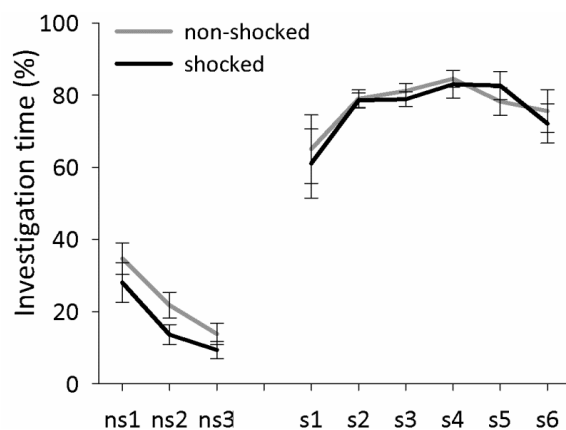


Figure 6. Exposure to five electric foot shocks in the absence of a social stimulus does not induce social fear.

Investigation of non-social (empty cages; ns1-ns3) and social (cages with mice; s1-s6) stimuli by non-shocked and shocked mice ($n = 8$ per group) during social fear extinction 1 day after foot-shock exposure (3 min exposure to stimulus, 3 min inter-exposure interval). Data represent mean percentage of investigation time \pm SEM.

No effect of foot-shock exposure on social fear

Exposure to foot shocks in the absence of the social stimulus did not alter social investigation 1 day later (Figure 6, Table 2).

Reversal of short- and long-term social fear by acute diazepam and chronic paroxetine treatment, respectively

Dose-dependent reversal of short-term social fear by diazepam

Acute diazepam dose-dependently reversed social fear in conditioned mice ($F_{(4,44)}=5.16$, $p=0.02$; Table 3), with doses higher than 0.5 mg/kg being unsuccessful in reversing social fear because of their sedative properties (Table 3). However, these higher doses were not sedative on the EPM and caused a dose-dependent anxiolysis (Table 3).

| Conditioning | Treatment | % NSI | % SI | % Sedative index | CA entries | Time OA |
|--------------|-----------|-----------|------------|------------------|------------|-------------|
| UC mice | Vehicle | 23.6±1.7 | 69.6±2.5 | 0±0 | 100±5.6 | 100±11.0 |
| | 0.5 Dia | 20.3±3.0 | 68.8±2.4 | n.a. | 88.5±10.9 | 97.8±6.0 |
| | 0.75 Dia | 7.6±1.8* | 40.1±3.2* | 28.4±4.8* | 99.5±7.7 | 120.2±9.0 |
| | 1.0 Dia | 12.2±5.3* | 31.8±12.2* | 34.3±13.9* | 75.1±11.5 | 151.1±66.0 |
| | 1.25 Dia | 4.7±3.7* | 7.9±2.3* | 51.8±8.0* | 115.6±13.8 | 238.7±34.3* |
| C mice | Vehicle | 22.0±3.9 | 28.8±4.9 | n.a. | — | — |
| | 0.5 Dia | 22.1±6.3 | 64.6±7.7* | n.a. | — | — |
| | 0.75 Dia | 18.3±4.6 | 27.0±12.2 | n.a. | — | — |
| | 1.0 Dia | 8.4±2.3 | 19.8±8.8 | n.a. | — | — |
| | 1.25 Dia | 13.9±7.1 | 10.1±5.8 | n.a. | — | — |

Table 3. Dose-dependent effects of diazepam in the social fear conditioning and the elevated plus-maze (EPM). Unconditioned (UC) and conditioned (C) mice were injected intraperitoneally with either vehicle (5 ml/kg saline) or diazepam (Dia; 0.5, 0.75, 1.0, or 1.25 mg/kg) 30 min before social fear extinction in the social fear conditioning paradigm or EPM testing. Data represent mean values ± SEM. NSI, non-social investigation; SI, social investigation; CA, closed arms of the EPM; OA, open arms of the EPM; n.a., not analysed; —, not performed. The sedative index is defined as the percentage of time lacking muscle tone and movement. * $p<0.05$.

Mice showed similar non-social anxiety before treatment during social fear conditioning on day 1 (Figure 7A, Table 2). Conditioned mice received a similar number of foot shocks during social fear conditioning (vehicle 2.3±0.15 vs. 0.5 mg/kg diazepam 2.2±0.19;

$T_{(18)}=0.42$, n.s.). During social fear extinction on day 2, the four groups showed similar non-social investigation. Although conditioned vehicle-treated mice showed reduced social investigation compared with all other groups ($p<0.05$; Figure 7B), in conditioned diazepam-treated mice social investigation returned to levels found in unconditioned mice. No difference between the mice was found during extinction recall on day 3 (Figure 7C).

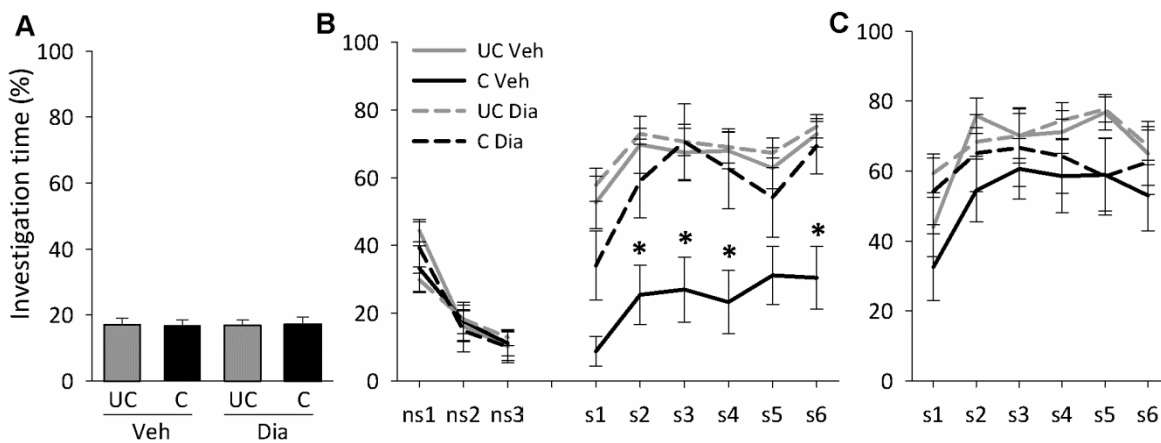


Figure 7. Acute diazepam (Dia) treatment reverses short-term social fear. (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 10$ per group). (B) Investigation of non-social (ns1 ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Unconditioned (UC) and conditioned (C) mice were injected intraperitoneally either with vehicle (Veh; 5 ml/kg saline) or with Dia (0.5 mg/kg) 30 min before social fear extinction. (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent mean percentage of investigation time \pm SEM. * $p<0.05$ vs. the other three groups.

Reversal of long-term social fear by paroxetine

Mice showed similar non-social anxiety before treatment during social fear conditioning on day 1 (Figure 8A, Table 2). Conditioned mice received a similar number of foot shocks during social fear conditioning (vehicle 2.38 ± 0.18 vs. paroxetine 2.31 ± 0.16 ; $T_{(14)}=0.26$, n.s.). During social fear extinction on day 15, the four groups showed similar non-social investigation. Although conditioned vehicle-treated mice showed reduced social investigation compared with all other groups ($p<0.05$; Figure 8B), in conditioned paroxetine-treated mice

social investigation returned to levels found in unconditioned mice. During extinction recall on day 16, conditioned vehicle-treated mice still showed reduced social investigation compared with all other groups ($p < 0.05$; Figure 8C).

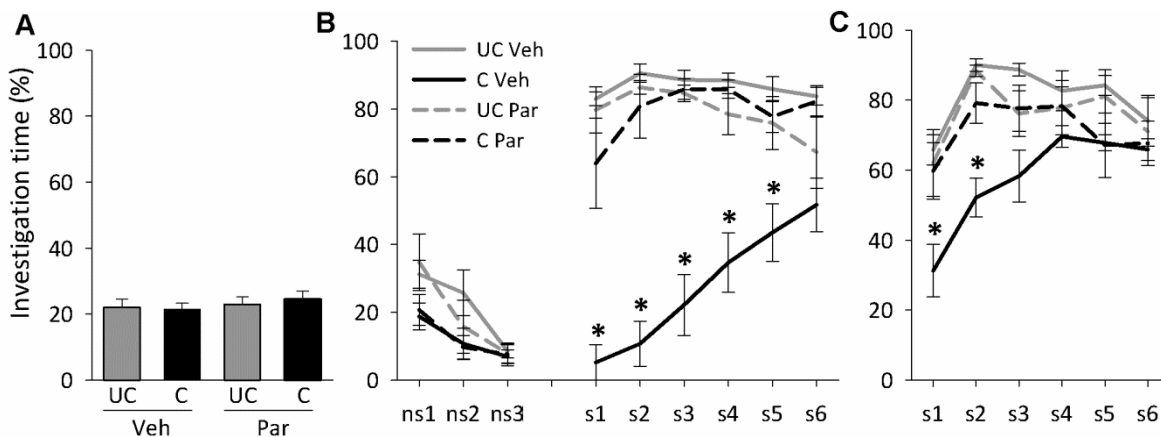


Figure 8. Chronic paroxetine (Par) treatment reverses long-term social fear. (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 8$ per group). Par (10 mg/kg/day) was administered over 14 days in the drinking water (Veh) of unconditioned (UC) and conditioned (C) mice starting 1 day after social fear conditioning. (B) Investigation of non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 15 (3 min exposure to stimulus, 3 min inter-exposure interval). (C) Investigation of social stimuli (s1-s6) during extinction recall on day 16. Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ vs. the other three groups.

DISCUSSION

To the best of our knowledge, this study describes the first animal model of SAD that specifically induces social fear without potentially confounding alterations in other behavioral measures. We show that the novel social fear conditioning model induces both short- and long-term specific fear of social stimuli, and that this fear sensitizes over time. Furthermore, social fear conditioning does not induce other behavioral changes that might account for the observed social fear, such as fear of novelty, increased general anxiety, depressive-like behavior, and impaired locomotion. We further show that social fear is dose-dependently reversed by acute diazepam, at a dose that is not anxiolytic in a non-social context. Finally,

we show that chronic paroxetine treatment reverses social fear, similar to the best outcomes in SAD patients, validating the social fear conditioning model. Therefore, the social fear conditioning model represents a unique and novel model to gain a better understanding of the underlying etiology of SAD and to test compounds with novel mechanisms of action that could provide better treatment outcome for patients.

Despite its prevalence and symptom severity, the etiology of SAD remains poorly understood, due in part to a lack of appropriate animal models. Currently, lasting social fear and avoidance in both rats and mice can be induced by two main traumatic stress procedures, namely social defeat and foot-shock exposure. Social defeat is used both acutely, i.e. one defeat by a dominant male and chronically, i.e. repeated defeat by several dominant males (Huhman, 2006; Yan et al., 2010). Foot-shock exposure is used as exposure to a single (Short and Maier, 1993; Siegmund and Wotjak, 2007) or to repeated foot shocks (Haller and Bakos, 2002; Louvart et al., 2005; Mikics et al., 2008a). Although social defeat and foot-shock exposure decrease social investigation, they also lead to behavioral alterations including increased general anxiety, depression, and impaired locomotion that might account for the observed social deficit (Denmark et al., 2010; Hollis et al., 2010). Furthermore, in the case of acute social defeat, the induced social avoidance is generally directed toward the con-specific that performed the defeat (Lai et al., 2005; Lukas et al., 2011). Although such models are useful and have improved our understanding of SAD, there is a need for animal models that lead to specific social fear, without any confounding behavioral alterations, to further elucidate the mechanisms underlying SAD.

Our social fear conditioning model is based on operant conditioning, where animals learn to associate a voluntary behavior with its consequences. When the consequence is favorable the behavior will occur more frequently, whereas when the consequence is unfavorable the behavior will occur less frequently (Thorndike, 1933; White, 1989). Social fear conditioning

implies punishing naïve mice when investigating a con-specific and results in fear and avoidance of social stimuli. The social fearful phenotype is expressed in reduced investigation of social stimuli and intense aversive responses toward them, such as freezing (absence of movement except that required for respiration; Fanselow, 1980), stretched approaches, and defensive burying (Table 4). Although all these behaviors have been linked with increased anxiety and fear, the reduced social investigation was the most robust indicator of social fear in our paradigm and, therefore, the one we focused on. Furthermore, as none of the unconditioned mice showed freezing, stretched approaches, or defensive burying, by using social investigation as the main readout of social fear direct comparison with unconditioned mice is possible.

| Experiment | Decreased SI | Freezing | Stretched approaches | Defensive burying |
|--|---------------|--------------|----------------------|-------------------|
| Short-term social fear (Figure 2) | 92.3% (12/13) | 61.5% (8/13) | 61.5% (8/13) | 38.5% (5/13) |
| Long-term social fear (Figure 3) | 100% (8/9) | 88.9(8/9) | 55.6% (5/9) | 22.2% (2/9) |
| Fear of novelty (Figure 5) | 100% (8/8) | 75% (6/8) | 50% (4/8) | 0% (0/8) |
| Foot-shock exposure (Figure 6) | 0% (0/8) | 0% (0/8) | 12.5% (1/8) | 0% (0/8) |
| Short-term social fear by diazepam (Figure 7) | 90% (9/10) | 90% (9/10) | 80% (8/10) | 30% (3/10) |
| Long-term social fear by paroxetine (Figure 8) | 100% (8/8) | 75% (6/8) | 75% (6/8) | 75% (6/8) |

Table 4. Behavioral parameters indicating social fear during social fear extinction. Decreased social investigation (SI) represents the percentage of conditioned mice in each experiment that showed a decrease of at least 50 % in investigation of the first social stimulus compared with the mean of their respective unconditioned mice. Freezing, stretched approaches, and defensive burying represent the percentage of conditioned mice that showed those behaviors when the social stimuli were in their home cage during social fear extinction. None of the unconditioned mice showed these behaviors. Data from Figure 7 and 8 includes only conditioned vehicle-treated mice.

To evaluate the effects of social fear conditioning on social fear, we used a modified version of the social approach/avoidance paradigm (Berton et al., 2006), where we first exposed mice to non-social stimuli, i.e. empty cages to exclude possible confounding effects due to fear of the cage itself. As the stimulus mouse used during social fear conditioning was enclosed in a cage that was identical to the cages used as non-social stimuli during social fear extinction, the cage additionally served as a cue and, therefore, elicited a fear response in conditioned mice (Table 5). However, this fear was extinguished by placing the empty cage in the home cage of the mice over night. As non-social investigation was not decreased in conditioned mice after extinguishing the fear of the cage, it is unlikely that fear renewal to the cage occurred and thereby decreased social investigation (Table 5). Furthermore, exposure to non-social stimuli during social fear extinction did not affect the level of social investigation in either conditioned or unconditioned mice (Table 5). Therefore, to allow for a within-individual assessment of non-social and social fear, mice were exposed to both non-social and social stimuli throughout the experiments. For assessing social investigation, mice were repeatedly exposed to different unfamiliar con-specifics. Different individuals were used for two reasons. First, we aimed to obtain a reversal of social fear in general and not a reversal of fear toward a specific individual. Second, we aimed to maintain high interest in the social stimuli as repeated exposure to the same con-specific has been shown to decrease social interest in both mice (Ferguson et al., 2002; Choleris et al., 2009) and rats (Thor et al., 1982; Popik and van Ree, 1998). This exposure-like paradigm is similar to exposure therapy during cognitive-behavioral therapy in humans, where patients are repeatedly exposed to the feared situation.

Social fear conditioning induced both short- and long-term specific fear of social stimuli. Social investigation increased with each exposure to the social stimuli, indicating gradual extinction of social fear, which is similar to the outcome during cognitive-behavioral therapy

in SAD patients (Clark et al., 2003), and extinction after cued and context fear conditioning in mice and rats (Myers and Davis, 2002). When the social fear extinction procedure was performed 1 day after social fear conditioning, social fear was completely reversed by the next day, during extinction recall. However, when the social fear extinction was performed 15 days after social fear conditioning, conditioned mice still showed social fear during extinction recall, indicating that the social fear sensitized over time. Previous studies also showed sensitization of fear responses over time after single or repeated foot-shock exposure (Siegmund and Wotjak, 2007; Mikics et al., 2008a), suggesting that the circuitry underlying the conditioned social fear not only remains stable, but may even strengthen over time. Moreover, the maintenance of social fear over both short- and long-term not only offers the possibility to test medication with fast onset of action, such as benzodiazepines, but also with a delayed onset of action, such as antidepressants (Katz et al., 2006; Mitchell, 2006; Priest, 2006).

Unlike acute social defeat, social fear conditioning induced a general fear of social stimuli, which was not limited to the stimulus to which the mouse has been conditioned, but extended to unfamiliar social stimuli as well. This general social fear is similar to the general social avoidance induced by chronic social defeat (Avgustinovich et al., 2005; Berton et al., 2006), where several dominant males are consecutively used to defeat a subordinate animal (Miczek, 1979; Rodgers and Randall, 1986; Kabbaj et al., 2001). However, chronic social defeat also increased general anxiety (Keeney and Hogg, 1999; Avgustinovich et al., 2005; Berton et al., 2006; Denmark et al., 2010), decreased locomotor activity (Koolhaas et al., 1997; Rygula et al., 2005), and induced a depressive-like phenotype (Avgustinovich et al., 2005; Rygula et al., 2005; Berton et al., 2006; Hollis et al., 2010). Correspondingly, these behavioral changes might account for the decreased social investigation observed. Unlike chronic social defeat, social fear conditioning induced a specific fear of social stimuli without inducing changes in

general anxiety on the EPM, depressive-like behavior in the FST, or in home cage and novel environment (EPM) locomotion. We could also show that the induced social fear was specific to a social stimulus, as novel objects that were similar in size and color to the social stimuli did not induce fear responses in conditioned mice. Thus, in future studies it would be interesting to compare treatments that are effective in the social fear conditioning paradigm in such social defeat models to determine their specificity.

The social fear conditioning model involves the use of aversive stimuli, in this case electric foot shocks. Previous studies have shown that foot-shock exposure alone decreased social investigation for both short- and long-term, however, only when assessed in a novel environment (Short and Maier, 1993; Haller and Bakos, 2002; Haller et al., 2003; Louvart et al., 2005; Leveleki et al., 2006; Siegmund and Wotjak, 2007; Mikics et al., 2008a,b). When social investigation was assessed in the home cage, no differences were found, suggesting that the former result is due to novelty-induced anxiety (Mikics et al., 2008a). Shock exposure also increased general anxiety and decreased locomotion and exploratory behavior in the open field and EPM (Van Dijken et al., 1992; Bruijnzeel et al., 2001; Pijlman and van Ree, 2002; Kavushansky et al., 2009), indicating that the decreased social investigation observed after foot-shock exposure may rather be due to an increase in general anxiety. Our paradigm assessed the experimental mice in their home cage, which reduces the possibility of novelty-induced anxiety. Moreover, we did not observe changes in home cage social investigation when mice were exposed to foot shocks in the absence of the stimulus mouse, indicating that the fear induced during social fear conditioning is a result of the association between the experienced pain and investigation of a con-specific rather than a direct result of foot-shock exposure.

| NS and S investigation | ns1 | ns2 | ns3 | s1 | s2 | s3 | s4 | s5 | s6 |
|--|-----------|-----------|-----------|-----------|------------|------------|------------|------------|------------|
| 1. Without over night extinction of fear of the empty cage | | | | | | | | | |
| 1.1. Without previous non-social exposure | | | | | | | | | |
| UC mice (n=9) | | | | 42.3±8.3 | 75.0±5.8 | 77.0±3.2 | 69.8±4.7 | 69.4±4.7 | 66.2±3.7 |
| C mice (n=9) | | | | 0.3±0.2* | 1.7±1.2* | 1.8±1.0* | 1.2±0.5* | 3.7±1.3* | 5.6±2.4* |
| 1.2. With previous non-social exposure | | | | | | | | | |
| UC mice (n=6) | 27.0±10.2 | 40.1±16.2 | 44.4±16.3 | 66.3±11.1 | 78.4±3.8 | 71.0±11.5 | 72.7±9.7 | 72.5±9.5 | 79.7±5.1 |
| C mice (n=6) | 0.8±0.4* | 0.6±0.4* | 1.3±0.7* | 1.2±0.5* | 2.7±1.3* | 1.7±1.1* | 2.6±1.6* | 2.4±1.6* | 3.2±1.3* |
| 2. With over night extinction of fear of the empty cage | | | | | | | | | |
| 2.1. Without previous non-social exposure | | | | | | | | | |
| UC mice (n=8) | | | | 72.4±7.7 | 80.7±3.0 | 85.8±3.0 | 81.1±3.0 | 82.0±4.0 | 79.7±5.1 |
| C mice (n=9) | | | | 5.3±3.2* | 18.7±9.5* | 32.0±11.9* | 37.7±12.5* | 40.7±13.2* | 3.2±1.3* |
| 2.2. With previous non-social exposure | | | | | | | | | |
| 2.2.1. Short-term social fear (Figure 2) | | | | | | | | | |
| UC mice (n=13) | 38.7±3.8 | 13.5±2.5 | 12.5±3.6 | 71.2±4.7 | 83.3±1.7 | 73.1±5.0 | 71.8±6.3 | 71.0±5.7 | 68.8±7.9 |
| C mice (n=13) | 32.4±5.6 | 21.2±7.1 | 7.5±2.0 | 13.1±6.3* | 35.9±9.9* | 42.8±9.5* | 48.0±8.7* | 56.2±8.9 | 42.0±8.0* |
| 2.2.2. Short-term social fear by diazepam (Figure 7) | | | | | | | | | |
| UC mice (n=10) | 44.4±3.4 | 16.2±4.5 | 10.2±4.2 | 52.6±7.7 | 69.7±8.4 | 67.5±8.3 | 68.0±5.4 | 62.8±6.1 | 72.9±3.8 |
| C mice (n=10) | 33.3±6.7 | 17.6±5.6 | 11.1±3.7 | 8.8±4.4* | 25.4±8.8* | 27.0±9.6* | 23.3±9.4* | 31.2±8.6 | 30.4±9.3* |
| 2.2.3. Long-term social fear (Figure 3) | | | | | | | | | |
| UC mice (n=9) | 46.8±10.2 | 48.3±10.6 | 37.7±8.7 | 73.5±8.0 | 75.9±7.0 | 79.3±3.6 | 77.6±5.1 | 76.8±4.7 | 75.7±3.7 |
| C mice (n=9) | 48.2±8.3 | 39.3±8.0 | 28.0±6.8 | 15.3±9.9* | 28.5±11.5* | 35.9±12.4* | 41.4±13.2* | 39.0±12.5* | 38.8±12.4* |
| 2.2.4. Long-term social fear by paroxetine (Figure 8) | | | | | | | | | |
| UC mice (n=8) | 31.2±4.1 | 25.8±6.7 | 8.5±1.9 | 83.0±2.1 | 90.5±2.7 | 88.6±2.9 | 88.5±2.1 | 85.8±3.7 | 83.8±2.6 |
| C mice (n=8) | 18.8±3.9 | 10.7±4.6 | 6.9±2.0 | 5.2±5.1* | 10.8±6.7* | 22.2±9.0* | 34.7±8.8* | 43.6±8.5* | 51.7±7.9* |

Table 5. Investigation of the non-social (NS; ns1-ns3) and social stimuli (S; s1-s6) during social fear extinction in relation with overnight exposure to the empty cage and to non-social stimuli during social fear extinction. Mice were exposed to social fear conditioning and the empty cage was either placed or not in their home cage over night. Social fear extinction was assessed 1 day or 15 days after social fear conditioning. Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ versus unconditioned (UC) mice. Statistical significance shown in points 2.2.2. and 2.2.4. represents those from all treatment groups, as shown in the results section. C; conditioned mice.

Having shown the specificity of the fear induced during social fear conditioning, we assessed the therapeutic efficacy of benzodiazepines (diazepam) and antidepressants (paroxetine) in reversing social fear in our model. Although β -blockers can be beneficial in humans, their efficacy is limited to performance anxiety (Faigel, 1991; Liebowitz et al., 1992), therefore we did not assess them in the social fear conditioning paradigm.

Acute diazepam treatment dose-dependently reversed short-term social fear, without further increasing social investigation in unconditioned mice. Doses higher than 0.5 mg/kg (0.75, 1.0, and 1.25 mg/kg) had sedative effects in the home cage and even reduced investigation in unconditioned mice, counter indicating, therefore, their use in the social fear conditioning paradigm (Table 3). Diazepam reversed social fear at a dose (0.5 mg/kg) that did not alter general anxiety on the EPM (Table 3). We could only demonstrate anxiolytic effects of diazepam on the EPM at a dose of 1.25 mg/kg, confirming recent findings (Hascoët et al., 2000a). The sedative effect of doses higher than 0.5 mg/kg diazepam might not have been observed in previous studies, as the increased arousal level in novel environments, such as the EPM, open field, and light-dark box, may have masked the sedative effect of diazepam.

Chronic paroxetine treatment, started 1 day after social fear conditioning to prevent possible confounding effects on fear memory consolidation, was also successful in reversing long-term social fear, without further increasing social investigation in unconditioned mice. However, acute SSRI treatment increased social fear in conditioned mice (data not shown). This is in

line with data showing that SSRI treatment reduces cued fear after chronic treatment, but increases cued fear after acute treatment (Burghardt et al., 2004, 2007). All in all, the reversal of social fear by medication used for SAD patients provides predictive validity to the social fear conditioning model.

Finally, it is of interest to note that the use of the social fear conditioning model is not restricted to male CD1 mice, as shown in this study. Preliminary data have shown that the paradigm can also be used successfully in inbred lines such as C57/B16, and in a different species, namely Wistar rats (data not shown). This further demonstrates the utility of the novel paradigm for gaining a better understanding of the etiology of SAD. Moreover, social fear conditioning has not been used to induce social fear in female mice or rats yet, which, however, would be promising given the higher prevalence of SAD in women (Schneier et al., 1992; Talepasand and Nokani, 2010).

In summary, we have established a novel social fear conditioning paradigm that induces specific and long-lasting fear of social stimuli in naïve mice, and shows both face and predictive validity to SAD. The induced social fear is specific to several social stimuli and not the result of fear of novelty, increased general anxiety, depressive-like behavior, or impaired locomotion. Our model might, therefore, be used to gain a better understanding of the underlying causes and mechanisms of SAD in humans and also to test compounds with novel mechanisms of action that could provide better treatment outcome for these patients.

Chapter 3

Social fear conditioning as an animal model of social anxiety disorder

Author's contribution:

Toth: wrote the manuscript

Neumann: revised the manuscript

Slattery: revised the manuscript

[adapted from: Toth I, Neumann ID, Slattery DA (under review) Social fear conditioning as an animal model of social anxiety disorder. Invited manuscript to Current Protocols in Neuroscience]

ABSTRACT

Social fear and avoidance of social situations represent the main behavioral symptoms of SAD, a disorder that is poorly elucidated and has rather unsatisfactory therapeutic options. Therefore, animal models are needed to study the underlying etiology of the disorder and possible novel treatment approaches. However, the current paradigms modeling SAD-like symptoms in rodents are not specific, as they induce numerous other behavioral deficits in addition to social fear and avoidance. Here, we describe the protocol for the social fear conditioning paradigm, an animal model of SAD that specifically induces social fear of unfamiliar con-specifics, without potentially confounding alterations in other behavioral measures. Theoretical and practical considerations for performing the social fear conditioning paradigm in both rats and mice, as well as for the analysis and interpretation of the obtained data, are described in detail.

INTRODUCTION

SAD is defined as a "marked and persistent fear of one or more social or performance situations in which the person is exposed to unfamiliar people or possible scrutiny by others" (American Psychiatric Association, 1994). SAD patients often avoid the feared social situations or else endure them with intense anxiety and distress. SAD is a common anxiety disorder with a lifetime prevalence of 12.1% (Kessler et al., 2005a), and is more prevalent in women than in men (Talepasand and Nokani, 2010). Two subtypes of SAD have been described, namely specific and generalized SAD. Specific SAD involves fear and avoidance of a particular social situation and includes performance anxiety (e.g. fear of public speaking, eating or drinking in public), interaction anxiety (fear of social interaction and observation situations), and fear of showing anxiety symptoms (Bögels et al., 2010). Patients with generalized SAD are more impaired as they fear and avoid a wide range of social situations (Ruipérez et al., 2002). The avoidant behavior is often the greatest impairment in these patients, plays a critical role in the maintenance of SAD, and prevents the reversal of fear in social situations (American Psychiatric Association, 1994).

Currently, the best treatment responses in SAD patients are obtained with a combination of psychotherapy and pharmacotherapy. One of the techniques used in psychotherapy is exposure therapy (Gould et al., 1997; Fedoroff and Taylor, 2001), which leads to a gradual fear extinction, i.e. a decline in the fear response as a result of repeated exposure to the feared situation. This psychotherapeutic technique is often combined with a rather unspecific pharmacotherapy, originally designed for depression or generalized anxiety, such as benzodiazepines and antidepressants (Gould et al., 1997; Fedoroff and Taylor, 2001). Many SAD patients fail to respond to the available treatment options, achieve only partial remission of symptoms, or show a high relapse rate after treatment discontinuation (Blanco et al., 2002), due at least in part to the insufficient understanding of the disorder and the unspecific

medication. Therefore, a better understanding of the etiology and underlying neurobiological mechanisms of social fear, particularly extinction of social fear, is needed. However, for these purposes, animal models of are needed.

Although human performance anxiety and fear of showing anxiety symptoms (see above) cannot be reliably modeled in laboratory animals, several paradigms have been shown to induce social avoidance and fear in rodents and can be used as animal models of human interaction anxiety. Such paradigms include social defeat (Huhman, 2006), foot-shock exposure (Haller and Bakos, 2002), maternal separation (Franklin et al., 2011), and restraint stress (Gehlert et al., 2005). While these paradigms have contributed a great deal of knowledge regarding the mechanisms underlying social avoidance and fear, they are rather unspecific with respect to the behavioral alteration they induce. Thus, increased general anxiety, depressive-like behavior, anhedonia, and numerous other changes have often been shown to accompany the induced social fear (Avgustinovich et al., 2005; Rygula et al., 2005). When using these paradigms, it is therefore not clear whether social fear is the major deficit induced, or whether it is a consequence of one of the aforementioned behavioral alterations. Given that social fear might either represent the major symptom of the disorder, as seen in SAD, or a co-morbid condition to other psychiatric disorders, such as depression and schizophrenia, animal models that induce specific social fear without any confounding behavioral alterations are required to study the etiology and underlying mechanisms of pure social fear, which might lead to a better understanding of the disorder and more specific and efficient medications for disorders associated with social deficits.

In this Unit we describe the *social fear conditioning paradigm*, which induces specific social fear in naïve animals and shows both face and predictive validity to SAD (Toth et al., 2012b). The social fear conditioning paradigm is based on operant conditioning, where animals learn to associate a voluntary behavior with its consequences. When the consequence is favorable

the behavior will occur more frequently, whereas when the consequence is unfavorable the behavior will occur less frequently. Social fear conditioning implies punishing naïve animals by administering mild electric foot shock when they investigate a con-specific and results in fear and avoidance of social stimuli. The socially-fearful phenotype is expressed as reduced investigation of social stimuli and intense aversive responses toward them, such as freezing, stretched approaches, and defensive burying. Importantly, the social fear conditioning induces a long-lasting (at least two weeks) social fear, which offers not only the possibility to test medication with fast, but also with delayed onset of action. Additionally, the induced social fear is not accompanied by alterations in general anxiety, novelty anxiety, and depressive-like behavior, as occurs in most of the animal models mentioned above.

MATERIALS

- Experimental animals: male or female rats or mice, 8 to 12 weeks of age unless otherwise wished
- Stimulus animals: same species, strain, sex, age, and weight as experimental animals

Calculate the number of stimulus animals needed as follows: 1 stimulus per 6 experimental animals during social fear conditioning + minimum 6 (6 different stimuli needed during social fear extinction). For example, if you plan an experiment with 6 animals, you need 7 stimulus animals; for an experiment with 12 animals or more you need at least 8 stimulus animals

- Experimental room with controlled temperature (22 - 24°C) and humidity (60 - 70 %) and 12-hr light/dark cycle

Unless otherwise wished, experiments are performed during the early light phase.

However, when other time-points of experimenting are wished, preliminary validation of

the paradigm is recommended

- Conditioning room close to the experimental room, where the computerized fear conditioning system is located

The experimental and conditioning room should be close by so that animals can be transported from one room to the other as quickly as possible

- Computerized fear conditioning system that allows manual administration of foot shocks, such as that from TSE Systems. The conditioning box needs to be equipped with a video camera that allows the visualization and recording of the experiment

The size of the conditioning box should be similar to the size of the observation cages (see below), i.e. large enough to allow the experimental animal to avoid the stimulus animal.

Care is needed that the conditioning box is not too large, especially when conditioning mice, as they might be distracted by the novelty of the conditioning box (see Figure 9)

- Standard cages with sawdust bedding for single- or group-housing of animals
- Transparent “observation” cages with sawdust bedding: for mice ca. 30 x 25 x 35 cm; for rats ca. 55 x 35 x 35 cm

These cages are needed to allow behavioral monitoring and videotaping. The observation cages need to be large enough to allow the experimental animal to avoid the stimulus animal. Calculate the approximate size of the observation cage as follows: length = width of the stimulus cage x 4 - 5; width = length of the stimulus cage x 1.5 - 3; height = height of the stimulus cage x 3 - 4.5 to allow unrestrained climbing on the stimulus cage by the experimental animal (see Figure 10)

- Stimulus cages: wire mesh cages or plastic cages with large perforations for encaging stimulus animals: for mice ca. 7 x 7 x 6 cm; for rats ca. 20 x 10 x 9 cm

These cages will be used either as non-social stimuli, i.e. they will remain empty, or as social stimuli, i.e. a stimulus animal will be enclosed in the cage. The size of the stimulus

cage needs to correspond to the size of the stimulus animals, so that stimulus animals are comfortably encaged but cannot move too freely and influence, thereby, the behavior of the experimental animal (see Figure 11). Calculate the number of stimulus cages needed as the number of animals tested per day + 2



Figure 9. Photograph depicting the conditioning box and an experimental mouse investigating the non-social (left) and social (right) stimuli during social fear conditioning. The conditioning box needs to be large enough to allow the experimental mouse to avoid the social stimulus.



Figure 10. Photograph depicting the observation cage and an experimental mouse investigating a social stimulus during social fear extinction (see section 16.2.). The observation cage needs to be large enough to allow the experimental mouse to avoid the social stimulus.



Figure 11. Photograph depicting the stimulus cages, which are used either as non-social stimuli (up), i.e. they remain empty, or as social stimuli (bottom); i.e. they are used for enclosing mice (left) and rats (right). The size of the stimulus cages needs to correspond to the size of the stimulus animals, so that stimulus animals are comfortably encaged but cannot move too freely.

- Video cameras with tripod. In case you intend to perform the experiments in the dark phase, video cameras with night-shot or an infra-red camera and source are needed

- Two differently-smelling detergents or cleaning solutions
- Behavioral analysis program that allows scoring several behaviors at a time, such as JWatcher or EventLog

These programs can be downloaded for free at www.jwatcher.ucla.edu and www.manageengine.com/products/eventlog/download

- Balance to weigh animals
- Stopwatches (1 for each animal tested in parallel per day)

NOTE: All protocols using live animals and fear conditioning procedures must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow the officially approved procedures for the care and use of laboratory animals.

Prepare the animals for the experiment

1. Order animals at least 7 days before they will be used. House experimental mice singly in standard cages for 7 days before social fear conditioning. House stimulus animals in groups of 4 to 6. If more experimental rooms are available, house experimental animals in a separate room from stimulus animals. If only one experimental room is available, avoid housing experimental and stimulus animals close to each other
2. Weigh experimental animals and house them singly in transparent observation cages 3 days before social fear conditioning. Split the animals into the required number of groups based on body weight, such that the mean weight of the groups does not differ
If experimental animals are housed longer than 3 days in the observation cages, they are generally aggressive towards the stimulus animals. You can avoid this by changing the sawdust bedding 2-3 days before social fear conditioning
3. Acclimatize stimulus animals to being enclosed in stimulus cages. Repeat the procedure 10 min each day for 3 days before social fear conditioning

Set up the conditioning environment and perform the social fear conditioning (experimental day 1)

4. Use detergent number 1 to clean 2 stimulus cages and the conditioning box before conditioning the first animal

Use one of the stimulus cages as a non-social stimulus, i.e. this cage will remain empty.

Use the other stimulus cage as a social stimulus, i.e. enclose a stimulus animal in this cage. Change this stimulus animal with a novel one after a maximum of 6 experimental animals have been conditioned. This is needed to maintain low stress levels in the stimulus animals. Mark the stimulus animals with a waterproof pen and if required use them again, however, only for social fear conditioning

IMPORTANT! Do not use detergent number 1 again! Smell serves as a cue and will become associated with the conditioning procedure, and will induce non-social and, therefore, unspecific fear

5. Use detergent number 2 to clean the rest of the stimulus cages

These cages will be placed over night in the home cage of the experimental animals, i.e. if you test 12 animals you need 12 stimulus cages

6. Set up the computerized fear conditioned system for manual application of 0.7 mA foot shocks using a pulsed current. Set up the video camera to record the experiment. Prepare a stopwatch

7. Protocol for conditioned animals

7.1. Take the experimental animal out of its home cage, place it in a standard cage with clean sawdust and transport it to the conditioning room. Turn the video camera on to record the experiment. Place the animal in the conditioning box and start the stopwatch

7.2. After a 30-s habituation time, place the non-social stimulus, i.e. empty stimulus cage, in a corner of the conditioning box for 3 min

7.3. After 3 min, take the non-social stimulus out of the conditioning box and place the social stimulus, i.e. the stimulus cage with the enclosed stimulus animal, in the same place where the non-social stimulus has previously been

7.4. Allow the experimental animal to sniff for 2 - 3 s at the social stimulus, then administer a short (ca. 1 s) foot shock. For reliable social fear conditioning, animals should receive at least 2 foot shocks (if more than 5 foot shocks are required, exclude the animal from the study; see section 7.4.1.1). Two possible scenarios apply here: the experimental animal will either approach the social stimulus again or not.

7.4.1. When the experimental animal approaches the social stimulus again and makes nasal contact with the social stimulus, administer immediately a short (ca. 1 s) foot shock

7.4.1.1. If, after the second approach, the experimental animal approaches the social stimulus for a third time within 2 min, administer immediately a short foot shock. Do not repeat this procedure for more than five times!

7.4.1.2. If, after the second approach, the experimental animal does not approach the social stimulus for 2 min, follow the steps described in point 7.5. and 7.6.

7.4.2. In rare cases, experimental animals do not approach the social stimulus for a second time. If the experimental animal does not approach the social stimulus for 5 min, follow the steps described in point 7.5. and 7.6.

7.5. Turn the video camera off. Remove the social stimulus. Take the experimental animal out of the conditioning box, place it in the standard cage and transport it back to its home cage. Place an empty stimulus cage (cleaned with detergent number 2!) in the home cage of the experimental animal over night

IMPORTANT! It is essential to place the empty stimulus cage over night in the home cage of the experimental animals, as the stimulus cage, just like the smell of detergent

number 1, serves as a cue and will become associated with the conditioning procedure.

By placing the stimulus cage over night in the home cage of the experimental animals, the fear of this cage will extinguish or not consolidate so that the fear responses observed later on are triggered only by the encaged stimulus animals

7.6. Use detergent number 1 to clean the conditioning box before conditioning the next animal

8. Protocol for unconditioned (control) animals

8.1. Follow the same steps as described in points 7.1. and 7.2.

8.2. After 3 min, take the non-social stimulus out of the conditioning box and place the social stimulus in the same place where the non-social stimulus has previously been, again for 3 min

8.3. After 3 min, follow the steps described in points 7.5. and 7.6.

IMPORTANT! In case you intend to assess short-term social fear, perform the social fear extinction one day after social fear conditioning. In this case, remove the empty stimulus cages from the home cage of the experimental animals 1 h before social fear extinction. In case you intend to assess long-term social fear, perform the social fear extinction 15 days after social fear conditioning. In this case, remove the empty stimulus cages 24 h after social fear conditioning

Set up the testing environment and perform the social fear extinction (day 2 or 15)

9. Use detergent number 2 to clean the stimulus cages

These cages can be used as both non-social and social stimuli. If you intend to test 6 experimental animals at a time you need 6 stimulus cages, 6 stimulus animals – different from those used during social fear conditioning, 6 video cameras each with a tripod, and 6 stopwatches. If you intend to test less than 6 experimental animals, you need the

corresponding number of video cameras and stopwatches. However, you will still need 6 stimulus animals, as each of the 6 social stimuli used during social fear extinction needs to be unfamiliar to the experimental animals

10. Prepare for recording the experiment. Place a video camera on a tripod in front of an observation cage, so that you obtain a ca. 45° angle to the long wall of the cage. Place a stopwatch next to the observation cage

11. Protocol for short-term social fear assessment (day 2)

- 11.1. One hour after removing the stimulus cages, start the social fear extinction procedure.

Turn the video camera on and place the non-social stimulus in the home cage of the experimental animal near a short wall of the cage. Start the stopwatch

- 11.2. After 3 min, remove the non-social stimulus and wait for 3 min

- 11.3. After 3 min, repeat the procedure 2 more times, so that you expose the experimental animal to 3 non-social stimuli for 3 min, with a 3-min inter-exposure interval

IMPORTANT! In case you do not have enough time to clean the non-social stimuli between exposures with detergent number 2, place each non-social stimulus always to the same experimental animal. This is essential to prevent unspecific fear responses to the smell of a different animal

- 11.4. After 3 min pause, place a social stimulus in the home cage for 3 min. Repeat the procedure 5 more times, so that you expose the experimental animal to 6 social stimuli for 3 min, with a 3-min inter-exposure interval

IMPORTANT! For each of the 6 social exposures use a different social stimulus! This is essential, as fear of general social stimuli is assessed. In case you use the same social stimulus for each of the 6 exposures, you can only draw conclusions about social fear towards this individual social stimulus (see section Animal considerations: Stimulus animals)

12. Protocol for long-term social fear assessment (day 15)

The social fear extinction procedure is performed exactly the same as described for short-term social fear in point 11. The only point that needs to be considered is that experimental animals have been housed in the observation cages for more than two weeks. In this case, clean the observation cage and change the sawdust bedding 2 - 3 days before social fear extinction. Alternatively, if the animals have been housed in standard cages, house them in observation cages 3 days before social fear extinction

Set up the testing environment and perform the extinction recall (day 3 or 16)

13. Set up the testing environment as described in points 9. and 10.
14. Turn the video camera on and place a social stimulus in the home cage of the experimental animal near a short wall of the cage. Start the stopwatch
15. After 3 min, remove the social stimulus and wait for 3 min. Repeat the procedure 5 more times, so that you expose the experimental animal to 6 different social stimuli for 3 min, with a 3-min inter-exposure interval

IMPORTANT! For each of the 6 social exposures use a different social stimulus, like during social fear extinction! (see section Animal considerations: Stimulus animals for more details)

Analysis and interpretation of the obtained data

16. Social fear conditioning data

The video of each experimental animal contains the 30 s habituation time, the 3 min with the non-social stimulus, and the 3 min with the social stimulus

- 16.1. Analyze and calculate the percentage of non-social investigation and exploration that both conditioned and unconditioned animals show during the 3 min with the non-social stimulus

Non-social investigation is defined as sniffing, touching, gnawing, and climbing on the non-social stimulus, i.e. the empty stimulus cage

Exploration is defined as all other behaviors showed, such as sniffing the air or walls of the conditioning box, rearing, cleaning, moving, resting, etc

- 16.2. Analyze and calculate the percentage of social investigation and exploration that unconditioned animals show during the 3 min with the social stimulus

As conditioned mice receive electric foot shocks when they investigate the social stimulus, analysis of social investigation is not needed

Social investigation is defined as sniffing, touching, biting the stimulus animal, and sniffing, touching, gnawing, and climbing on the stimulus cage containing the stimulus animal

Exploration is defined as in point 16.1.

- 16.3. Compare the non-social investigation between conditioned and unconditioned animals

This will be your parameter of pre-conditioning non-social anxiety and should not be different between conditioned and unconditioned animals

- 16.4. Compare the non-social and social investigation of the unconditioned animals

This will be your indicator that the animals you are using show social preference, i.e. an increased percentage of social compared with non-social investigation

17. Social fear extinction data

The social fear extinction data of one experimental animal contains nine 3-min videos, 3 with the non-social stimuli and 6 with the social stimuli

IMPORTANT! If a video contains less than 3 min, it has to be disregarded

- 17.1. Analyze and calculate the percentage of non-social and social investigation, defensive burying, freezing, and exploration that experimental animals show during each of the nine 3-min videos. Count the number of attempt approaches

Non-social investigation, social investigation, and exploration are defined as in points 16.1. and 16.2. Reduced non-social and social investigation are indicators of non-social and social fear, respectively

Defensive burying is defined as pushing the sawdust with the forelimbs in the direction of the non-social or social stimulus, and is an indicator of fear

Freezing is defined as absence of movement except that required for respiration, and is an indicator of fear

Attempt approaches are defined as approaches towards the non-social or social stimulus with elongation of the body and forward movement of the forepaws, while the hind paws are not moved. After an attempt approach, animals often run away. Attempt approaches are an indicator of fear

- 17.2. Compare the non-social investigation between conditioned and unconditioned animals

This will be your parameter of non-social fear and should not be different between conditioned and unconditioned animals. The animals that do not show non-social investigation or show aversive responses towards the non-social stimuli, i.e. defensive burying, freezing, or attempt approaches, should be removed from the study, as they are probably still afraid of the stimulus cage and the fear they show is not specific to the social stimulus

- 17.3. Compare the social investigation between conditioned and unconditioned animals

This will be your parameter of social fear and should be different between conditioned and unconditioned animals, with conditioned animals showing reduced levels of social investigation

18. Extinction recall data

The extinction recall data of one experimental animal contains six 3-min videos with the social stimuli. Analyze and interpret the data as described in point 17.

COMMENTARY

Background information

Although a number of paradigms have been shown to induce social fear and avoidance in animals (Haller and Bakos, 2002; Gehlert et al., 2005; Huhman, 2006; Franklin et al., 2011), most of these paradigms also induce a number of other behavioral alterations, which might contribute to the observed social avoidance. These alterations include increased general anxiety, depressive-like behavior, and motivation-related alterations such as anhedonia. It should be kept in mind, however, that for studying the etiology and the underlying mechanisms of pure social fear, animal models that induce specific social fear without any confounding behavioral alterations are required. These animal models might lead to a better understanding of social fear and, in turn, more specific and efficient medications for patients suffering from disorders associated with social deficits, such as SAD, autism spectrum disorders, and possibly post-traumatic stress disorder due to social trauma. Additionally, by understanding the basic neural circuitries important for the expression of adaptive social fear and the way how these circuitries are altered in patients displaying pathological social fear, patients that present social fear and anxiety as a co-morbid symptom to other psychiatric disorder, such as schizophrenia and depression might also benefit.

Critical parameters and Troubleshooting

Animal considerations: Sex of experimental animals

Most of the studies on social fear and anxiety have been performed on male rodents for two main reasons. First, males lack the potential confounding hormonal fluctuations associated with the estrous cycle, which might interact with other experimental parameters. Second, the social defeat paradigms, which are the most reliable models to induce social fear and avoidance in rodents, and are based on territoriality and establishment of dominant-subordinate relationships between con-specifics, cannot be reliably used in females of most rodent species, except the California mice and Syrian hamsters. This is because females are usually not territorial and do not form typical dominant-subordinate relationships with other females (Palanza, 2001). The social fear conditioning paradigm can be reliably used to induce social fear in females that are not territorial, and the behavioral phenotype induced is very specific and comparable to the behavioral phenotype induced in males (Toth et al., 2012b). It should be noted, however, that preliminary studies should assess the effects of the estrous cycle-associated hormonal fluctuations on female social fear conditioning and extinction. Nevertheless, the etiology of social fear and the efficacy of medication needs to be studied both in male and female rodents, given the higher prevalence of anxiety disorders, including SAD, in women (Talepasand and Nokani, 2010), and the different responses to medication between men and women (Franconi et al., 2007). The social fear conditioning paradigm might be, therefore, used to assess possible sex differences in both the etiology and treatment of social fear.

Animal considerations: Species-specific factors

When using the social fear conditioning model, a second point that needs to be considered is the species used. Although the paradigm has been originally reported to induce social fear in

the CD1 outbred mouse line (Toth et al., 2012b), which might represent a more relevant animal model of SAD due to the higher genetic variability, preliminary data have shown that the social fear conditioning paradigm can also be reliably used in inbred lines, such as C57/Bl6 mice and Wistar rats (Toth et al., unpublished). Although the CD1 and C57/Bl6 are both highly social species, we found differences in social behavior, with C57/Bl6 mice showing lower levels of social investigation, less social motivation following repeated social exposure, and less aggressive behavior towards social stimuli. Therefore, preliminary studies should assess the basal level of social investigation and intra-species aggression when using different breeding lines for the social fear conditioning experiments. Given the possible inter-species differences in social behavior, the use of different breeding lines as experimental and stimulus animals may lead to inconsistent results (see next section).

Animal considerations: Stimulus animals

A highly important aspect that needs to be considered is the selection of stimulus animals, namely the age, weight, sex, and strain.

The use of age- and weight-matched social stimuli is recommended to minimize unspecific social avoidance, especially when using male individuals, as previous studies identified increased levels of terpenic constituents in the urine of single-housed, dominant or aggressive males that work as ‘aversion signals’ for other males and discourage investigation of areas marked with this urine (Jones and Nowell, 1973; Novotny et al., 1990). Although females of most rodent species do not form typical dominant-subordinate relationships, preliminary studies should assess the effects of using larger females as social stimuli on social investigation.

The sex of the stimulus animals may affect not only the level of social investigation, but also the neural circuitries recruited by the task. Due to sexually-motivated behaviors, it is expected

that male experimental animals spend more time investigating receptive stimulus females and, therefore, the social fear will be extinguished faster. If the purpose of the study is not related to sexual behavior, the use of ovariectomized females as social stimuli should be considered. However, it should be taken into account that investigation of male versus ovariectomized female social stimuli by male experimental animals recruits separate neural circuitries (Lukas, Toth et al., in press).

The strain of the stimulus animals should also be taken into account, as different strains of rats and mice show different levels of social motivation, social preference, and aggressive behavior (Miczek et al., 2001; Lim and Young, 2006). When using genetically-modified animals as both experimental and stimulus animals, preliminary screening should assess the level of social investigation showed by and directed towards these animals. If not specifically otherwise intended, using con-specifics of the same age, weight, sex, and strain as social stimuli is, therefore, the best option for the social fear conditioning paradigm to prevent any unspecific alterations in social avoidance or investigation.

Depending on the type of social fear studied, different unknown con-specifics or a single con-specific can be used as social stimuli during social fear extinction. In this Unit, we described the social fear conditioning protocol employed to induce and assess general social fear, such as observed in SAD patients. For this purpose, different unknown con-specifics are used for social fear conditioning and each of the 6 social exposures during social fear extinction and extinction recall. On the other hand, individual social fear, i.e. fear of a specific individual can be induced and assessed by using a single con-specific for both social fear conditioning, social fear extinction, and extinction recall. In this case, however, it has to be considered that, although social investigation might gradually increase in conditioned animals during social fear extinction as a result of decreased social fear, a decrease in social investigation does not necessary reflect social fear. Repeated exposure to the same con-specific has been shown to

decrease social interest in rodents (Thor et al., 1982; Ferguson et al., 2002), and is rather an indicator of social memory.

The protocol described in this Unit implies that single-housed experimental animals show a short-lasting social memory, i.e. less than 24 h, which allows the use of the same 6 stimulus animals for both social fear extinction and extinction recall. Single-housed rats and mice typically show a short-term social memory, i.e. up to 1 h, whereas group-housed mice show a long-term social memory, i.e. for 24 h up to 7 days (Kogan et al., 2000; Noack et al., 2010). Although the social fear conditioning paradigm implies single-housing the experimental animals for 7 days before social fear conditioning, preliminary studies should assess long-term social memory to exclude any bias on social investigation. If these preliminary studies reveal long-term social memory in experimental animals, different unknown con-specifics should be used during social fear extinction and extinction recall.

Impact of housing conditions

The importance of the size of the observation cage has been briefly discussed in the Materials section and plays a relevant role during social fear extinction. Given that exposure to social stimuli triggers intense fear responses and avoidance of social stimuli in conditioned animals, the home cage where the social fear extinction procedure is performed needs to be large enough to allow the free manifestation of these behaviors. The size of the home cage directly influences the speed of social fear extinction, i.e. in small cages social fear will be extinguished faster, as conditioned animals cannot appropriately avoid the social stimuli, whereas in large cages social fear will be extinguished slower, as conditioned animals avoid social stimuli when possible (unpublished observations).

The housing conditions prior to and after social fear conditioning are very important. To stimulate social motivation, experimental animals should be single-housed for several days

prior to social fear conditioning (Niesink and Van Ree, 1982). Single-housing of experimental animals after social fear conditioning is essential, as group-housing extinguishes social fear and is also known to attenuate the behavioral effects of other stressors, such as social defeat, foot-shock exposure, and restraint in water (Ruis et al., 1999; Cherg et al., 2010).

Given that housing in novel environments triggers short-term increases in locomotor activity and arousal, experimental animals should be housed in observation cages at least 24 h prior to social fear conditioning to prevent possible novelty-induced anxiety and unspecific anxiety responses during social fear extinction. For the best behavioral results, the sawdust bedding should be changed 2 - 3 days prior to social fear conditioning. Housing the animals on the same bedding and performing the social fear conditioning experiment without changing the sawdust bedding should be avoided due to potential increases in territorial-motivated aggressive behavior.

Specificity of the behavioral effects

The specificity of the behavioral effects induced by social fear conditioning has been verified and published for male CD1 mice (Toth et al., 2012b). When using other breeding lines, however, preliminary studies should assess the effects of social fear conditioning on basal behavioral parameters, such as general anxiety, depressive-like behavior, novelty-induced anxiety, and locomotor behavior to rule out other possible confounding behavioral alterations. When using the stimulus cages during both social fear conditioning and extinction, a highly relevant aspect is that during social fear conditioning the stimulus cage becomes associated with the conditioning procedure and triggers fear responses, which lead to unspecific social fear during social fear extinction. A reliable way to ensure that social fear is only triggered by social stimuli has been described in the method presented and consists of extinguishing the fear of the stimulus cage by exposing the experimental animals to this cage over night.

Another possibility is to use two different types of cages during social fear conditioning and social fear extinction/extinction recall. For this approach, however, unspecific anxiety responses to the novel stimulus cage used during social fear extinction need to be ruled out in preliminary studies.

Anticipated results

The effects of social fear conditioning on both short- and long-term social investigation in male CD1 mice have been recently published (Toth et al., 2012b). The social fear conditioning induces both short- and long-term social fear, which is expressed as decreased social investigation (see Figure 12 for a set of expected results). During social fear extinction, the social fear is gradually extinguished as a result of repeated social exposure. While short-term social fear can be completely extinguished during the social fear extinction procedure, long-term social fear is still expressed during extinction recall, indicating that social fear sensitizes over time (see Figure 12 for a set of expected results).

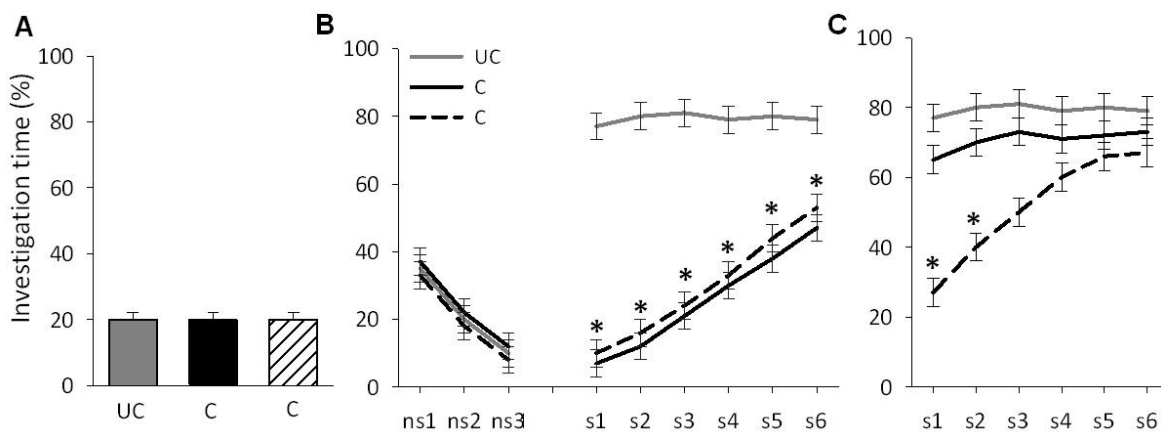


Figure 12. Social fear conditioning induces short- and long-term social fear. (A) Pre-conditioning investigation of the non-social stimulus (empty cage) by unconditioned (UC) and conditioned (C) mice during social fear conditioning on day 1. (B) Investigation of non-social (ns1–ns3) and social (cages with mice; s1–s6) stimuli during social fear extinction on day 2 (black line) or on day 15 (dotted line; 3-min exposure to stimulus, 3-min inter-exposure interval). (C) Investigation of social stimuli (s1–s6) during extinction recall on day 3 (black line) or on day 16 (dotted line). Adapted from Toth et al., 2012b.

Time Considerations

This section assumes that one experimenter is performing all the procedures. Actual performance of the social fear experiment requires a minimum of 3 days, depending on the number of experimental animals and the type of social fear assessed, i.e. short- or long-term. Long-term social fear studies require ≥ 16 days, depending on the experimental design. In more detail, setting up the testing environment for social fear conditioning, social fear extinction, and extinction recall requires ca. 30 min each. Social fear conditioning requires a maximum of 10 to 15 min per animal. Social fear extinction and extinction recall require cca. 1 h and 40 min per animal, however, several animals can be tested in parallel. Analysis of the obtained data requires ca. 1 h per animal.

Unlike other animal models that induce social fear and avoidance, social fear conditioning experiments are fast, reliable, and do not require large animal numbers to reach statistical significance. However, at least 7 social stimuli and special resources are required, such as computerized fear conditioning system, video cameras, and appropriate observational and stimulus cages.

Chapter 4

Brain oxytocin in social fear conditioning and its extinction: involvement of the lateral septum

Author's contribution:

Toth: designed research, performed research, analyzed data, wrote the manuscript

Slattery: designed research, revised the manuscript

Neumann: designed research, revised the manuscript

[adapted from: Toth I, Neumann ID, Slattery DA (under review) Brain oxytocin in social fear conditioning and its extinction: involvement of the lateral septum. Biological Psychiatry]

ABSTRACT

Central OT has anxiolytic and pro-social properties both in humans and rodents, and has been recently proposed as a potential therapeutic agent for disorders associated with social deficits, such as SAD. Here, we aimed to verify and localize the effects of OT on social fear in a mouse model of SAD, the social fear conditioning, and to determine whether social fear is accompanied by alterations at the level of the brain OT system. Central infusion of synthetic OT, but not of the closely related neuropeptide AVP, reversed social fear via the OTR without decreasing general anxiety or increasing locomotion. Social fear was accompanied by alterations in the OT system, namely by increased OTR binding in the bilateral dorso-lateral septum (DLS), right CeA, right dentate gyrus (DG), and right cornu ammonis 1 (CA1). These alterations normalized after social fear was extinguished and suggest that the DLS, amygdala, and the hippocampus might be part of a brain network involved in the development and/or neural support of social fear. Infusion of OT into this network, namely into the DLS reversed social fear, confirming the involvement of the DLS in social fear. These results demonstrate that OT has the potential to reverse social fear in male mice by local effects within the DLS, and suggest OT as potential add-on drug in the treatment of disorders associated with social deficits, such as SAD.

INTRODUCTION

The appropriate display of social behaviors is essential for the well-being and survival of social species, and disorders associated with social deficits, such as SAD are highly debilitating (Blanco et al., 2002; Labuschagne et al., 2010). Therefore, substantial research has been performed to identify the neuronal circuitries involved in the control of complex social behaviors, and to determine how these circuitries are affected in subjects displaying social deficits. Given that social fear and avoidance of social situations are the main behavioral symptoms of SAD, the best treatment outcomes are obtained with cognitive-behavioral therapy (Fedoroff and Taylor, 2001), which leads to a gradual fear extinction, i.e. a decline in the fear response as a result of repeated exposure to the feared situation. The pharmacotherapy for SAD is limited to medication originally designed for depression or generalized anxiety, such as antidepressants and benzodiazepines. Even with therapy, many SAD patients achieve only partial remission of symptoms, or show a high rate of relapse after treatment discontinuation (Blanco et al., 2002). Therefore, the development of approaches that combine psychotherapy with more specific pharmacotherapies is still needed.

One target that has recently received attention is OT, a neuropeptide synthesized within the PVN and SON of the hypothalamus (Neumann, 2007). Despite these discrete synthesis sites, OTR are widespread throughout the brain (Gimpl and Fahrenholz, 2001). The reason for the interest in OT includes the fact that synthetic OT has been shown to facilitate social encounters and to decrease anxiety and stress in humans (Bartz and Hollander, 2006; Heinrichs et al., 2009). Similar effects were found in rodents, where both synthetic and endogenous OT were shown to exert pro-social (Popik and van Ree, 1991; Ferguson et al., 2001), anxiolytic (Bale et al., 2001; Waldherr and Neumann, 2007; Blume et al., 2008), and stress-attenuating (Windle et al., 1997; Neumann et al., 2000) effects. More relevant for SAD, we have recently shown that brain OT is essential for social preference in rats and mice and

synthetic OT reverses the social avoidance induced by acute social defeat (Lukas et al., 2011). However, given that acute social defeat induced a rather individual social fear, the effects of OT need to be verified in an animal model that induces general social fear and mimics the behavioral symptoms of SAD more accurately.

Therefore, in the present study we aimed to verify and localize the effects of OT on social fear in a mouse model of SAD, the social fear conditioning (Toth et al., 2012b), which was shown to induce a behavioral phenotype similar to SAD. Furthermore, we aimed to determine whether the social fear induced by social fear conditioning is accompanied by alterations in the brain OT system.

MATERIALS AND METHODS

Animals

Male CD1 mice (Charles River, Sulzfeld, Germany, 30 - 35 g) were individually housed for 1 week and transferred to observation cages (30 x 23 x 36 cm) 3 days before experiments started. Age- and weight-matched male CD1 mice were used as social stimuli. Mice were kept under standard laboratory conditions (12:12 light/dark cycle, lights on at 06:00, 22°C, 60% humidity, food and water ad libitum). Experiments were performed during the light phase, between 08:00 and 12:00, in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz and the guidelines of the National Institutes of Health.

Social fear conditioning paradigm

Social fear conditioning (day 1). Mice were placed in the conditioning chamber (45 x 22 x 40 cm) and, after a 30-s adaptation period, an empty wire mesh cage (7 x 7 x 6 cm) was placed as

a non-social stimulus near one of the short walls. After 3 min, the non-social stimulus was replaced by an identical cage containing an unfamiliar male mouse. Unconditioned (UC) mice were allowed to investigate this social stimulus for 3 min. Conditioned (C) mice were given a 1-s electric foot shock (0.7 mA, pulsed current) each time they investigated the social stimulus. Mice received between 2 and 5 foot shocks, with a variable inter-shock interval, depending on when social contact was made. Mice were returned to their home cage when no further social contact was made for 2 min. The time investigating the non-social stimulus was considered as a pre-conditioning measure of non-social anxiety.

Social fear extinction (day 2). One day after social fear conditioning, mice were exposed in their home cage to 3 different non-social stimuli, i.e. empty cages, to assess non-social investigation as a parameter of non-social fear. Mice were then exposed to 6 different unfamiliar social stimuli, i.e. male mice, each in a different 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-exposure interval.

Extinction recall (day 3). One day after social fear extinction, mice were exposed in their home cage to 6 different unfamiliar social stimuli for 3 min, with a 3-min inter-exposure interval.

Elevated plus-maze

General anxiety was tested on the EPM as previously described (Toth et al., 2012b). Increased percentage of time spent on the open arms (110 lx) indicated reduced anxiety. The number of entries into the closed arms (25 lx) during the 5-min testing period indicated locomotor activity.

Home cage locomotion

Locomotion was measured for 1 h using the Noldus system (Noldus Information Technology, Wageningen, The Netherlands) after a 1-min removal of the mice from the home cage (Toth et al., 2012b,c).

Stereotaxic cannula implantation

Implantation of guide cannulas (21 G, 8 mm length; Injecta GmbH, Germany) for intracerebroventricular (icv) or local infusions was performed under isoflurane anesthesia (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany) as previously described (Toth et al., 2012c), either 2 mm above the right lateral ventricle (from Bregma: + 0.2 mm, lateral: + 1.0 mm, depth: + 1.4 mm) or bilateral 1 mm above the DLS (from Bregma: - 0.3 mm, lateral: \pm 0.5 mm, depth: + 1.6 mm; Paxinos and Franklin, 1997). To avoid post-surgical infections, mice received subcutaneous antibiotics (s.c.; 3 mg/30 μ l Baytril®, Bayer Vital GmbH, Leverkusen, Germany). After surgery, mice were handled for 5 days before experiments started.

Intracerebral infusions

Mice received icv or DLS infusions of either vehicle (sterile Ringer solution; icv 2 μ l, DLS 0.2 μ l/side), synthetic OT (Sigma-Aldrich, Germany; icv: 0.1 or 0.5 μ g/2 μ l; DLS: 5 ng/0.2 μ l/side), synthetic AVP (Sigma-Aldrich, Germany; icv: 0.1 or 0.5 μ g/2 μ l), or a selective OTR antagonist (OTR-A; desGly-NH₂,d(CH₂)₅[Tyr(Me)₂,Thr₄]OVT; icv: 2 μ g/2 μ l) via an infusion cannula connected via polyethylene tubing to a Hamilton syringe.

The correct infusion site was histologically verified after the experiment. One DLS-infused mouse was removed from the study. Drug doses and timing were selected based on previous studies (Lukas et al., 2011; Kessler et al., 2011; Toth et al., 2012c).

OTR autoradiography

To determine the influence of social fear and its extinction on OTR binding, the following groups were studied: 1) **naïve** mice; 2) **UC-ext**: unconditioned mice without social fear extinction; 3) **UC+ext**: unconditioned mice with prolonged social fear extinction, i.e. exposure to 12 social stimuli instead of 6; 4) **C-ext**: conditioned mice without social fear extinction; 5) **C+ext**: conditioned mice with prolonged social fear extinction to ensure complete extinction of social fear in all mice. We have previously shown that social fear is completely extinguished 24h after the social fear extinction procedure (Toth et al., 2012b).

Forty-eight hours after social fear conditioning (Litvin et al., 2011) mice were killed using CO₂, brains were removed, snap frozen, and stored at -20°C. Brains were cut in 16 µm coronal sections targeting the CeA, basolateral (BLA) and medial (MeA) amygdala, DG, CA1, CA3, IL, PL, cingulate cortex (CC), medial preoptic area (MPOA), ventro-lateral septum (VLS), DLS, nucleus accumbens (NAc), and BNST (see Figure 13). These brain regions were selected based on their role in conditioned fear and social behavior (LeDoux and Muller, 1997; Neumann, 2008). OTR autoradiography was performed as previously described (Caughey et al., 2011). OTR binding was expressed as grey density and calculated for each mouse by taking the mean of 4 to 6 sections per region of interest. Left and right regions were scored separately.

Statistical analysis

PASW/SPSS (Version 17) was used. Data were analysed by Student's t-tests, one- or two-way ANOVA for repeated measures, followed by a Bonferroni post-hoc analysis whenever appropriate. Statistical significance was set at $p < 0.05$. Overall statistics are shown in Table 6 and 7.

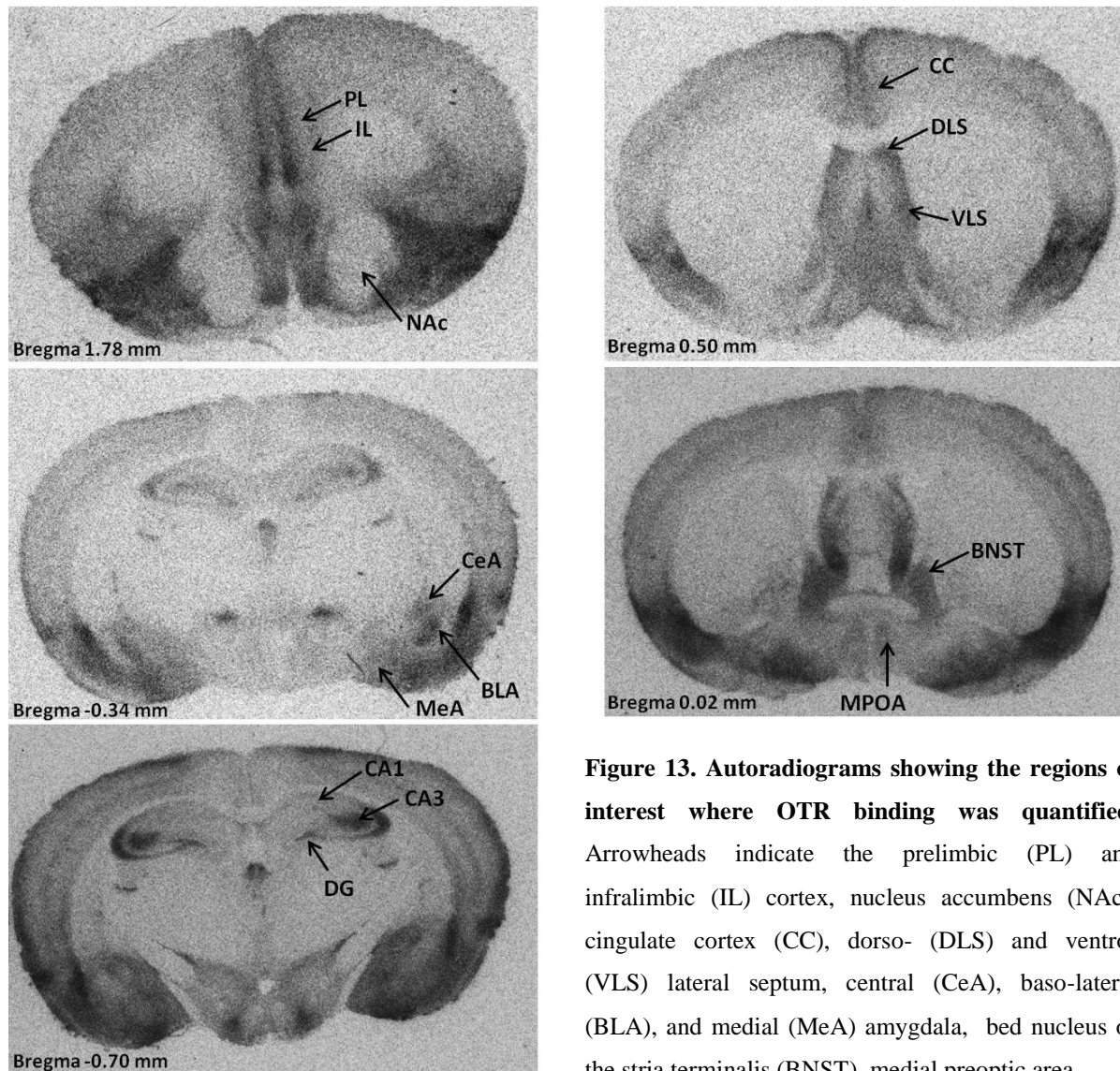


Figure 13. Autoradiograms showing the regions of interest where OTR binding was quantified. Arrowheads indicate the prelimbic (PL) and infralimbic (IL) cortex, nucleus accumbens (NAc), cingulate cortex (CC), dorso- (DLS) and ventro- (VLS) lateral septum, central (CeA), baso-lateral (BLA), and medial (MeA) amygdala, bed nucleus of the stria terminalis (BNST), medial preoptic area

(MPOA), cornu ammonis 1 (CA1) and 3(CA3), dentate gyrus (DG).

| Experiment | Group effect | Group \times stimulus or time effect |
|----------------------------------|---------------------------------|--|
| Effects of icv OT on: | | |
| Social fear (Figure 14) | | |
| Social fear conditioning (day 1) | $F_{(5,46)}=0.15$; $p=0.98$ | |
| Social fear extinction (day 2) | $F_{(5,46)}=15.36$; $p<0.01^*$ | $F_{(40,368)}=5.31$; $p<0.01^*$ |
| Extinction recall (day 3) | $F_{(5,46)}=3.44$; $p=0.01^*$ | $F_{(25,230)}=1.45$; $p=0.08$ |
| General anxiety (EPM; Figure 19) | $F_{(2,19)}=0.42$; $p=0.66$ | |
| EPM locomotion (Figure 19) | $F_{(2,19)}=0.13$; $p=0.88$ | |
| Home cage locomotion (Figure 19) | $F_{(2,17)}=6.88$; $p=0.01^*$ | $F_{(10,85)}=1.38$; $p=0.20$ |

| Experiment | Group effect | Group \times stimulus or time effect |
|----------------------------------|---------------------------------|--|
| Effects of OTR-A on: | | |
| Social fear (Figure 15) | | |
| Social fear conditioning (day 1) | $F_{(4,30)}=0.26$; $p=0.90$ | |
| Social fear extinction (day 2) | $F_{(4,30)}=7.54$; $p<0.01^*$ | $F_{(32,240)}=2.61$; $p<0.01^*$ |
| Extinction recall (day 3) | $F_{(4,30)}=3.87$; $p=0.01^*$ | $F_{(20,150)}=0.67$; $p=0.85$ |
| General anxiety (EPM; Figure 19) | $T_{(10)}=0.61$; $p=0.56$ | |
| EPM locomotion (Figure 19) | $T_{(10)}=1.54$; $p=0.16$ | |
| Home cage locomotion (Figure 19) | $F_{(1,18)}=0.07$; $p=0.79$ | $F_{(5,90)}=0.41$; $p=0.84$ |
| Effects of icv AVP on: | | |
| Social fear (Figure 16) | | |
| Social fear conditioning (day 1) | $F_{(3,19)}=0.39$; $p=0.76$ | |
| Social fear extinction (day 2) | $F_{(3,19)}=7.10$; $p<0.01^*$ | $F_{(24,152)}=3.97$; $p<0.01^*$ |
| Extinction recall (day 3) | $F_{(3,19)}=3.47$; $p=0.04^*$ | $F_{(15,95)}=0.64$; $p=0.84$ |
| General anxiety (EPM; Figure 19) | $F_{(2,19)}=19.76$; $p<0.01^*$ | |
| EPM locomotion (Figure 19) | $F_{(2,19)}=4.39$; $p=0.03^*$ | |
| Home cage locomotion (Figure 19) | $F_{(2,15)}=3.34$; $p=0.06$ | $F_{(10,75)}=3.17$; $p<0.01^*$ |
| Effects of DLS OT on: | | |
| Social fear (Figure 18) | | |
| Social fear conditioning (day 1) | $F_{(2,20)}=1.00$; $p=0.38$ | |
| Social fear extinction (day 2) | $F_{(2,20)}=11.47$; $p<0.01^*$ | $F_{(16,160)}=5.58$; $p<0.01^*$ |
| Extinction recall (day 3) | $F_{(2,20)}=7.10$; $p=0.01^*$ | $F_{(10,100)}=2.17$; $p=0.03^*$ |
| General anxiety (EPM; Figure 19) | $T_{(15)}=-0.64$; $p=0.53$ | |
| EPM locomotion (Figure 19) | $T_{(15)}=-0.90$; $p=0.38$ | |

Table 6. Overall statistics for the behavioral data. Icv, intracerebroventricular; OT, oxytocin; EPM, elevated plus-maze; OTR-A, OT receptor antagonist; AVP, arginine vasopressin; DLS, dorso-lateral septum. Stimulus effect refers to both non-social and social stimuli during social fear extinction, while only to social stimuli during extinction recall. Time effect refers to the time-bins during home cage locomotion measurements. Student's t-tests, one-way or two-way ANOVA for repeated measures followed by Bonferroni post-hoc test; * $p<0.05$.

| Brain region | Student's t-test | Conditioning effect | Extinction effect | Conditioning x extinction effect |
|--------------|-----------------------------|------------------------------|---------------------------------|----------------------------------|
| DLS left | $T_{(10)}=-0.60$; $p=0.57$ | $F_{(1,19)}=0.37$; $p=0.55$ | $F_{(1,19)}=0.13$; $p=0.73$ | $F_{(1,19)}=4.67$; $p=0.04^*$ |
| DLS right | $T_{(10)}=-0.48$; $p=0.64$ | $F_{(1,19)}=0.93$; $p=0.35$ | $F_{(1,19)}=0.02$; $p=0.89$ | $F_{(1,19)}=5.71$; $p=0.03^*$ |
| CeA left | $T_{(9)}=-0.35$; $p=0.74$ | $F_{(1,18)}=0.27$; $p=0.61$ | $F_{(1,18)}=0.30$; $p=0.59$ | $F_{(1,18)}=0.89$; $p=0.36$ |
| CeA right | $T_{(9)}=0.33$; $p=0.75$ | $F_{(1,18)}=0.41$; $p=0.53$ | $F_{(1,18)}=0.003$; $p=0.96$ | $F_{(1,18)}=5.56$; $p=0.03^*$ |
| DG left | $T_{(9)}=-0.69$; $p=0.51$ | $F_{(1,19)}=1.84$; $p=0.19$ | $F_{(1,19)}=0.18$; $p=0.68$ | $F_{(1,19)}=0.84$; $p=0.37$ |
| DG right | $T_{(9)}=0.37$; $p=0.72$ | $F_{(1,19)}=2.14$; $p=0.16$ | $F_{(1,19)}=0.002$; $p=0.96$ | $F_{(1,19)}=10.3$; $p=0.01^*$ |
| CA1 left | $T_{(8)}=0.50$; $p=0.63$ | $F_{(1,19)}=1.35$; $p=0.26$ | $F_{(1,19)}=0.51$; $p=0.48$ | $F_{(1,19)}=1.67$; $p=0.21$ |
| CA1 right | $T_{(8)}=-0.14$; $p=0.89$ | $F_{(1,19)}=3.67$; $p=0.07$ | $F_{(1,19)}=0.01$; $p=0.91$ | $F_{(1,19)}=5.18$; $p=0.04^*$ |
| MPOA left | $T_{(6)}=-0.09$; $p=0.97$ | $F_{(1,13)}=1.54$; $p=0.24$ | $F_{(1,13)}=53.66$; $p<0.01^*$ | $F_{(1,13)}=0.18$; $p=0.68$ |
| MPOA right | $T_{(6)}=1.13$; $p=0.30$ | $F_{(1,13)}=0.30$; $p=0.59$ | $F_{(1,13)}=47.61$; $p<0.01^*$ | $F_{(1,13)}=0.62$; $p=0.44$ |

Table 7. Overall statistics for the oxytocin receptor autoradiography. DLS, dorso-lateral septum; CeA, central amygdala; DG, dentate gyrus; CA1, cornu ammonis 1; MPOA, medial preoptic area. Student's t-test refers to statistical comparison between naïve and UC-ext groups. Two-way ANOVA for repeated measures (comparison between UC-ext, UC+ext, C-ext and C+ext groups) followed by Bonferroni post-hoc test; * $p<0.05$.

RESULTS

Icv OT reverses social fear

To verify whether OT reverses social fear, conditioned and unconditioned mice ($n = 6$ to 10 per group) were infused icv with either vehicle or OT (0.1 or 0.5 μg) 10 min before social fear extinction on day 2.

Mice showed similar non-social anxiety during social fear conditioning (Figure 14A, Table 6). Conditioned mice received a similar number of foot shocks during social fear conditioning (vehicle 2.0 ± 0.3 ; 0.1 μg OT 2.1 ± 0.2 ; 0.5 μg OT 2.5 ± 0.2 ; $F_{(2,23)}=0.79$, $p=0.47$). During social fear extinction, all groups showed similar non-social investigation. While conditioned

vehicle-treated mice showed reduced social investigation compared with all other groups ($p \leq 0.01$; Figure 14B) reflecting social fear, both doses of OT increased social investigation to levels found in unconditioned mice. During extinction recall on day 3, conditioned vehicle-treated mice still showed reduced social investigation compared with unconditioned vehicle- and OT-treated (0.1 μg) mice ($p < 0.05$; Figure 14C).

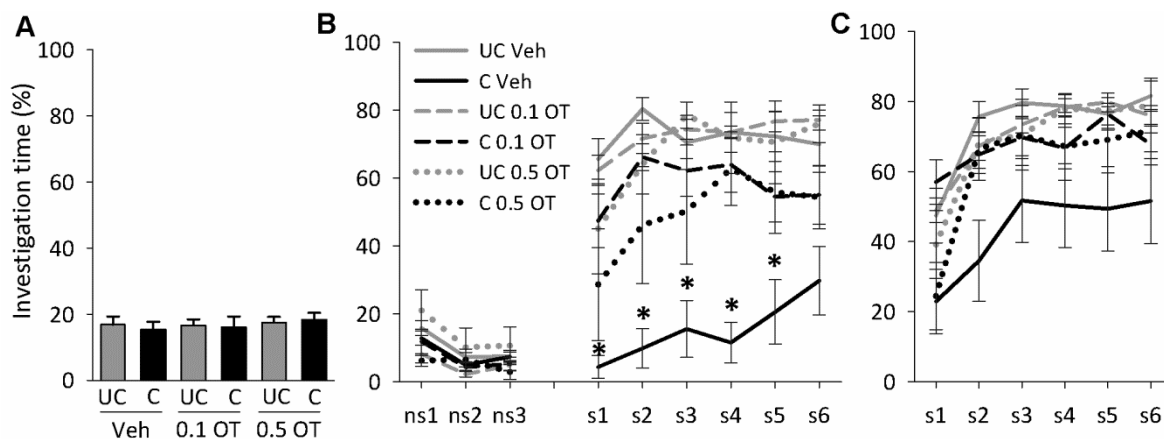


Figure 14. Intracerebroventricular oxytocin (OT) reverses social fear. (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 6$ to 10 per group). (B) Investigation of the non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Unconditioned (UC) and conditioned (C) mice were infused icv with either vehicle (Veh; 2 μl) or OT (0.1 or 0.5 $\mu\text{g}/2 \mu\text{l}$) 10 min before social fear extinction. (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent means \pm SEM. * $p < 0.05$ vs. all groups.

OT reverses social fear via OTR and is required for social investigation

To verify the involvement of brain OTR in the effects of OT on social fear, conditioned and unconditioned mice ($n = 7$ per group) were infused icv with either vehicle or OTR-A 40 min before social fear extinction on day 2. Thirty min later, vehicle-treated mice were infused with vehicle, while OTR-A-treated mice were infused with either vehicle or OT (0.1 μg).

Mice showed similar non-social anxiety during social fear conditioning (Figure 15A, Table 6). Conditioned mice received a similar number of foot shocks during social fear conditioning

(Veh/Veh 2.3 ± 0.4 ; OTR-A/Veh 2.1 ± 0.3 ; OTR-A/OT 2.3 ± 0.2 ; $F_{(2,18)}=0.07$, $p=0.93$). During social fear extinction, all groups showed similar non-social investigation. All groups, including the unconditioned OTR-A/Veh-treated mice, showed reduced social investigation compared with unconditioned Veh/Veh-treated mice ($p<0.05$; Figure 15B). During extinction recall on day 3, conditioned Veh/Veh-treated mice still showed reduced social investigation compared with unconditioned Veh/Veh-treated mice ($p<0.05$; Figure 15C).

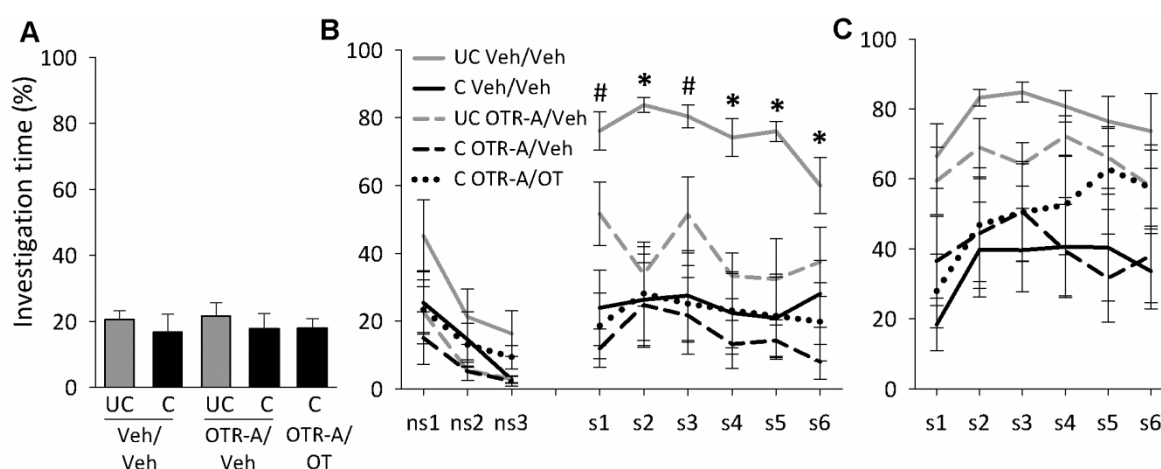


Figure 15. Oxytocin (OT) reverses social fear via OT receptor (OTR) and is required for social investigation. (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 7$ per group). (B) Investigation of the non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Unconditioned (UC) and conditioned (C) mice were infused icv with either vehicle (Veh; 2 μ l) or OTR antagonist (OTR-A; 2 μ g/2 μ l) 40 min before social fear extinction. Thirty min later, Veh-treated mice were infused with Veh, while OTR-A-treated mice were infused with either Veh or OT (0.1 μ g/2 μ l). (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent means \pm SEM. $p<0.05$ *vs. all groups; # vs. all groups except UC OTR-A/Veh.

Icv AVP does not reverse social fear

To confirm peptide-specificity of OT effects, conditioned and unconditioned mice ($n = 5$ to 6 per group) were infused icv with either vehicle or AVP 10 min before social fear extinction on day 2.

Mice showed similar non-social anxiety during social fear conditioning (Figure 16A, Table 6). Conditioned mice received a similar number of foot shocks during social fear conditioning (vehicle 2.6 ± 0.4 vs. AVP 2.5 ± 0.2 ; $T_{(9)}=0.23$, $p=0.82$). During social fear extinction, conditioned mice showed similar non-social investigation, but reduced social investigation compared with unconditioned mice, independent of treatment ($p<0.05$; Figure 16B). During extinction recall on day 3, there was a main group effect (Figure 16C), but post-hoc analysis revealed no significant differences between the groups.

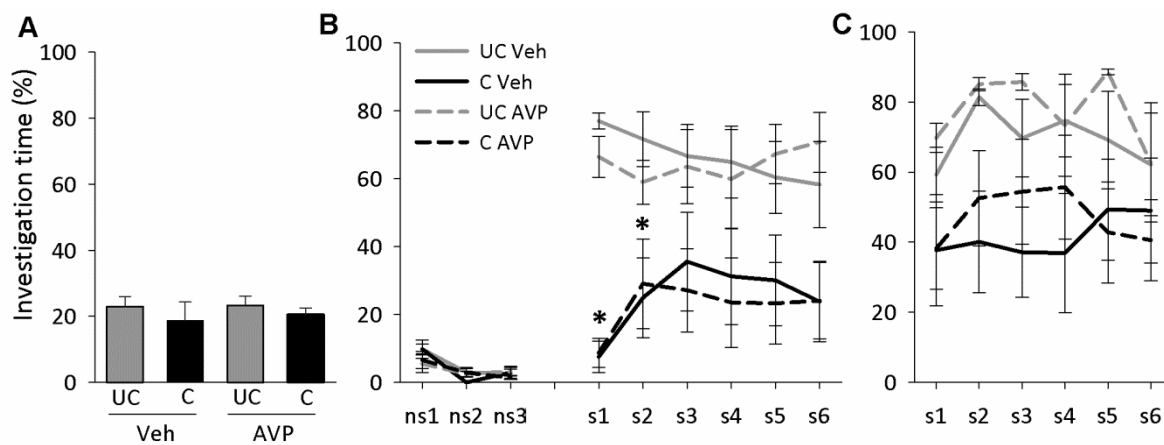


Figure 16. Intracerebroventricular arginine vasopressin (AVP) does not reverse social fear. (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 5$ to 6 per group). (B) Investigation of the non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Unconditioned (UC) and conditioned (C) mice were infused icv with either vehicle (Veh; 2 μ l) or AVP (0.1 μ g/2 μ l) 10 min before social fear extinction. (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent means \pm SEM. * $p<0.05$ vs. UC Veh.

Social fear and its extinction alter OTR binding

Given the role of OT in both social investigation (Lukas et al., 2011) and social fear reversal (Figure 14), we determined whether social fear conditioning and social fear extinction alter OTR binding in brain regions associated with conditioned fear and social behavior. Student's t-tests revealed no difference between naïve and UC-ext mice in any of the brain regions

investigated (Figure 17A-E, Table 7). Therefore, two-way ANOVA with factors conditioning and extinction was performed between UC-ext, C-ext, UC+ext and C+ext mice.

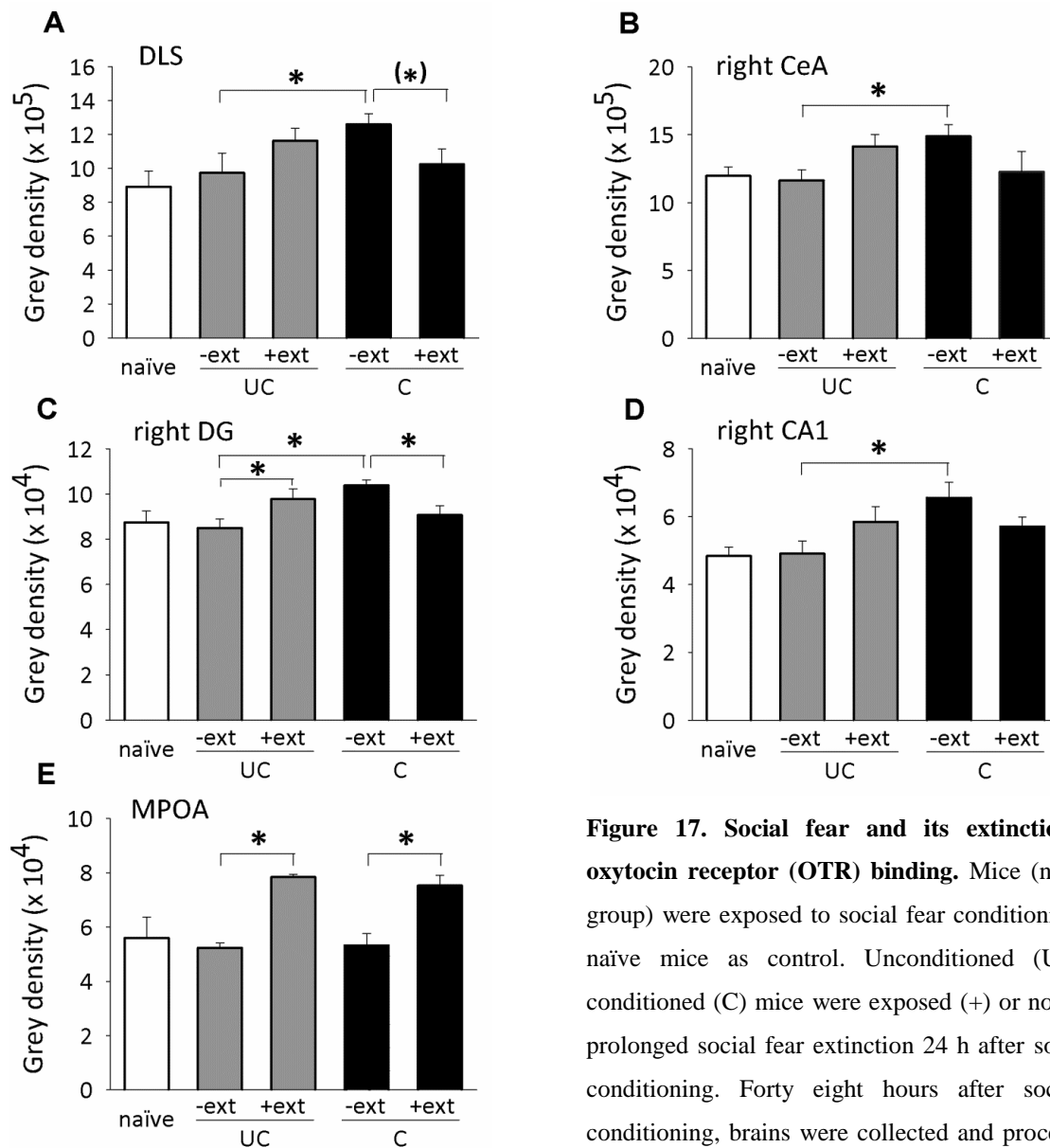


Figure 17. Social fear and its extinction alter oxytocin receptor (OTR) binding. Mice ($n = 6$ per group) were exposed to social fear conditioning, with naïve mice as control. Unconditioned (UC) and conditioned (C) mice were exposed (+) or not (-) to a prolonged social fear extinction 24 h after social fear conditioning. Forty eight hours after social fear conditioning, brains were collected and processed for OTR autoradiography. DLS, dorso-lateral septum;

CeA, central amygdala; DG, dentate gyrus; CA1, cornu ammunis 1; MPOA, medial preoptic area; Data represent means \pm SEM. *,(*) $p < 0.05$; * Bonferroni post-hoc; (*) Mann-Whitney-U-test.

A significant conditioning \times extinction interaction effect was found bilaterally in the DLS, right CeA, right DG, and right CA1, where conditioned mice without social fear extinction (C-ext) showed an increased OTR binding compared with their respective unconditioned

controls (UC-ext; Figure 17A-D). After social fear extinction, however, OTR binding no longer differed between conditioned and unconditioned mice (C+ext vs. UC+ext). Separate statistics (Mann-Whitney-U-test) revealed a decreased OTR binding bilaterally in the DLS of conditioned mice after social fear extinction (C-ext vs. C+ext).

Moreover, a significant extinction effect was observed within the MPOA and right DG (Figure 17C,E). In more detail, prolonged social fear extinction increased OTR binding in both conditioned and unconditioned mice in the MPOA, whereas in the right DG it increased OTR binding in unconditioned mice (UC-ext vs. UC+ext), while it decreased OTR binding in conditioned mice (C-ext vs. C+ext).

There were no group differences in OTR binding in the BLA, MeA, CA3, IL, PL, CC, VLS, NAc, and BNST (data not shown).

OT reverses social fear within the DLS

To determine whether alterations in OTR binding in the DLS are involved in social fear, mice ($n = 7$ to 8 per group) were infused bilaterally into the DLS with either vehicle or OT 10 min before social fear extinction on day 2.

Mice showed similar non-social anxiety during social fear conditioning (Figure 18A, Table 6). Conditioned mice received a similar number of foot shocks during social fear conditioning (vehicle 2.8 ± 0.3 vs. OT 2.8 ± 0.4 ; $T_{(14)} = 0.0$, $p = 1.0$). During social fear extinction, all groups showed similar non-social investigation. While conditioned vehicle-treated mice showed reduced social investigation compared with both groups ($p < 0.01$; Figure 18B) reflecting social fear, OT increased social investigation to levels found in unconditioned mice. During extinction recall on day 3, conditioned vehicle-treated mice still showed reduced social investigation compared with both groups ($p < 0.05$; Figure 18C).

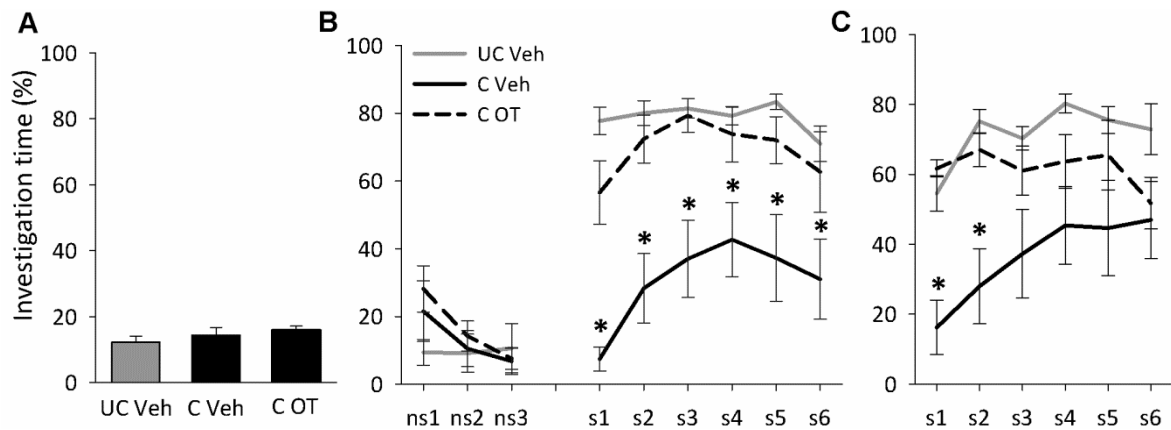


Figure 18. Oxytocin (OT) reverses social fear within the dorso-lateral septum (DLS). (A) Investigation of the non-social stimulus (empty cage) during social fear conditioning on day 1 ($n = 7$ to 8 per group). (B) Investigation of the non-social (ns1-ns3) and social (cages with mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). Unconditioned (UC) and conditioned (C) mice were infused bilaterally into the DLS with either vehicle (Veh; $0.2 \mu\text{l}/\text{side}$) or OT ($5 \text{ ng}/0.2 \mu\text{l}/\text{side}$) 10 min before social fear extinction. (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent means \pm SEM. * $p < 0.05$ vs. both groups.

Effects of OT, OTR-A, and AVP on general anxiety and locomotion

To verify the specificity of these effects on social fear conditioning, separate groups of mice ($n = 6$ to 9 per group) were infused icv or within the DLS with either vehicle, OT, OTR-A, or AVP at the same doses as for the social fear conditioning experiments, and tested either on the EPM 10 min later, or in the home cage immediately thereafter.

Neither icv nor DLS infused OT affected general anxiety or locomotion on the EPM (Figure 19, Table 6). Home cage locomotion was increased by 0.5 , but not by $0.1 \mu\text{g}$ icv OT, as previously reported (Toth et al., 2012c). OTR-A did not affect general anxiety, EPM and home cage locomotion. Both doses of AVP increased general anxiety, while 0.5 , but not $0.1 \mu\text{g}$ AVP decreased EPM locomotion, and tended to decrease home cage locomotion.

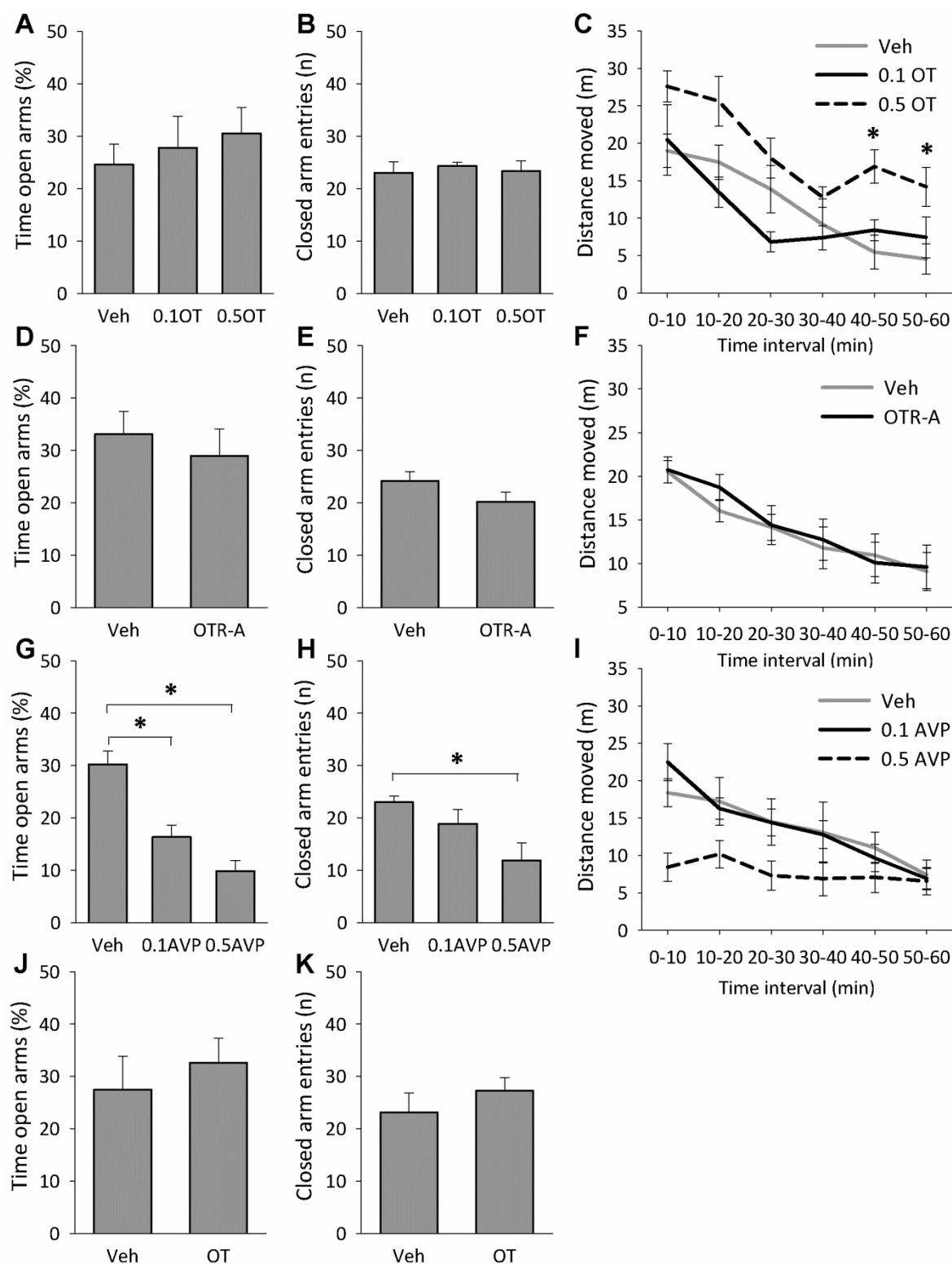


Figure 19. Effects of oxytocin (OT; intracerebroventricular [icv] A-C; dorso-lateral septum [DLS]: J,K), OT receptor antagonist (OTR-A; D-F), arginine vasopressin (AVP; G-I) on general anxiety on the elevated plus-maze (EPM, time open arms), locomotor activity on the EPM (closed arm entries) and in the home cage. Mice were infused icv or bilaterally into the DLS with either vehicle (Veh; icv 2 μ l; DLS 0.2 μ l/side), OT (icv 0.1 or 0.5 μ g/2 μ l; DLS 5 ng/0.2 μ l/side), OTR-A (icv 2 μ g/2 μ l), or AVP (icv 0.1 or 0.5 μ g/2 μ l) and tested either on the EPM 10 min later, or in the home cage immediately thereafter. Data represent means \pm SEM. * p <0.05.

DISCUSSION

The present study demonstrates that OT has the potential to reverse social fear of unknown con-specifics in male mice through OTR-mediated actions, without causing any confounding behavioral alterations in general anxiety or locomotor activity. Social fear was accompanied by an increased OTR binding in the bilateral DLS, right CeA, right DG, and right CA1. These alterations normalized after extinction of social fear and suggest that the DLS, amygdala, and the hippocampus might be part of a brain network involved in the development and/or neural support of social fear. Importantly, infusion of OT within the DLS reversed social fear, thereby localizing the effects of OT on social fear. Taken together, these results suggest that the DLS is an important region in the expression and extinction of social fear and that OT may be a potential add-on drug for the treatment of disorders associated with social deficits, such as SAD.

We have previously shown that OT reverses avoidance of a dominant con-specific induced by social defeat in male rats (Lukas et al., 2011). Here, we extend these findings by showing that OT not only reverses social avoidance towards a specific previously encountered con-specific, but also general social fear of several unknown con-specifics, making the translation of these findings to humans more applicable. Importantly, OT only increased social investigation in conditioned, but not in unconditioned mice, as we have previously shown in male rats (Lukas et al., 2011). Similarly, in male goldfish, icv infused isotocin, the teleost correspondent of OT (Acher et al., 1995), increased social approach only in individuals with low sociability (Thompson and Walton, 2004). Although synthetic OT decreased general anxiety when infused into the PVN (Blume et al., 2008) or into the CeA (Bale et al., 2001), icv OT did not alter general anxiety on the EPM, as previously reported in rats (Slattery and Neumann, 2010), indicating that the reversal of social fear by OT is unlikely due to its general anxiolytic properties. Furthermore, the increased social investigation in conditioned OT-treated mice

was not due to increased locomotion, as neither EPM nor home cage locomotion was altered after OT (0.1 μ g) treatment. However, the higher OT dose (0.5 μ g) increased home cage locomotion, while slightly decreasing social investigation in both conditioned and unconditioned mice, indicating that, if anything, increases in locomotion have a rather distracting effect on social investigation. Interestingly, OT infused icv 10 min prior to social fear extinction seems to specifically reverse social fear, but not fear in general, as OT infused icv at a comparable time-point delayed extinction in a cued fear conditioning paradigm, where the feared stimulus is a tone (Toth et al., 2012c). Therefore, the effect of OT on social fear might be related to the social aspect of the social fear conditioning paradigm and the pro-social properties of OT.

We also showed that OT reversed social fear via the OTR, as pre-administration of an OTR-A blocked its effects. Interestingly, OTR-A decreased social investigation in unconditioned mice without inducing non-specific alterations in locomotion, general anxiety, or typical signs of social fear as those seen in conditioned mice, i.e. freezing, attempt approaches, and defensive burying (Toth et al., 2012b). This suggests that endogenous OT is required for naturally-occurring social investigation, and confirms our previous study in male rats and mice (Lukas et al., 2011).

We could show that the reversal of social fear was specific to OT, as the closely related neuropeptide AVP did not reverse social fear; however, its anxiogenic effect on the EPM could be confirmed (Bhattacharya et al., 1998; Kessler et al., 2011). High doses of AVP (0.5 μ g) decreased locomotion, contraindicating their use in the social fear conditioning experiment. As AVP and OT often exert opposite effects on behavior (Rooszendaal et al., 1992; Thompson and Walton, 2004), the use of AVP receptor antagonists could be considered for future social fear conditioning experiments.

Considering these findings and previous studies showing social interaction- (Murakami et al., 2011) and social defeat- (Litvin et al., 2011) induced alterations in OTR expression, we determined the effect of social fear conditioning on OTR binding in brain regions involved in social behavior and conditioned fear (LeDoux and Muller, 1997; Neumann, 2008). Whereas a single 3-min exposure to a social stimulus was not sufficient to alter OTR binding in any of the brain regions investigated, alterations in OTR binding were found as a result of either social fear conditioning or repeated social exposure during the prolonged social fear extinction procedure. In more detail, OTR binding was increased in the MPOA of both conditioned and unconditioned mice in response to repeated social exposure. This result is in accordance with previous studies showing increased c-Fos expression in the MPOA in response to same-sex social stimuli (Ferguson et al., 2001; Richter et al., 2005). The increased OTR binding in the MPOA might be, therefore, relevant for recognition of social stimuli, as OT infusion into the MPOA facilitated social recognition (Popik and van Ree, 1991).

OTR binding was increased in the right CeA, right DG, right CA1, and bilaterally in the DLS of conditioned mice without social fear extinction. Importantly, this increase in OTR binding normalized after extinction of social fear, suggesting that alterations at the level of the brain OT system might be involved in the development and/or neural support of social fear. Interestingly, we found no alterations in OTR binding in the rest of the brain regions investigated, suggesting that these alterations are specific to brain regions involved in processing social fear-related information and in the display of social fear.

The CeA coordinates the behavioral and physiological correlates of fear expression (LeDoux et al., 1988), and both local infusion (Roozendaal et al., 1992; Viviani et al., 2011) and evoked axonal release of OT (Knobloch et al., 2012) attenuated the fear response in rodents. Similar to our conditioned mice, Rhesus monkeys with exaggerated defensive and fear-related behavior towards human intruders show increased activity in the right amygdala (Kalin et al.,

1998). Furthermore, SAD patients show hyperactivity of the amygdala in response to threatening faces (Labuschagne et al., 2010), which can be attenuated by nasal OT (Kirsch et al., 2005; Labuschagne et al., 2010). The increased OTR binding in the CeA might be, therefore, relevant for processing threatening stimuli and for the display of social fear.

The DG and CA1 are important for memory processing both in humans (Nadel et al., 2007) and rodents (Corcoran and Maren, 2001) and support, together with the amygdala, contextual fear conditioning (Kim et al., 1993). The increased OTR binding in the hippocampus might be, therefore, relevant for processing context-dependent information related to social fear.

The increased OTR binding in the DLS of conditioned mice without social fear extinction is in accordance with a previous study showing increased OTR mRNA in the LS of chronically-defeated mice (Litvin et al., 2011), and suggests that the DLS might be relevant for recognition of stimuli associated with the conditioning procedure. In support, increased c-Fos expression was found in the LS after cued fear conditioning (Calandreau et al., 2007), while pre-conditioning inactivation of the LS disrupted fear conditioning (Calandreau et al., 2007), indicating a role of the LS in fear learning. The LS was also involved in social and associative memory (Igelstrom et al., 2010), where the OT system plays a crucial role, with OT facilitating (Popik et al., 1992) and OTR-A impairing social recognition (Lukas, Toth et al., in press).

Considering the same pattern of OTR binding alterations found in the CeA, hippocampus and DLS of conditioned mice and the interconnectivity between these regions (Gallagher et al., 1995; Sheehan et al., 2004), it can be suggested that these limbic areas might be part of a brain network involved in the development and/or neural support of social fear. Given that the LS receives input from both the hippocampus (Gallagher et al., 1995) and the amygdala (Sheehan et al., 2004), and that the DLS was the only region where the OTR binding

alterations occurred bilaterally, we infused OT into the DLS and demonstrated its potential to reverse social fear. Similar to its icv effects, DLS-infused OT did not increase social investigation through unspecific alterations in general anxiety and locomotion. Although we have shown here that OT reverses social fear when infused into the DLS, it is not excluded that similar effects would be found when infused into the CeA, DG, or CA1.

The findings of increased OTR binding in the DLS of conditioned mice with social fear, which might indicate a more efficient OT neurotransmission, and OT reversing social fear when infused into the DLS is intriguing. Two possible explanations are that OTR are up-regulated in these mice to compensate for a decreased local OT release and thus extracellular availability or for an impaired OTR signaling. These hypotheses are not mutually exclusive, but whether social fear decreases OT release or impairs OTR signaling remains to be verified.

Although most human studies focus on the amygdala in relation to social fear (Kirsch et al., 2005; Domes et al., 2007b; Labuschagne et al., 2010), our study suggests the involvement of a more complex brain network in the development and/or neural support of social fear. Given the possibility of intranasal OT delivery in humans, it might seem relevant for future studies to focus on the lateral septum as well.

In summary, we have shown that the brain OT system is altered in conditioned mice without social fear extinction, and that these alterations normalized after extinction of social fear. We have also shown that OT has the potential to reverse social fear, an effect that we localized within the DLS. Our findings suggest OT as potential add-on drug in the treatment of disorders associated with social deficits, such as SAD.

Chapter 5

Central administration of oxytocin receptor ligands affects cued fear extinction in rats and mice in a time-point-dependent manner

Author's contribution:

Toth: designed research, performed research, analyzed data, wrote the manuscript

Neumann: designed research, revised the manuscript

Slattery: designed research, revised the manuscript

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ABSTRACT

OT has been proposed as a potential therapeutic agent for PTSD. In this study, we aimed to verify whether pharmacological manipulation of the brain OT system affects cued fear conditioning and extinction. Male rats and mice were administered icv synthetic OT (rats: 0.1 or 1.0 $\mu\text{g}/5 \mu\text{l}$; mice: 0.1 or 0.5 $\mu\text{g}/2 \mu\text{l}$) and/or OTR-A (rats: 0.75 $\mu\text{g}/5 \mu\text{l}$) either before cued fear conditioning or cued fear extinction. Preconditioning administration of OT did not affect fear conditioning in rats, but decreased fear expression and facilitated fear extinction. In contrast, preconditioning blockade of OT neurotransmission by OTR-A did not affect fear conditioning or fear expression, but impaired fear extinction. When administered before cued fear extinction, OT impaired fear extinction in both rats and mice, indicating that the effects of OT on fear extinction are conserved across species. This impairment was OTR-mediated, as the inhibitory effect of OT on fear extinction was abolished by prior treatment with OTR-A. The impaired fear extinction was not a result of reduced locomotion in rats, whereas an apparent decrease in fear expression and facilitation of fear extinction with the higher OT dose in mice was the result of behavioral hyperactivity. These results suggest that increasing OT neurotransmission during traumatic events is likely to prevent the formation of aversive memories. In contrast, OT treatment before cued fear extinction, which would be the comparable time-point for psychotherapy in PTSD patients, rather delays fear extinction and, therefore, caution is needed before recommending OT for the treatment of PTSD.

INTRODUCTION

Pavlovian fear conditioning is a form of learning in which an association between a stimulus and its aversive consequences is made. Cued fear conditioning has been used in laboratory animals as a model of PTSD and involves the presentation of a neutral stimulus, such as a tone or light (CS) in association with an aversive stimulus, such as a mild foot shock (US). Through repeated CS-US associations, animals learn that the CS predicts the US, and a conditioned response, such as freezing (Fanselow, 1980), is elicited in the absence of the US. Fear extinction is regarded as a form of new learning (for reviews see Cammarota et al., 2007; Quirk et al., 2010) and is defined as the attenuation of the conditioned response by repeated exposure to the CS without the US. Inability to extinguish fear memories was shown to involve hyperactivity of the amygdala (Rauch et al., 2000; Stein et al., 2002; Dilger et al., 2003) and is a core symptom in several psychiatric disorders, such as specific phobia, generalized anxiety disorder, SAD, panic disorder, and PTSD. The current treatment for PTSD consists of psychotherapy, often combined with antidepressant, benzodiazepine, and antipsychotic treatment, with SSRIs providing the best response rates (Marshall and Pierce, 2000; Stein et al., 2006, 2009). However, treatment-resistant PTSD patients achieve only partial symptom remission or show a high rate of relapse after treatment discontinuation (Davidson et al., 2004; Bisson et al., 2007; Brunello et al., 2001; Ipser et al., 2006). Therefore, the development of approaches that combine psychotherapy with novel pharmacotherapy is still needed.

Neuropeptides, which have discrete synthesis, release, and receptor sites in the brain (Landgraf and Neumann, 2004; Wotjak et al., 2008), have emerged as viable research candidates with respect to both pathophysiology and treatment of PTSD (Gülpinar and Yegen, 2004; Viero et al., 2010). One such neuropeptide, OT, which is synthesized in the PVN and SON of the hypothalamus and centrally released within these hypothalamic and

other limbic regions, including the septum, hippocampus, and CeA in response to various stressful stimuli (Ebner et al., 2005; Neumann, 2007), has been recently proposed as a potential therapeutic agent for PTSD (Olf et al., 2010). Both synthetic and endogenous OT exert anxiolytic properties in rodents (McCarthy et al., 1996; Waldherr and Neumann, 2007; Blume et al., 2008) and inhibit the activity of the HPA axis (Windle et al., 1997; Neumann et al., 2000). Comparable effects were also found in humans (Heinrichs et al., 2003), as OT was shown to reduce the activation of the amygdala to threatening faces, thereby reducing the autonomic and behavioral manifestation of fear in healthy volunteers and SAD patients (Kirsch et al., 2005; Labuschagne et al., 2010). More indirect evidence for the anxiolytic and antistress effects of OT in humans comes from nursing mothers who are calmer and less anxious during stressful situations, possibly due to high brain OT activity (Heinrichs et al., 2001; Carter et al., 2001; Slattery and Neumann, 2008). Given that PTSD is marked by deficits in anxiety/stress regulation and hyperactivity of the amygdala (Rauch et al., 2000; Shin et al., 2004a), OT might be a good candidate for the treatment of PTSD (Olf et al., 2010; Viviani et al., 2011). Therefore, we aimed to study in detail whether OT affects fear conditioning and fear extinction and whether such effects depend on the timing of administration. The classical fear conditioning paradigm involves acquisition, expression, and extinction of fear memories, and drugs can differentially affect these processes (Lattal and Abel, 2001; Makkar et al., 2010). Therefore, we manipulated the OT system by icv administration of synthetic OT and/or OTR-A either before cued fear conditioning (also referred to as acquisition) or cued fear extinction. In order to be able to draw more general conclusions, we performed the experiments in both rats and mice and hypothesized that OT would facilitate cued fear extinction in both species.

MATERIALS AND METHODS

Animals

Male Wistar rats (280 - 300 g) and male CD1 mice (35 - 40 g) were purchased from Charles River, Sulzfeld, Germany. Animals were group-housed in polycarbonate cages (rats: 55 × 22 × 18 cm; mice: 42 × 26 × 15 cm) for 1 week before surgery and kept under standard laboratory conditions (12:12 light/dark cycle, lights on at 06:00, 22 °C, 60 % humidity, food and water ad libitum). After surgery, animals were single-housed in observation cages (rats: 40 × 24 × 36 cm; mice: 30 × 23 × 36 cm). All behavioral procedures took place during the light phase and were conducted in accordance with the local government of the Oberpfalz (Bavaria, Germany) and the guidelines of the National Institutes of Health.

Stereotaxic cannula implantation

Guide cannula implantation was performed under isoflurane anesthesia (Forene®, Abbott GmbH & Co. KG, Wiesbaden, Germany). To avoid post-surgical infections, all animals received antibiotics (s.c.; 3 mg/30 µl Baytril®, Bayer Vital GmbH, Leverkusen, Germany). Animals were mounted on a stereotaxic frame, and a guide cannula (21 G; rats: 12 mm length; mice: 8 mm length; Injecta GmbH, Klingenthal, Germany) was implanted above the right lateral ventricle (rats: from Bregma + 1.0 mm, lateral + 1.6 mm, depth + 1.8 mm; mice: from Bregma: + 0.2 mm, lateral + 1.0 mm, depth + 1.4 mm). The guide cannula was fixed with two stainless steel screws using dental cement (Kallocryl, Speiko-Dr. Speier GmbH, Münster, Germany) and closed by a stainless steel dummy cannula. After surgery, animals were handled daily (stroking, holding, and cleaning of dummy cannulas) for 5 days before experiments started.

Intracerebral infusions

Animals received icv infusions of either vehicle (sterile Ringer solution; rats: 5 µl; mice: 2 µl), synthetic OT (Sigma-Aldrich, Munich, Germany; rats: 0.1 or 1.0 µg/5 µl; mice: 0.1 or 0.5 µg/2 µl - from this point onward referred to as lower and higher OT doses for rats and mice, respectively), or a selective OTR-A (desGly-NH₂,d(CH₂)₅[Tyr(Me)₂,Thr₄]OVT; rats: 0.75 µg/5 µl) (Manning et al., 2008) via an infusion cannula (25 G, extended 2 mm beyond the guide cannula) connected via polyethylene tubing to a Hamilton syringe. The infusion system was left in place for 30 s following the infusion to allow diffusion of the solution.

To verify the infusion site, animals were killed using CO₂ and ink was infused icv before removal of the brain. Brains were cut coronally and checked for staining of the ventricle. Drug doses and timing were selected based on previous studies in our laboratory (Waldherr and Neumann, 2007; Bosch and Neumann, 2008; Lukas et al., 2011).

Cued fear conditioning apparatus

The cued fear experiments were performed in two different contexts, A and B, which differed in visual, tactile, and olfactory cues as previously described (Toth et al., 2012a). Briefly, cued fear conditioning occurred in context A, which consisted of a transparent Perspex box (rats: 45 × 45 × 40 cm; mice: 23 × 23 × 36 cm) with an electric grid floor. Context A was cleaned with water containing a small amount of a neutral-smelling detergent before each trial. Cued fear extinction and extinction recall occurred in context B, which consisted of a black Perspex box (rats: 45 × 45 × 40 cm; mice: 23 × 23 × 36 cm) with a smooth floor. Context B was cleaned with water containing a small amount of a lemon-scented detergent before each trial. The boxes were enclosed in a wooden chamber to reduce external noise and visual stimulation. A low level of background noise was produced by ventilation fans within the chamber. Illumination (300 lx for context A and 20 lx for context B) was provided by four

white light-emitting diodes. Auditory stimuli were delivered through a speaker attached 30 cm above the floor of the box. Freezing, defined as the absence of all movement except that required for respiration (Fanselow, 1980), was measured with the TSE fear conditioning system (TSE System GmbH, Bad Homburg, Germany). The conditioning chamber contained two horizontal detection fields, each with 32 (rats) or 16 (mice) infrared light beams set 1.3 cm apart. Inactivity was measured by the infrared beams and defined as no light beam interruption for at least 3 s (rats) or 1 s (mice). We have previously shown that such measurements are comparable with hand-scoring by an experienced observer (Toth et al., 2012a).

Cued fear conditioning procedure

The procedure was adapted from the literature (Muigg et al., 2008) and has been shown to induce a robust cued fear conditioning in our laboratory (Toth et al., 2012a).

Cued fear conditioning (day 1). Animals were placed in the conditioning chamber (context A) and, after a 5-min adaptation period, exposed to five CS-US pairings with a 2-min inter-stimulus interval. The CS was an 80-dB, 4.5-kHz (rats) or 8-kHz (mice), 30-s white noise, which co-terminated with a mild electric foot shock (US; 0.7 mA, pulsed current, 2 s). Animals were returned to their home cage 5 min after the last CS-US pairing.

Cued fear extinction (day 2). One day after cued fear conditioning, animals were placed in context B and, after a 5-min adaptation period, exposed to 30 (rats) or 20 (mice) CS presentations (30 s white noise, 5 s inter-stimulus interval). Animals were returned to their home cage 5 min after the last CS presentation. Freezing during the cued fear extinction session increased until the sixth CS; therefore, this period was defined as fear expression. After the sixth CS, freezing decreased; therefore, this period was defined as fear extinction.

These CS presentations were collapsed into ten blocks with the mean freezing percentage during three or two CS presentations represented in each block for rats and mice, respectively.

Extinction recall (day 3). One day after cued fear extinction, animals were again placed in context B, and after a 5-min adaptation period, were exposed to five CS presentations (30 s white noise, 5 s inter-stimulus interval). Animals were returned to their home cage after the last CS presentation. These CS presentations were then collapsed into one block.

Home cage locomotion

In separate groups of rats and mice, locomotion was assessed immediately after OT administration in the home cage for 1 h using the Noldus Ethovision XT 5.1 program (Noldus Information Technology, Wageningen, The Netherlands), as previously described (Slattery et al., 2012; Toth et al., 2012b).

Statistical analysis

PASW/SPSS (Version 17) was used to perform all statistical analyses. Cued fear conditioning and extinction data were analyzed using repeated measures ANOVA, followed by a Bonferroni post hoc analysis whenever appropriate. Extinction recall and home cage locomotion data were analyzed using a one-way ANOVA, followed by a Bonferroni post hoc analysis whenever appropriate. The criterion for significance was $p \leq 0.05$. Overall statistics are shown in Table 8.

RESULTS

Effects of preconditioning manipulation of the OT system on cued fear in rats

To determine whether preconditioning manipulation of the OT system influences cued fear, rats were infused icv with either vehicle (n = 12), OT (1.0 µg/5 µl; n = 13), or OTR-A (n = 13) 10 min before cued fear conditioning.

| Experiment | Group effect | Group × CS effect |
|--|---------------------------------|----------------------------------|
| Preconditioning manipulation of the OT system (Figure 20) | | |
| Cued fear conditioning (day 1) | $F_{(2,35)}=0.48$, $p=0.63$ | $F_{(8,140)}=0.83$; $p=0.58$ |
| Cued fear extinction (day 2) | $F_{(2,35)}=11.50$, $p<0.01^*$ | $F_{(18,315)}=2.40$, $p<0.01^*$ |
| Extinction recall (day 3) | $F_{(2,35)}=6.95$, $p<0.01^*$ | |
| Pre-extinction manipulation of the OT system in rats (Figure 21) | | |
| Cued fear conditioning (day 1) | $F_{(2,24)}=0.07$, $p=0.94$ | $F_{(8,96)}=0.23$, $p=0.99$ |
| Cued fear extinction (day 2) | $F_{(2,24)}=3.40$, $p=0.05^*$ | $F_{(18,216)}=1.22$, $p=0.25$ |
| Extinction recall (day 3) | $F_{(2,24)}=2.88$, $p=0.08$ | |
| Pre-extinction manipulation of the OT system in mice (Figure 22) | | |
| Cued fear conditioning (day 1) | $F_{(2,42)}=0.08$, $p=0.92$ | $F_{(8,168)}=0.27$, $p=0.97$ |
| Cued fear extinction (day 2) | $F_{(2,42)}=24.33$, $p<0.01^*$ | $F_{(18,378)}=1.67$, $p=0.04^*$ |
| Extinction recall (day 3) | $F_{(2,42)}=0.32$, $p=0.73$ | |
| OTR-A prior to OT (Figure 23) | | |
| Cued fear conditioning (day 1) | $F_{(2,21)}=0.02$, $p=0.98$ | $F_{(8,84)}=0.02$, $p=1.0$ |
| Cued fear extinction (day 2) | $F_{(2,21)}=0.42$, $p=0.66$ | $F_{(18,189)}=1.01$, $p=0.45$ |
| Extinction recall (day 3) | $F_{(2,21)}=0.99$; $p=0.39$ | |
| Effects of OT on home cage locomotion | | |
| In rats (Figure 24A) | $F_{(2,19)}=0.22$, $p=0.80$ | |
| In mice (Figure 24B) | $F_{(2,17)}=6.88$, $p=0.01$ | |

Table 8. Overall statistics for the behavioral data. OT, oxytocin; OTR-A, OT receptor antagonist; One-way ANOVA or repeated measures ANOVA followed by Bonferroni post-hoc test; * $p<0.05$.

Cued fear conditioning was successful in all groups, as the level of freezing increased across trials ($F_{(4,140)}=42.77$; $p<0.01$; Figure 20A). There was no difference in conditioning between treatment groups (Table 8). During cued fear extinction on day 2, there was a significant difference in fear extinction between treatment groups, with OT-treated rats showing lower CS-elicited freezing during blocks 2 and 3 compared with vehicle-treated rats, while OTR-A-treated rats showed higher freezing during blocks 6-10 compared with vehicle-treated rats ($p<0.05$; Figure 20B). During extinction recall on day 3, there was a significant difference between treatment groups (Figure 20C), with OTR-A-treated rats showing higher CS-elicited freezing compared with both vehicle- and OT-treated rats ($p<0.05$). There was no difference between vehicle- and OT-treated rats.

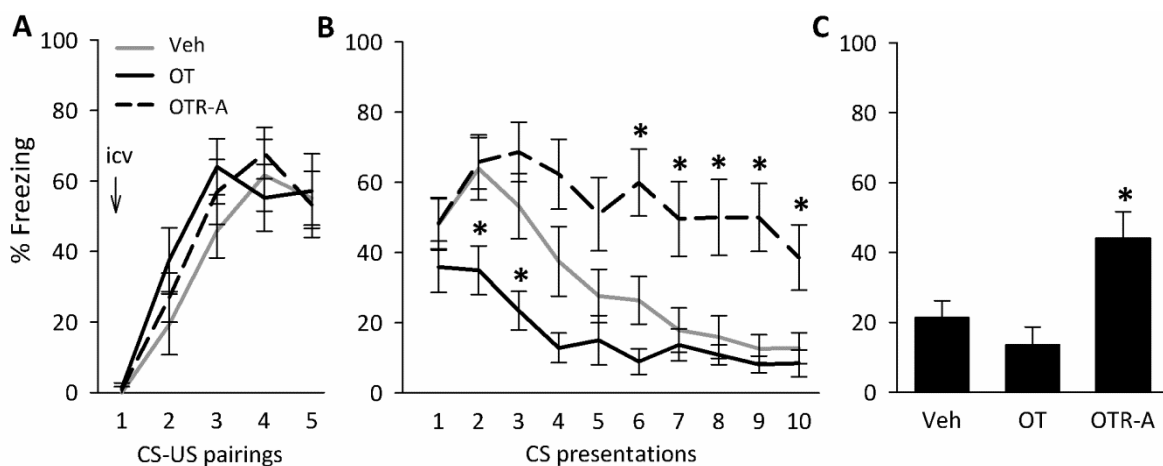


Figure 20. Oxytocin (OT) facilitates, whereas OT receptor antagonist (OTR-A) impairs cued fear extinction when infused before cued fear conditioning in rats. (A) Rats were infused icv with either vehicle (Veh; 5 μ l; $n = 12$), OT (1.0 μ g/5 μ l; $n = 13$), or OTR-A (0.75 μ g/5 μ l; $n = 13$) 10 min before cued fear conditioning. (B) On day 2, cued fear extinction was assessed. (C) On day 3, extinction recall was assessed. Data represent the mean time of CS-elicited freezing \pm SEM. * $p<0.05$ vs. Veh-treated rats.

Effects of OT administered before cued fear extinction on cued fear in rats and mice

To determine whether OT administered before cued fear extinction influences cued fear, rats and mice were infused icv with either vehicle (rats: $n = 9$; mice: $n = 21$), a lower OT dose

(rats: $n = 6$; mice: $n = 8$), or a higher OT dose (rats: $n = 12$; mice: $n = 16$) 10 min before cued fear extinction.

Cued fear conditioning was successful in both rats and mice, as the level of freezing increased across trials (rats: $F_{(4,96)}=14.84$; $p<0.01$; Figure 21A; mice: $F_{(4,168)}=22.15$; $p<0.01$; Figure 22A). There was no difference in conditioning between groups the day before treatment (Table 8). During cued fear extinction on day 2, there was a significant difference between treatment groups in both rats (Figure 21B) and mice (Figure 22B). While both OT doses increased CS-elicited freezing compared with vehicle in rats (0.1 μg , blocks 7, 10; 1.0 μg , blocks 7-10), OT exhibited a dose-dependent effect in mice. More specifically, the lower OT dose increased (block 9; $p<0.05$), while the higher dose decreased (blocks 1-7, 9; $p<0.05$) CS-elicited freezing compared with the vehicle-treated group (Figure 22B). During extinction recall on day 3, there was a tendency towards an increased CS-elicited freezing in OT-treated rats compared with vehicle-treated rats (Figure 21C), while no difference was found between treatment groups in mice (Figure 22C).

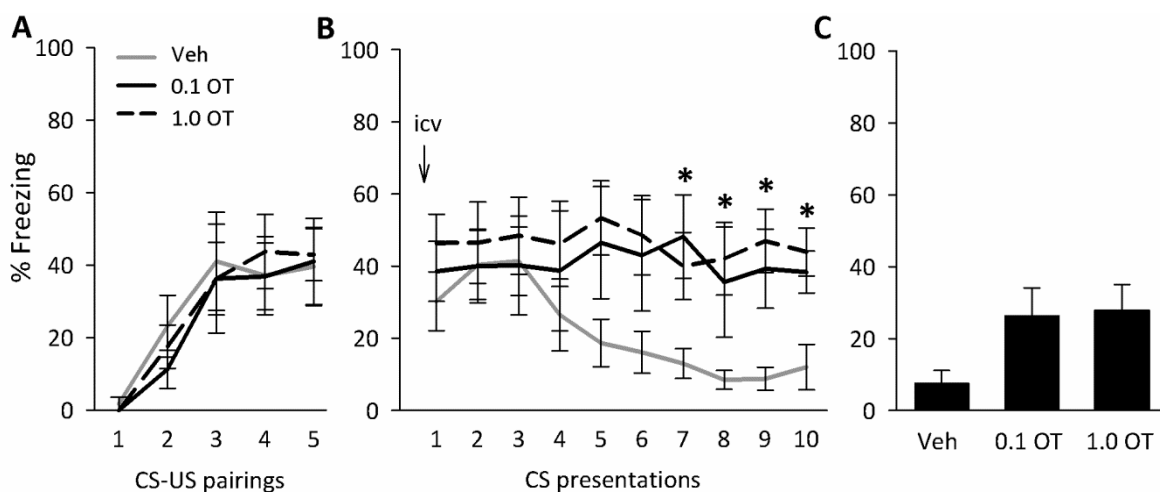


Figure 21. Oxytocin (OT) impairs cued fear extinction when infused before cued fear extinction in rats. (A) On day 1, rats were cued fear conditioned. (B) On day 2, 10 min before cued fear extinction, rats were infused icv with either vehicle (Veh; 5 μl ; $n = 9$), a lower OT dose (0.1 $\mu\text{g}/5 \mu\text{l}$; $n = 6$), or a higher OT dose (1.0 $\mu\text{g}/5 \mu\text{l}$; $n = 12$). (C) On day 3, extinction recall was assessed. Data represent the mean time of CS-elicited freezing \pm SEM. * $p<0.05$ vs. Veh-treated rats.

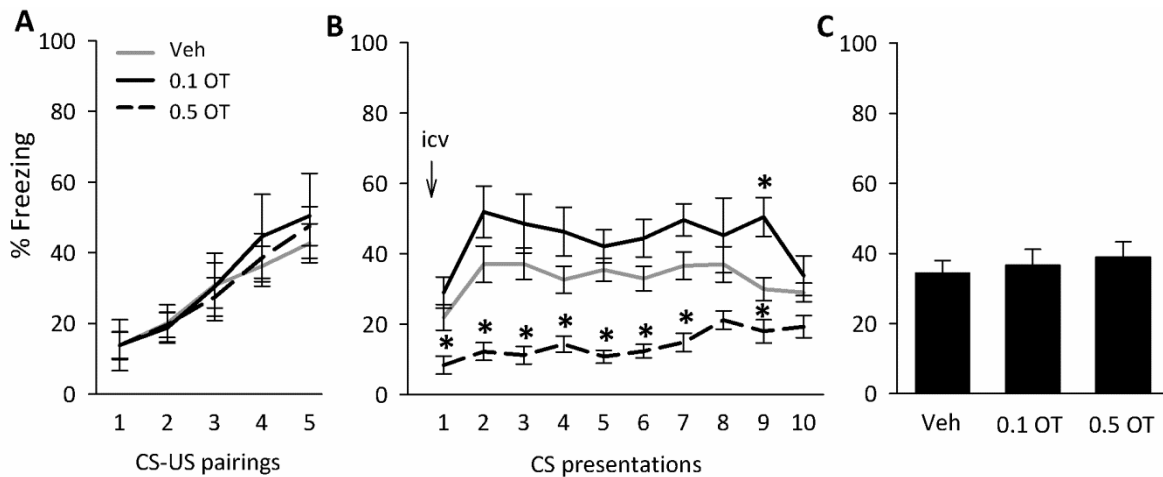


Figure 22. Oxytocin (OT) impairs cued fear extinction when infused in a low dose before cued fear extinction in mice. (A) On day 1, mice were cued fear conditioned. (B) On day 2, 10 min before cued fear extinction, mice were infused icv with either vehicle (Veh; 2 μ l; n = 21), a lower OT dose (0.1 μ g/2 μ l; n = 8), or a higher OT dose (0.5 μ g/2 μ l; n = 16). (C) On day 3, extinction recall was assessed. Data represent the mean time of CS-elicited freezing \pm SEM. * p <0.05 vs. Veh-treated mice.

Effects of OTR-A alone and on OT-induced delay in cued fear extinction in rats

To determine whether OTR-A infusion itself facilitates cued fear extinction and whether synthetic OT impairs cued fear extinction by binding to the OTR, rats were infused icv with either vehicle (n = 8) or OTR-A (n = 16) 40 min before cued fear extinction. Thirty minutes later, vehicle-treated rats were infused icv with vehicle, while OTR-A-treated rats were infused with either vehicle (n = 8) or OT (1.0 μ g/5 μ l; n = 8).

Cued fear conditioning was successful in all groups, as the level of freezing increased across trials ($F_{(4,84)}=14.75$; $p<0.01$; Figure 23A). There was no difference in conditioning between groups the day before treatment (Table 8). On day 2, cued fear extinction was successful in all treatment groups, as the high levels of freezing during the first trials decreased substantially by the last trial ($F_{(9,189)}=8.29$; $p<0.01$; Figure 23B). There was no difference in cued fear extinction between treatment groups. During extinction recall on day 3, there was no difference between treatment groups (Figure 23C).

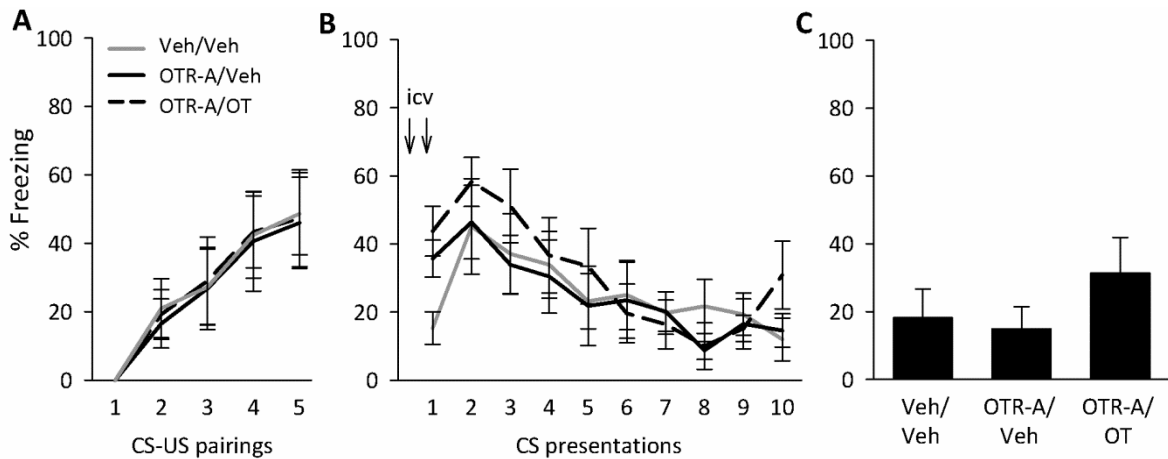


Figure 23. Oxytocin (OT) impairs fear extinction via OT receptor (OTR) in rats. (A) On day 1, rats were cued fear conditioned. (B) On day 2, 40 min before cued fear extinction, rats were infused icv with either vehicle (Veh; 5 μ l; n = 8) or OTR antagonist (OTR-A; 0.75 μ g/5 μ l; n = 16). Thirty minutes later, Veh-treated rats were infused again with 5 μ l Veh (Veh/Veh), while OTR-A-treated rats were infused with either Veh (OTR-A/Veh; 5 μ l; n = 8) or OT (OTR-A/OT; 1.0 μ g/5 μ l; n = 8). (C) On day 3, extinction recall was assessed. Data represent the mean time of CS-elicited freezing \pm SEM.

Effects of OT on home cage locomotion in rats and mice

To determine whether the doses of OT used for the cued fear experiments affect locomotion, separate groups of rats and mice were infused icv with either vehicle (rats: n = 8; mice: n = 6), a lower OT dose (rats: n = 7; mice: n = 7), or a higher OT dose (rats: n = 7; mice: n = 7) and home cage locomotion was measured immediately for 1 h.

There was no difference in home cage locomotion between groups in rats (Figure 24A). In mice, however, there was a significant difference between groups (Figure 24B), with the higher OT dose increasing locomotion compared with both vehicle (p =0.03) and the lower OT dose (p =0.01). The lower OT dose, however, did not affect home cage locomotion.

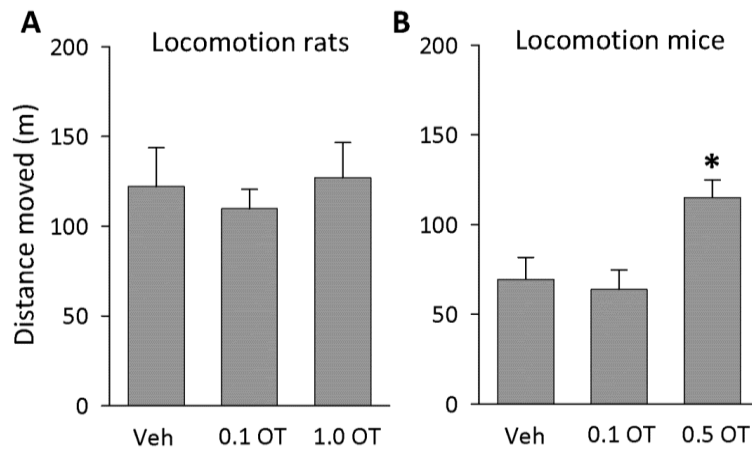


Figure 24. Oxytocin (OT) effects on home cage locomotion in rats (A) and mice (B).

Separate groups of rats and mice were infused icv with either vehicle (Veh; rats: 5 μ l; n = 8; mice: 2 μ l; n = 6), a lower OT dose (rats: 0.1 μ g/5 μ l; n = 7; mice: 0.1 μ g/2 μ l; n = 7), or a higher OT dose (rats: 1.0 μ g/5 μ l; n = 7; mice: 0.5 μ g/2 μ l; n = 7) immediately before home cage locomotion was monitored. Data represent the distance moved within 1 h \pm SEM.

*p<0.05 vs. Veh-treated mice.

DISCUSSION

The present study demonstrates that modulation of the central OT system affects cued fear extinction in a timepoint-dependent manner. In more detail, we could show that, when administered before cued fear conditioning, OT did not affect fear conditioning, but decreased fear expression during cued fear extinction and facilitated fear extinction. In contrast, OTR-A administered at the same time-point did not affect cued fear conditioning or fear expression, but impaired fear extinction. In contrast, when administered before cued fear extinction, OT impaired fear extinction, while OTR-A had no effect, suggesting a lack of involvement of the endogenous OT system at this timepoint. These findings could be observed both in rats and mice, indicating that the effects of OT on cued fear extinction are conserved across species, making the translation of these findings to humans more applicable. OT impaired cued fear extinction by binding to the OTR, as the inhibitory effect of icv OT on fear extinction was abolished by prior treatment with icv OTR-A. However, the impaired cued fear extinction was not a result of reduced locomotion, as neither rats nor mice showed changes in locomotion after OT treatment. These findings suggest that, while elevated OT levels at the

time of a traumatic event prevent the formation of aversive memories, caution is needed before recommending OT for the treatment of PTSD.

Preconditioning manipulation of the OT system

According to our hypothesis, preconditioning administration of OT decreased expression of cued fear and facilitated fear extinction, without directly affecting fear conditioning. In contrast, OTR-A administration impaired both fear extinction and extinction recall, indicating that an elevated activity of the endogenous OT during cued fear conditioning is required for successful fear extinction.

A possible explanation for these effects is the modulatory effect of OT on corticosterone (CORT) secretion. In female rats, chronic OT reduced stress-induced CORT release (Windle et al., 1997), while OTR-A increased CORT secretion into the blood in both male and female rats via an activation of the HPA axis (Neumann et al., 2000). Previous studies demonstrated that decreasing CORT concentration before conditioning by glucocorticoid synthesis inhibitors, such as metyrapone (Loscertales et al., 1997; Cordero et al., 2002) or dehydroepiandrosterone (Fleshner et al., 1997), or by blocking CORT activity through a glucocorticoid receptor antagonist (Cordero and Sandi, 1998) attenuated fear expression. Although CORT activation before exposure to tasks that involve acquisition of information has been shown to impair cognitive processing (Conrad et al., 1996; Kirschbaum et al., 1996; Lupien and McEwen, 1997), CORT release during the actual learning process facilitates cognitive processing (for reviews see Sandi, 1998; de Kloet et al., 1999). However, whether alterations in available CORT mediate the facilitatory effects of preconditioning OT on cued fear extinction remain to be verified.

As OT and OTR-A treatment did not alter cued fear conditioning itself, the observed effects on cued fear extinction are unlikely to be due to the antinociceptive properties of OT.

However, several studies have shown that the OT system modulates pain perception (Yang et al., 2007, 2011; Condés-Lara et al., 2009), with OT increasing and OTR-A decreasing the pain threshold in a dose-dependent manner (Uvnäs-Moberg et al., 1992; Lundeberg et al., 1994; Yang et al., 2011).

Although the mechanisms underlying the facilitatory effect of preconditioning OT on cued fear extinction are yet unknown, these findings suggest that activation of the endogenous OT system is beneficial during traumatic experiences to protect against the development of traumatic memory pathologies, such as PTSD.

Manipulation of the OT system before cued fear extinction

Contrary to our hypothesis, icv administration of OT before cued fear extinction impaired fear extinction as reflected by increased CS-elicited freezing. This was observed both in rats and mice, indicating that the inhibitory effects of OT on cued fear extinction are conserved across species. However, while we could show that the impairing effects of OT were mediated via the OTR as preadministration of an OTR-A blocked its effects, OTR-A treatment alone did not facilitate fear extinction, indicating that the endogenous OT system is not involved in cued fear extinction at this timepoint. The enhanced OT-induced freezing to the CS was tone-specific and not generalized as neither rats nor mice froze before tone onset nor did they show increased freezing responses to the tone prior to its association with the shock. Taken together, these results suggest that OT treatment before cued fear extinction delays the extinction of cued fear. Considering that cued fear extinction is regarded as a form of new learning (for reviews see Cammarota et al., 2007; Quirk et al., 2010), when animals learn that the CS no longer predicts the US, drugs that interfere with the acquisition of fear learning should also block the acquisition of extinction memories when administered before the extinction procedure (Myers and Davis, 2002). This might explain why OT decreased fear

expression and facilitated fear extinction when administered before cued fear conditioning and impaired fear extinction when administered before cued fear extinction.

We propose that CORT is a possible mediator of the pre-extinction effects of OT on cued fear extinction, similar to its preconditioning effects. While decreasing CORT concentrations before conditioning attenuates fear expression (Fleshner et al., 1997; Loscertales et al., 1997; Cordero and Sandi, 1998; Cordero et al., 2002), decreasing CORT concentrations before extinction by icv and BLA administration of metyrapone (Barrett and Gonzalez-Lima, 2004; Yang et al., 2006) blocks fear extinction. In contrast, glucocorticoid receptor agonists were shown to facilitate fear extinction when administered before the extinction procedure (Yang et al., 2006, 2007).

Several studies have shown that OT facilitated, rather than impaired, fear extinction when administered before the extinction procedure directly into the CeA (Roosendaal et al., 1992; Viviani et al., 2011), a brain region that coordinates the behavioral and physiological correlates of fear expression (LeDoux et al., 1988). In our study, however, OT was administered icv, which is likely to explain the discrepant results. While OT administered into the cerebral ventricles may reach the CeA, it may not do so in a concentration sufficient to facilitate fear extinction. Moreover, it is likely to reach brain areas which increase fear responses, such as the BLA. The BLA, a storage site for fear memories, is thought to mediate the initial acquisition of extinction (Herry et al., 2006, 2008; Sotres-Bayon et al., 2007) and the expression of extinction memory via inhibition of CeA output neurons (Quirk et al., 2003; Likhtik et al., 2008). However, whether OT impairs fear extinction when administered into the BLA remains to be verified.

In support of this region-dependent hypothesis, several studies have shown that OT facilitated the extinction of passive avoidance behavior, when applied either icv into the hippocampal

dentate gyrus or into the dorsal raphe nucleus immediately after the learning trial (Bohus et al., 1978; Kovács et al., 1979; de Wied et al., 1991). However, when applied into the dorsal septal nucleus, OT impaired the extinction of passive avoidance (Kovács et al., 1979), suggesting that OT affects extinction memory in a region-dependent manner. Although both passive avoidance and cued fear conditioning use foot shocks as the aversive sensory stimuli, several studies utilizing knockout mice have shown deficits in cued fear conditioning, while passive avoidance behavior was normal (Weeber et al., 2000; Takao et al., 2010; Kaidanovich-Beilin et al., 2009). The subtle differences between the two paradigms and the different timepoints of OT administration might also account for the different effects of central OT on extinction of cued fear versus passive avoidance behavior.

Despite previous studies showing that OT causes sedation at high doses in rats (Uvnäs-Moberg et al., 1994), neither dose of OT used in the present study altered home cage locomotion in rats. This indicates that the impairment of fear extinction by OT in rats is not due to nonspecific alterations in locomotion. In contrast, the higher OT dose employed in mice resulted in behavioral hyperactivity, defined as increased home cage locomotion and excessive scratching and grooming, confirming previous findings (Delanoy et al., 1979; Meisenberg and Simmons, 1982). This behavioral hyperactivity likely reflects the apparent decrease in fear expression and facilitation of fear extinction caused by the higher OT dose in mice as such behaviors would mask any underlying fear-related behaviors. However, the lower OT dose, which did not alter home cage locomotion, actually impaired fear extinction in mice. This is in agreement with the rat studies and strongly implies that OT administered before cued fear extinction has a detrimental outcome on fear extinction.

In summary, we have shown that icv OT decreases fear expression and facilitates fear extinction when administered before cued fear conditioning, which might have a beneficial effect during traumatic events. In contrast, when applied before cued fear extinction, which

would be the comparable time-point for psychotherapy in PTSD patients, OT delays fear extinction. Considering that a more specific and local administration of OT is not possible in patients, caution is needed before recommending OT for the treatment of PTSD.

Chapter 6

General discussion

6.1. Summary of results

The main goals of the present thesis were to develop a specific animal model of SAD and to assess the therapeutic efficacy of OT in reversing social and cued fear. In order to achieve this, I established the social fear conditioning paradigm (chapter 2; Toth et al., 2012b) in a way that is very similar in both the conditioning and extinction procedure with the cued fear conditioning paradigm described in the literature (Muigg et al., 2008; Toth et al., 2012a). The similarity between these two conditioning paradigms offers the possibility of comparing drug effects on conditioned fear in social versus non-social contexts. Afterwards, I administered OT to conditioned mice and/or rats and verified the potency of OT to reverse social (chapter 4; Toth et al., under review) and cued (chapter 5; Toth et al., 2012c) fear.

In chapter 2, I described the social fear conditioning paradigm in male mice, a model that shows both face and predictive validity to SAD. In more detail, I could show that social fear conditioning induces a specific and long-lasting (at least two weeks) social fear of unknown con-specifics without inducing other behavioral alterations that might account for the observed social fear, such as fear of novelty, general anxiety, depressive-like behavior, and impaired locomotion. Moreover, I could show that this social fear is reversed by acute diazepam and chronic paroxetine treatment, medication that currently provide the best response rates in SAD patients. Therefore, I propose the social fear conditioning model as an attractive tool to study the mechanisms underlying social fear, which will lead to better understanding of the etiology and treatment of SAD.

In chapter 3, I described step by step how to perform the the social fear conditioning paradigm in male and female rodents. I also described theoretical and practical aspects to be considered when performing the experiments, and how to analyze and interpret the obtained data.

In chapter 4, using the social fear conditioning paradigm to induce social fear, I could demonstrate that social fear is accompanied by alterations in the brain OT system at the level of the limbic system, namely by an increased OTR binding in the bilateral DLS, right CeA, right DG, and right CA1. I could also show that these alterations normalized after extinction of social fear, suggesting that OTR expression within these brain regions might be involved in the development and/or neural support of social fear. Moreover, I could show that while the endogenous OT system is needed for naturally-occurring social investigation, exogenous OT administered into the ventricular system reverses social fear through OTR-mediated actions. Additionally, based on the OTR alterations, I could localize these effects within the brain and demonstrate that the DLS mediates the effects of OT on social fear. These results demonstrate the therapeutic efficacy of OT in disorders associated with social anxiety and fear, such as SAD and possibly PTSD due to social trauma.

In chapter 5, using the cued fear conditioning paradigm to induce cued fear, I could show that modulation of the OT system differentially affects extinction of cued fear depending on the time-point of administration. In more detail, I could show that when administered before cued fear conditioning OT facilitates, whereas OTR-A impairs cued fear extinction 24 h later, indicating that an activated endogenous OT system during traumatic events is likely to attenuate formation of fear memories. In contrast, when administered before cued fear extinction, which would be the comparable time-point for psychotherapy in PTSD patients, OT impairs cued fear extinction, while OTR-A has no effect, suggesting a lack of involvement of the endogenous OT system at this time-point. As both the conditioning and extinction procedure involve learning, these results suggest that exogenous OT impairs learning processes that occur during non-social fearful situations and indicate that caution is needed before recommending OT for the treatment of PTSD due to non-social trauma.

In summary, after establishing a specific animal model of SAD, I provided evidence for a differential effect of synthetic OT on social versus cued fear in rodents. Not only could I show that OT has the potential to reverse social fear, while it rather delays extinction of cued fear, but I could also identify a part of the brain network involved in mediating OT effects on social fear, i.e. the DLS. These studies raise attention to the importance of the feared situation when recommending OT for the treatment of SAD and PTSD. Furthermore, the social fear conditioning model represents an attractive animal model for studying the brain mechanisms underlying social fear, which might lead to a better understanding of the etiology of SAD and, in turn, more specific medication for these patients.

6.2. Social fear conditioning as an animal model of SAD

Many psychiatric disorders are associated with social anxiety symptoms, which cannot be appropriately treated by current medication. Social anxiety might either represent the major symptom of the disorder, as seen in SAD, or a comorbid condition to other psychiatric disorders, as seen in depression and schizophrenia. In order to develop medications that specifically target and might be, therefore, more efficient in reversing social anxiety symptoms, animal models that induce specific social fear are needed to understand the basic mechanisms of how social anxiety develops. Several paradigms, including acute and chronic social defeat have been shown to induce lasting social avoidance and fear in rodents (see section 1.7.1.), and have contributed a great deal of knowledge regarding the mechanisms underlying social fear. However, they are rather unspecific with respect to the behavioral alterations that they induce, as increased general anxiety, depressive-like behavior, fear of novelty, and impaired locomotion were shown to accompany the induced social fear (see section 1.7.1.). Therefore, when using the social defeat paradigms, it is not clear whether social avoidance and fear are the major deficits induced, or whether they result as comorbid conditions from the above mentioned behavioral alterations. Unlike the social defeat

paradigms, the social fear conditioning paradigm described in chapter 2 and 3 induces a very specific type of social fear without these potentially confounding behavioral alterations. Importantly, all these behavioral measures were performed one day after social fear conditioning. Given that at least one other psychiatric disorder is present in the majority of SAD patients, such as agoraphobia (Magee et al., 1996), depression (Schneier et al., 1992; Regier et al., 1998), or substance abuse (Schneier et al., 2010; Regier et al., 1998), and that SAD generally precedes all these disorders (see section 1.2.), it is not excluded that other behavioral alterations might occur in conditioned mice several weeks after social fear conditioning. This is an attractive aspect that has not been investigated in the present thesis and needs further elucidation. An interesting question that arises is whether treating social anxiety symptoms might improve general anxiety and depression symptoms in an animal model like chronic social defeat or such symptoms that might develop in conditioned mice several weeks after social fear conditioning.

6.3. Relevance of the social fear conditioning model for future research

Although the specificity of the behavioral effects and the validity of the social fear conditioning model have been repeatedly discussed in the present thesis, I would like to highlight several additional advantages of the social fear conditioning paradigm. First, the simplicity of the paradigm needs mentioning. While chronic social defeat (Avgustinovich et al., 2005; Berton et al., 2006) requires defeating animals repeatedly over several days by different dominant aggressive males, social fear conditioning is performed within a few minutes using a single stimulus animal. The behavioral impairment, however, is as severe as when induced by chronic social defeat, and it lasts for at least two weeks (chapter 2). This long-term maintenance of social fear offers not only the possibility to test medication with fast, but also with a delayed onset of action, such as antidepressants (Katz et al., 2006;

Mitchell, 2006; Priest, 2006). Additionally, the sensitization of the induced social fear over time offers the possibility to assess acute drug effects on a more severe type of social fear.

Second, the social fear conditioning paradigm closely resembles the cued fear conditioning paradigm with respect to both the conditioning and extinction procedure. These similarities between the two conditioning paradigms offers the possibility for comparison between conditioned fear in social versus non-social contexts, being at the level of neuronal circuitry, underlying molecular mechanisms, or therapeutic approaches. However, these paradigms also differ in at least three aspects, namely in the type of learning they imply, in the controllability of the aversive stimuli, and in the rewarding properties of the conditioned stimuli. The type of learning they imply, namely learning the association between a behavior and its consequence, i.e. operant conditioning (Thorndike, 1933) in the case of social fear conditioning, and learning the association between two stimuli, i.e. classical conditioning (Pavlov, 1927) in the case of cued fear conditioning, might recruit slightly different neuronal circuitries. The controllability of the aversive stimuli, namely the possibility to avoid the foot shocks by stopping to approach the social stimulus during social fear conditioning, and the impossibility to avoid the foot shocks during cued fear conditioning, might also recruit different neuronal circuitries. The rewarding properties of the conditioning stimuli, namely highly rewarding social stimuli versus less rewarding cued stimuli, might influence the speed of extinction, as observed in the present studies, with extinction of social fear occurring faster than extinction of cued fear. It is possible, therefore, that differences in drug sensitivity and underlying mechanisms not necessarily related to the type of fear per se, but rather due to the above mentioned differences, might occur when studying social and cued fear by using these two paradigms.

Third, the reliability of the social fear conditioning paradigm needs mentioning, as both conditioned and unconditioned mice show highly stable levels of social investigation between

experiments. Although the model needs to be replicated in other laboratories to demonstrate its transferability, the robustness of the induced social fear suggests that the social fear conditioning paradigm might be successfully reproduced in different laboratories.

Another important aspect that needs mentioning is the possibility to use the social fear conditioning paradigm not only in male rodents, as demonstrated in this thesis, but also in females (Figure 25). Although in several rodent species, such as California mice (*Peromyscus californicus*) and Syrian hamsters (*Mesocricetus auratus*), females show aggressive territorial behavior similar to males, in most rodent species females are not territorial and do not typically form dominant-subordinate relationships with other females. Therefore, social defeat models which are based on territoriality and establishment of dominant-subordinate relationships cannot be reliably used in females. Under certain circumstances, such as when defending their pups female rats and mice show high levels of aggressive behavior (Lonstein and Gammie, 2002; Bosch and Neumann, 2012), which can be used as a model of social defeat in females. However, exposure to repeated maternal defeat induces a rather unspecific socially fearful phenotype in defeated females. Although it does not increase general anxiety, as the chronic social defeat in males, repeated maternal defeat induces a depressive-like behavior in defeated females (Shimamoto et al., 2011; Bourke and Neigh, 2012). The behavioral phenotype induced by the social fear conditioning paradigm, on the other hand, is very specific and comparable to the behavioral phenotype induced in males, which allows its reliable use in females as well. Given the higher prevalence of anxiety disorders including SAD in women (Schneier et al., 1992; Talepasand and Nokani, 2010), and the different responses to medication between men and women (Franconi et al., 2007; Keers and Aitchinson, 2010), the etiology of anxiety disorders and the efficacy of medication needs to be studied both in male and female rodents.

Importantly, all the studies using the social fear conditioning paradigm described in this thesis and performed so far induced only male-male (chapter 2 and 4) or female-female (Figure 25) social fear, in order to exclude any sexually-motivated behaviors. However, given that most SAD patients fear and avoid individuals of both sexes, further studies need to investigate the underlying mechanisms and treatment responses in rodents conditioned to the opposite sex.

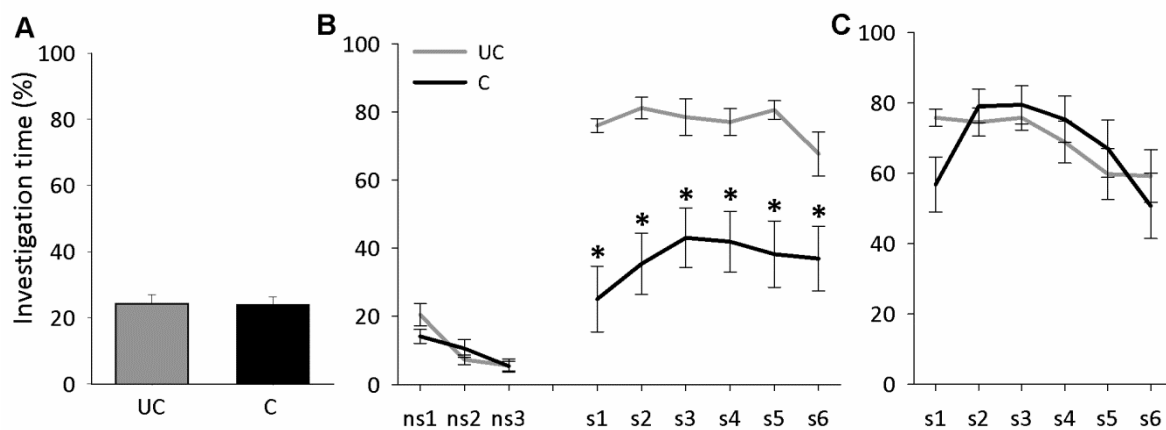


Figure 25. Social fear conditioning induces short-term social fear in female CD1 mice. (A) Investigation of the non-social stimulus (empty cage) by unconditioned (UC) and conditioned (C) mice during social fear conditioning on day 1 ($n = 12$ per group). (B) Investigation of non-social (ns1-ns3) and social (cages with female CD1 mice; s1-s6) stimuli during social fear extinction on day 2 (3 min exposure to stimulus, 3 min inter-exposure interval). (C) Investigation of social stimuli (s1-s6) during extinction recall on day 3. Data represent mean percentage of investigation time \pm SEM. * $p < 0.05$ vs. UC mice.

6.4. Effects of central OT system manipulation on social and cued fear

6.4.1. Effects of exogenous OT on social and cued fear

Having established the social (chapter 2; Toth et al., 2012b) and cued (Toth et al., 2012a) fear conditioning paradigms in our laboratory, in chapter 4 and 5 I assessed the therapeutic efficacy of exogenous OT in social and cued fear, respectively, given its pro-social, anxiolytic, and stress-attenuating effects and its potential therapeutic role in SAD and PTSD (see section 1.6.). In more detail, I could demonstrate that icv administered OT before social fear extinction has the potential to reverse social fear. On the other hand, icv administered OT

at this same time-point in the cued fear conditioning paradigm impaired extinction of cued fear, suggesting that OT has differential effects on social versus cued fear when administered before the extinction procedure. These results indicate that while OT might be a promising therapeutic approach in patients with social fear, such as SAD or PTSD due to social trauma, caution is needed before recommending OT for the treatment of PTSD due to non-social trauma. However, the time-point of OT administration in the cued fear conditioning study is an important aspect that needs to be considered. When translated to humans, the administration of OT before the extinction procedure in rodents would correspond to its administration before psychotherapy in PTSD patients, which, based on my results in rats and mice, should not be recommended due to the delaying effects of OT on cued fear extinction. However, drugs can also be administered after the psychotherapy session in PTSD patients to facilitate the consolidation of the “safety” memory trained during psychotherapy, which implies that OT might decrease cued fear responses when administered immediately after a short extinction procedure. In support of this possibility, I have shown that the mGluR7 allosteric agonist N,N'-dibenzylhydriyl-ethane-1,2-diamine dihydrochloride (AMN082) delays cued fear extinction when administered i.p. before extinction, while it decreases fear expression when administered immediately after a short extinction procedure (Toth et al., 2012a). A similar approach might be considered before contra-indicating OT as an adjuvant in the treatment of PTSD due to non-social trauma.

6.4.2. Effects of exogenous OT on learning

The impairing effect of icv administered OT on cued fear extinction suggests that OT might either prolong the expression of the conditioned fear response or interfere with the new learning process that occurs during the extinction procedure (Cammарota et al., 2007; Quirk et al., 2010). In an attempt to verify which of these hypotheses is true, I could demonstrate that OT decreases fear expression and facilitates cued fear extinction when administered before

cued fear conditioning. This suggests that OT impairs the learning processes that occur not only during extinction, but also during acquisition of cued fear. In support of my results, although not completely comparable due to the local administration, OT impaired fear learning when administered into the ventral hippocampus before acquisition of active avoidance behavior; i.e. learning to exit from a chamber in which an aversive stimulus was delivered (Ibragimov, 1990). On the other hand, OT might either have no effect on or even facilitate learning in social contexts. In more detail, in chapter 4 I have shown that icv administered OT completely reversed social fear starting from the first exposure to a social stimulus and did not gradually facilitate extinction, suggesting that OT is unlikely to reverse social fear by facilitating learning. In non-fearful social contexts, however, OT was shown to be both needed for and to facilitate social learning and memory. In more detail, icv administered OT facilitated (Benelli et al., 1995), whereas OTR-A impaired (Engelmann et al., 1998; Lukas, Toth et al., in press) recognition of a previously encountered con-specific. Furthermore, both OT and OTR knockout mice show deficits in recognition of previously encountered con-specifics, deficits which could be reversed by icv administered OT before, but not immediately after, the initial social encounter (Ferguson et al., 2000; Choleris et al., 2003; Takayanagi et al., 2005). These effects were mediated through the OTR in OT knockout mice, and through the AVP receptor 1A in OTR knockout mice (Sala et al., 2011). All these studies indicate that OT is needed for social learning and memory in non-fearful contexts. However, whether OT exerts different effects on social versus non-social learning in fearful contexts needs further elucidation and implies administration of OT before social fear conditioning.

6.4.3. Effects of endogenous OT on social and cued fear

As all the behavioral effects described above were mediated via OT actions on the OTR, i.e. administration of OTR-A before OT blocked OT effects on both social and cued fear, the

involvement of the endogenous OT system in social and cued fear needs mentioning. In the context of social fear conditioning, I have shown that icv administered OTR-A decreased social investigation in unconditioned mice (chapter 4). This suggests that endogenous OT is required for naturally-occurring social investigation in unconditioned mice and confirms our previous study in male rats and mice (Lukas et al., 2011). Importantly, even though social investigation decreased in unconditioned mice when the activity of the endogenous OT system was blocked, I observed no typical signs of social fear, such as freezing, attempt approaches, and defensive burying, which suggests that the endogenous OT system modulates social preference and motivation, but not social fear. Importantly, the endogenous OT system does not appear to regulate social investigation in conditioned mice, probably due to the socially-fearful phenotype and to the already low level of social investigation. In the cued fear conditioning, however, blockade of the endogenous OT system before cued fear conditioning impaired fear extinction, whereas before cued fear extinction had no affect on fear extinction. These results indicate that, at least in non-social contexts, the endogenous OT system plays a crucial role during traumatic experiences and protects against the development of traumatic memory pathologies. After the development of traumatic memories, however, the endogenous OT system has little modulatory effect on both social and cued fear memory. An interesting aspect that needs further elucidation is whether the endogenous OT system might also protect against the development of traumatic memory pathologies in social contexts and implies administration of OTR-A before social fear conditioning.

6.5. Neurocircuitry of social and cued fear

The differential role of the OT system in modulating social and cued fear goes beyond differential effects on behavior and learning processes, and might be more complex than outlined above. Although the brain regions involved in mediating the effects of OT on social and cued fear are quite similar, the directionality of OT effects within these brain regions

differs (Figure 26). Substantial research has been performed to identify the neuronal circuitry involved in social fear; however, little is known from the literature about the brain regions that actually mediate the effects of OT on social fear. In chapter 4, I have shown that social fear is accompanied by alterations in the OT system, namely by an increased OTR binding in the bilateral DLS, right CeA, and right hippocampus (DG, CA1). These alterations normalized after extinction of social fear, and suggest that OTR expression and, therefore, OT neurotransmission within this limbic network might be involved in the development and/or neural support of social fear. In support of this hypothesis, pharmacological induction of social avoidance by chronic administration of phencyclidine was shown to increase OTR binding in the CeA (Lee et al., 2005), whereas chronic social defeat increased OTR mRNA expression in the LS of defeated mice (Litvin et al., 2011). The involvement of the LS in mediating OT effects on social fear is strengthened by the fact that OT reversed social fear when administered into the DLS of conditioned mice (chapter 4). Although the DLS and CeA showed the same pattern of OTR binding alterations, OT did not reverse social defeat-induced social fear when administered into the CeA (Lukas et al., 2011). Two possible theories might explain the lack of OT effects within the CeA of acutely social defeated rats, namely the possible region-dependent effects of OT on social fear and the severity of the induced social fear. The possible region-dependent effects of OT on social fear suggests that OT might exert different effects within distinct brain regions that mediate its effects on social fear, and would imply that OT reverses social fear when administered into the DLS, but not when administered into the CeA. This hypothesis is supported by the fact that OT exerts differential effects on extinction of passive avoidance, i.e. refraining from entering a chamber in which an aversive stimulus was previously delivered, when administered into the CeA, hippocampal DG, dorsal raphe nucleus, and the LS (see below; Kovács et al., 1979; Roozendaal et al., 1992; Viviani et al., 2011). The severity of the induced social fear, namely fear of general

social stimuli in the case of social fear conditioning and fear of a specific individual in the case of acute social defeat, might involve recruitment of different brain regions to mediate the effects of OT. This hypothesis is supported by the fact that additional brain regions are progressively recruited as the complexity of behaviors increase to encode the processed information (Fanselow and Ponnusamy, 2008), and would imply that the CeA mediates the effects of OT on a general and, therefore, more severe type of social fear, such as that induced by social fear conditioning, but not on an individual type of social fear induced by acute social defeat. These hypotheses are not mutually exclusive, but whether OT reverses social fear induced by social fear conditioning when administered into the CeA remains to be verified.

In contrast with the limited knowledge about the brain regions that mediate the effects of OT on social fear, much more is known about the brain regions that mediate the effects of OT on cued fear. Thus, transgenic mice with decreased OTR binding within the LS, CeA, and hippocampus after post-natal day 21 show decreased fear responses during cued fear conditioning (Lee et al., 2008; Pagani et al., 2011). Several studies using the cued fear conditioning and passive avoidance paradigms have also demonstrated that the CeA (Roozendaal et al., 1992; Viviani et al., 2011), hippocampal DG, dorsal raphe nucleus, and the dorsal septum (Kovács et al., 1979) mediate OT effects on cued fear. Interestingly, differential effects were found when OT was administered into these regions, i.e. OT facilitated extinction of cued fear and passive avoidance when administered into the CeA (Roozendaal et al., 1992; Viviani et al., 2011), hippocampal DG, and into the dorsal raphe nucleus (Kovács et al., 1979), but impaired extinction of passive avoidance when administered into the dorsal septum (Kovács et al., 1979). This not only suggests that quite similar brain regions modulate the effects of OT on social versus cued fear, but that OT affects cued fear extinction in a region-dependent manner. Probably the most striking

difference is the fact that OT differentially modulates social and cued fear at the level of the LS, i.e. reverses social fear (chapter 4), but impairs cued fear extinction (Kovács et al., 1979). Behavioral differences also occur when OT is infused into the CeA, i.e. OT does not reverse social defeat-induced social avoidance (see above; Lukas et al., 2011), but facilitates cued fear extinction (Roozendaal et al., 1992; Viviani et al., 2011). Future studies will assess the effect of CeA and DG administered OT on social fear induced by social fear conditioning, and the involvement of the dorsal raphe nucleus in social fear.

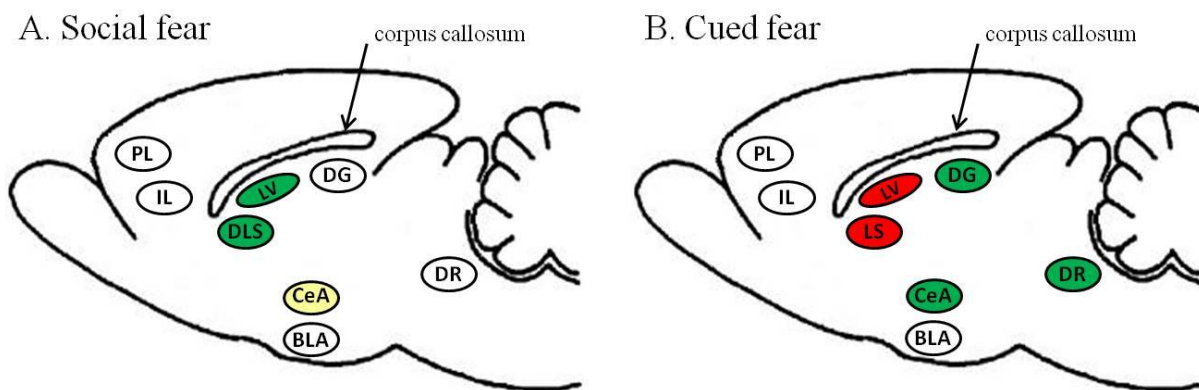


Figure 26. Diagram depicting the effects of exogenous oxytocin (OT) within brain regions mediating social (A) and cued (B) fear. Brain regions where OT infusion dampens (green), promotes (red), or does not affect (yellow) fear expression. Brain regions depicted in white represent regions involved in fear expression where OT was not administered to date. PL, prelimbic cortex; IL, infralimbic cortex; DLS, dorso-lateral septum; LS, lateral septum; LV, lateral ventricle; DG, dentate gyrus; CeA, central amygdala; BLA, basolateral amygdala; DR, dorsal raphe nucleus. Summarized from Kovács et al., 1979; Roozendaal et al., 1992; Lukas et al., 2011; Viviani et al., 2011; Toth et al., 2012c; chapter 4.

6.6. A possible mechanism underlying OT effects on social and cued fear

Although the behavioral effects of OT on social and cued fear assessed so far are quite straightforward, the underlying mechanisms of these effects are not clear yet. However, considering the behavioral data obtained in the present thesis, I could at least demonstrate that the effects observed on social and cued fear are unlikely due to the general anxiolytic properties of OT, as general anxiety was not affected by the doses of OT used in the fear

conditioning experiments (Figure 19; Slattery and Neumann, 2010). A possible mechanism that might explain the effects of OT on both social and cued fear is through the modulatory effect on CORT secretion. While chronic OT has been shown to reduce stress-induced CORT release in female rats (Windle et al., 1997), OTR-A increased CORT release in both male and female rats (Neumann et al., 2000). As high CORT levels during learning processes facilitate cognitive processing (Sandi, 1998; de Kloet et al., 1999), and OT impairs the learning processes that occur during both cued fear conditioning and extinction, this hypothesis seems plausible for the effects of OT on cued fear. In the case of social fear, it has been suggested that by reducing the behavioral and neuroendocrine responses to social stress, OT may inhibit defensive behaviors and enable animals to overcome their natural avoidance of proximity (Carter and Altemus, 1997; Carter, 1998; Unväs-Moberg, 1998). If this is the case, it seems likely that by reducing CORT release prior to social fear extinction, OT facilitates social approach, and thereby reverses social fear.

Another aspect that needs mentioning is the apparent lateralization of the OTR binding alterations found in socially-fear conditioned mice, which is difficult to interpret at the moment due to the sparse evidence in rodents, and the amygdala-focused research in humans. Although neuroimaging studies have provided substantial evidence for the amygdala as one of the core regions involved in mediating the fear response in the human brain, the contribution of the two hemispheres in the fear response is not clear yet. While some studies report increased activity in the right, but not the left amygdala during fear conditioning in healthy volunteers (Furmark et al., 1997), and symptom provocation in SAD (Tillfors et al., 2001), specific phobia (Veltman et al., 2004; Ahs et al., 2009), and PTSD (Pissiota et al., 2002) patients, other studies report increased activity both in the right and the left amygdala (Schienle et al., 2007; Petrovic et al., 2008b; Flemingham et al., 2010; Labuschagne et al.,

2010; Brühl et al., 2011; Wood et al., 2012). A recent study in mice has shown that observational fear learning (see section 1.1.) is controlled by the right, but not the left anterior cingulate cortex (Kim et al., 2012), suggesting that asymmetrical hemispheric mechanisms are underlying fear learning. Importantly, although I found significant increases in OTR binding only in the right CeA and hippocampus (DG, CA1) of conditioned mice, the direction of alterations was similar in both hemispheres, however, without reaching significance in the left hemisphere. Although it has been proposed that the right hemisphere controls aversive conditioning (Hugdahl, 1995), it is not yet clear whether the left-right differences found in the human amygdala activity and in the OTR distribution in the CeA and hippocampus of conditioned mice might reflect a functional segregation between the left and right brain regions in mediating fear and aversively-motivated behaviors.

Probably the most intriguing aspect is that although the effects of OT on human behavior and amygdala activity have been repeatedly shown (see section 1.6.), the only OTR autoradiography studies in post-mortem human brains reported high densities of OTR in the VLS, less in the DLS, but none in the hippocampus and amygdala (Loup et al., 1989, 1991). Given the similarity of the behavioral effects of OT between humans and rodents, it might be possible that in humans the behavioral effects of OT are mediated by OTR found in brain regions upstream of the hippocampus and amygdala. However, considering that the distribution of the OTR is quite well preserved between mammalian species (O'Connell and Hofmann, 2012), the assessment of OTR distribution with more sensitive techniques, such as radioligand binding studies in combination with positron emission tomography (PET) or single photon emission computed tomography (SPECT), might prove useful to gain more accurate information about the distribution of OTR in the human brain.

6.7. Relevance of the present thesis

The results described in this thesis are highly relevant due to the increasing interest in using OT in combination with cognitive-behavioral therapy in SAD and PTSD patients. Human studies demonstrated the efficacy of OT in reducing conditioned social fear in healthy volunteers (Petrovic et al., 2008a) and some symptoms of social anxiety in psychiatric disorders, such as SAD (Guastella et al., 2008; Labuschagne et al., 2010), autism spectrum disorder (Baron-Cohen et al., 2001; Andari et al., 2010), schizophrenia (Feifel et al., 2010; Goldman et al., 2011), and fragile X syndrome (Hall et al., 2012). The similarity between the behavioral effects observed after intranasal administration of OT in humans and icv administration of OT in conditioned mice, as shown in the present thesis, increases the possibility that similar brain circuitries and molecular mechanisms underlie the social deficits found in patients and in social fear conditioned rodents. The social fear conditioning model might bring us closer to understanding the etiology of SAD, identifying the neural circuitries altered in SAD, the molecular mechanisms underlying this disorder, and hopefully more specific medication for SAD patients.

The results described in this thesis demonstrate that the DLS and possibly CeA and hippocampus are important brain region mediating OT effects on social fear in rodents. To my knowledge, there are no human studies investigating the involvement of the septum or the hippocampus in social fear. Given the possibility of intranasal administration of OT in humans, my results provide a strong rationale for future studies to determine the involvement of these brain regions in social anxiety and fear in humans.

In conclusion, with this thesis, I raised attention to the importance of the feared situation when recommending OT for the treatment of SAD and PTSD and to the implications of the time-point of OT administration in report to psychotherapy. I also tried to contribute to a

better understanding of the etiology of SAD by identifying alterations in the OT system that might serve as possible therapeutic targets for SAD.

6.8. Perspective for future studies

The findings described in this thesis open up a number of additional research questions, which will lead to a better understanding of the etiology of SAD and PTSD and of the underlying mechanisms responsible for the differential effects of OT on social and cued fear. These future studies can be divided into the following sections:

- Icv manipulation studies to further elucidate the effects of the OT system on social and cued fear

Given that an activated OT system during cued fear conditioning attenuates fear learning in a non-social context (Figure 20), administration of OT and OTR-A before social fear conditioning would determine whether manipulation of the OT system might also affect fear learning in a social context. As blockade of fear consolidation attenuates subsequent fear expression, administration of OT and OTR-A immediately after social and cued fear conditioning would determine whether manipulation of the OT system affects fear consolidation in social and non-social contexts. Although OT impaired cued fear extinction when administered before the extinction procedure (Figure 21 and 22), administration of OT immediately after a short extinction procedure would determine whether OT facilitates the consolidation of extinction memory, and, thereby, might represent a relevant therapeutic approach in patients with PTSD due to non-social trauma.

Given that OT reverses social fear starting from the first exposure to a social stimulus (Figure 14), it is not clear whether OT impairs expression and/or recall of social fear, or completely erases the fear memory. Administration of OT 24 h after social fear conditioning, followed by the social fear extinction procedure 24 h later, would reveal whether OT erases the fear

memory. This approach would also reveal whether exposure to social stimuli is required for OT to reverse the social fear. Alternatively, OT might induce a rapid extinction of social fear as a result of social exposure, which might be observed only by analyzing multiple time bins in the first 3 min of exposure to a social stimulus.

- OTR binding studies in socially- and cued-fear conditioned animals

To compare the alterations at the level of the OT system in socially- and cued-fear conditioned animals, it would be important to perform a similar OTR binding study in the cued fear conditioning experiments as described in chapter 4. This study might reveal differential alterations in OTR expression induced by social and cued fear in brain regions involved in the fear circuitry, and might also identify additional brain regions that might mediate the effects of OT on cued fear.

- Local manipulation studies to further elucidate the effects of the OT system on social and cued fear

Given that the OT system has region-dependent effects on cued fear (section 6.5.; Figure 26), future studies should locally manipulate the OT system in the regions of interest revealed by the OTR binding study mentioned above, to further understand the modulatory effects of the OT system on cued fear. Additionally, local manipulations of the PL, IL, and BLA (Figure 26) are needed to determine whether and how OT affects cued fear at the level of these brain regions. As OT administered icv and into the DLS before the extinction procedure has differential effects on social versus cued fear, it would be important to perform these local manipulations in both socially- and cued-fear conditioned animals. Similar to the icv manipulations mentioned above, it would be important to consider different time-points of local manipulation, i.e. before and after fear conditioning, and before fear extinction, to provide more detailed information about the role of these brain regions in mediating the

effects of OT on different stages of social and cued fear learning and memory, i.e. acquisition, consolidation, expression, and extinction.

- OT release and signaling studies in socially- and cued-fear conditioned animals

The significance of the OTR up-regulation in socially-fear conditioned mice is not clear so far, however, it might represent a compensatory mechanism for a decreased local OT release and, thus, extracellular availability, and/or for an impaired OTR signaling. Therefore, measuring and comparing OT release between unconditioned mice and conditioned mice before and after extinction of social fear in brain regions that mediate the effects of OT on social fear, such as those revealed in chapter 4, i.e. the DLS, and possibly the CeA, DG, and CA1, would provide important information about the extracellular availability of OT in social fear. A similar OT release study should be employed in the cued-fear conditioned animals in brain regions that mediate OT effects on cued fear, such as the LS, CeA, and DG (Figure 26), but also in brain regions revealed by the OTR binding and local manipulation studies mentioned above.

Given the role of extracellular signal-regulated kinases (ERK) in learning and memory (Davis and Laroche, 2006), social behavior, and anxiety-related behavior (Sato et al., 2011; Jurek et al., 2012), and the fact that OT has been shown to activate this pathway (Jurek et al., 2012), investigation of the c-Raf - MEK1/2 - ERK1/2 mitogen-activated protein kinase (MAPK) signaling cascade in relevant brain regions (see above) in conditioned mice might provide more detailed information about OTR signaling in social fear, and might explain the differences in OTR expression found in these mice. An interesting question that arises is whether OTR expression and signaling is differentially affected by social and cued fear, which might explain the differential behavioral effects of central OT on social and cued fear.

- Genetic and viral studies

Although OTR knock-out mice do not show any alterations in social or cued fear when compared with OTR heterozygous or wild-type mice (Toth et al., unpublished), probably due to compensatory mechanisms, the use of OT knock-out mice for the social and cued fear conditioning experiments is appealing, given the effects of OT described in this thesis. It would be important to determine whether OT knock-out mice show altered social and cued fear learning and extinction, and whether these possible deficits might be rescued by icv and local OT. Given the possibility to up-regulate OTR expression by using the AAV-OTR-IRES-Venus vector (Sato et al., 2009), it would be interesting to determine whether social fear might be induced in naïve mice by up-regulating OTR in the DLS, CeA, DG, and CA1, as OTR were up-regulated in these brain regions in conditioned mice. It would be also important to verify whether the alterations in social and cued fear learning and extinction which might be observed in OT knock-out mice might be rescued with this viral vector.

- Studies to elucidate the effects on OT on different types of social fear

A relevant aspect concerning OT effects on social fear is whether these effects depend on how social fear was acquired, and, thus, how severe the social fear is, i.e. fear of a specific individual and no additional behavioral deficits in the case of acute social defeat, fear of general social stimuli and no additional short-term behavioral deficits in the case of social fear conditioning, and fear of general social stimuli and severe additional behavioral deficits in the case of chronic social defeat. Although icv administered OT reversed both acute social defeat- (Lukas et al., 2011) and social fear conditioning- (chapter 4) induced social fear, the effects of OT on chronic social defeat-induced social fear have not been assessed so far. The question arises whether treating the social fear might also improve general anxiety and depression symptoms observed in chronically social-defeated rodents. Furthermore, as the severity of

social fear increases, additional brain regions might be recruited to process the increasing amount of information and/or functional alterations might appear in an increasing number of brain regions involved in the fear circuitry. A comparative study between social fear induced by social fear conditioning, acute and chronic social defeat, implying icv and local manipulation of the OT system, assessment of OTR expression and signaling, as described above, would provide important information about the therapeutic potency of OT and the neurocircuitry involved and/or altered in social fear depending on the severity of symptoms.

- Studies to further characterize the social fear conditioning paradigm

Given that in SAD patients at least one other psychiatric disorder is present, and that SAD generally precedes all these disorders (see section 1.2.), it would be important to verify whether conditioned mice show additional behavioral deficits, such as increased general anxiety or depressive-like behavior, several weeks after social fear conditioning.

Although conditioned mice show intense social fear, it is not clear so far which aspect(s) of the social stimulus, i.e. sight, smell, sound, movement, or all together, trigger(s) the fear response in these mice. Encaging the stimulus mice in sound- and smell-isolated transparent cages would reveal whether social fear is triggered by the sight of a con-specific. By using a con-specific odor, recordings of con-specifics, i.e. positive and/or negative ultrasound vocalizations (Knutson et al., 2002), or anesthetized mice as social stimuli, it could be revealed whether the smell, the sound, and/or the movement of a con-specific are/is necessary to trigger the fear response.

Given that the social fear conditioning studies performed so far induced only male-male (chapter 2 and 4) and female-female (Figure 25) social fear, and that most SAD patients fear and avoid individuals of both sexes, future studies could investigate social fear in rodents conditioned to the opposite sex. These studies might also investigate the connection between

social fear and the reward system, given the sexually-motivated behaviors. Further studies might investigate the underlying mechanisms and treatment responses in rodents conditioned to the opposite sex.

- Comparative studies between male and female rodents

Given the sexual dimorphism of the OT system (Uhl-Bronner et al., 2005) and the fact that OT has been shown to reverse the acute social defeat-induced social fear in male (Lukas et al., 2011), but not in female (Lukas et al., unpublished) rats, comparative social and cued fear conditioning studies between male and female rodents are appealing. These studies should imply icv and local manipulation of the OT system at different time-points during the conditioning procedure, assessment of OTR binding and signaling (see above), and would provide important information about the involvement of the OT system in social and cued fear in both sexes.

- Studies on other possible targets to treat social and cued fear

Additional therapeutic approaches in social and cued fear might involve manipulation of the AVP and corticotropin releasing hormone (CRH) systems. Administration of AVP receptor 1b antagonists might prove relevant, given that OT and AVP often exert opposite effects on behavior (Roozendaal et al., 1992; Thompson and Walton, 2004), and that these antagonists have been shown to attenuate the chronic social defeat-induced social fear (Litvin et al., 2011). Administration of CRH receptor type 1 antagonists might be relevant, since they have been shown to reduce the behavioral and endocrine responses to stressors (French et al., 2007; Ising and Holsboer, 2007), and to exert anxiolytic effects (Ising and Holsboer, 2007). Administration of NPS might also be interesting, since this neuropeptide has been shown to exert anxiolytic effects and to reduce fear responses by facilitating fear extinction and attenuating contextual fear (Pape et al., 2010).

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Abbreviations

| | |
|--------|---|
| AMN082 | N,N'-dibenzylhydriyl-ethane-1,2-diamine dihydrochloride |
| ANOVA | analysis of variance |
| AVP | arginine vasopressin |
| BA | basal amygdala |
| BLA | basolateral amygdala |
| BNST | bed nucleus of the stria terminalis |
| C | conditioned mice |
| CA | closed arms of the elevated plus-maze |
| CA1 | cornu ammonis 1 |
| CA3 | cornu ammonis 3 |
| CC | cingulate cortex |
| CeA | central amygdala |
| CORT | corticosterone |
| CRH | corticotropin releasing hormone |
| CS | conditioned stimulus |
| DG | dentate gyrus |
| Dia | diazepam |
| DLS | dorso-lateral septum |
| dmPFC | dorsal division of the medial prefrontal cortex |
| DSM | Diagnostic and Statistical Manual of Mental Disorders |
| EPM | elevated plus-maze |
| ERK | extracellular signal-regulated kinase |
| FST | forced swim test |
| GABA | gamma-aminobutyric acid |
| HPA | hypothalamic-pituitary-adrenal |

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|-------|---|
| i.e. | latin <i>id est</i> , meaning “that is” |
| i.p. | intraperitoneal |
| icv | intracerebroventricular |
| IL | infralimbic cortex |
| ITC | intercalated cell masses |
| LA | lateral amygdala |
| LS | lateral septum |
| MAPK | mitogen-activated protein kinase |
| MeA | medial amygdala |
| mPFC | medial prefrontal cortex |
| MPOA | medial preoptic area |
| mRNA | messenger ribonucleic acid |
| n.a. | not analysed |
| n.s. | not significant |
| NAc | nucleus accumbens |
| NPS | neuropeptide S |
| ns | non-social stimulus |
| NSI | non-social investigation |
| OA | open arms of the elevated plus-maze |
| OT | oxytocin |
| OTR | oxytocin receptor |
| OTR-A | oxytocin receptor antagonist |
| Par | paroxetine |
| PET | positron emission tomography |
| PL | prelimbic cortex |
| PTSD | post-traumatic stress disorder |
| PVN | paraventricular nucleus |

| | |
|-------|--|
| s | social stimulus |
| s.c. | subcutaneous |
| SAD | social anxiety disorder |
| SEM | standard error of the mean |
| SI | social investigation |
| SON | supraoptic nucleus |
| SPECT | single photon emission computed tomography |
| SSRI | selective serotonin reuptake inhibitor |
| UC | unconditioned mice |
| US | unconditioned stimulus |
| Veh | vehicle |
| VLS | ventro-lateral septum |
| vmPFC | ventral division of the medial prefrontal cortex |

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Curriculum Vitae

Personal information

Date of birth: 15 April 1984
Place of birth: Timisoara, Romania
Nationality: Romanian

Education

| | |
|--------------|--|
| 2009 to date | PhD student in Neurobiology Department of Behavioral and Molecular Neurobiology, University of Regensburg. Supervisors: Prof. Dr. Inga Neumann, Dr. David Slattery |
| 2007 – 2009 | M.Sc. in Experimental and Clinical Neurosciences (ECN) University of Regensburg. Master thesis: Role of oxytocin in social memory and social approach behavior in male C57BL/6 mice. Overall grade: 1.2 |
| 2002 – 2006 | B.Sc. in Biology Western University of Timisoara Overall grade: 9.75 (scale 5-10; 10 maximum) |
| 1998 – 2002 | Colegiul National Banatean High-school, Timisoara |
| 1994 – 1998 | Scoala Generala nr. 18 Gymnasium, Timisoara |
| 1990 – 1994 | Nikolaus Lenau Elementary School, Timisoara |

Awards / Scholarships / Fellowships

| | |
|--------------|--|
| October 2012 | Young Scientists Award - ECNP, Vienna, Austria |
| Sept 2012 | EBBS Fellowship - Frontiers in Stress and Cognition: From Molecules to Behavior, Ascona, Switzerland |
| March 2012 | Travel Award to ECNP Workshop on Neuropsychopharmacology, Nice, France |
| 2009 - 2012 | Graduate Research Fellowship, Bayerische Forschungstiftung (BFS), Germany |
| 2009 | Final Degree Award from University of Regensburg Alumni (Studienabschlusspreis der EsdUR), Germany |
| 2007 - 2009 | Study Fellowship for Graduates, Deutscher Akademischer Austausch Dienst (DAAD), Germany |
| 2005 - 2006 | Excellence Scholarship, Western University of Timisoara, Romania |
| 2002 - 2005 | Merit Scholarship, Western University of Timisoara, Romania |

Publications

Toth I, Neumann ID, Slattery DA (under review) Social fear conditioning as an animal model of social anxiety disorder. Invited manuscript to Current Protocols in Neuroscience

Toth I, Neumann ID, Slattery DA (under review) Brain oxytocin in social fear conditioning and its extinction: involvement of the lateral septum. Biological Psychiatry

Lukas M*, **Toth I***, Veenema AH, Neumann ID (in press) Oxytocin mediates rodent social memory within the lateral septum and the medial amygdala depending on the relevance of the social stimulus: juvenile versus adult female. Psychoneuroendocrinology

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* equal contribution to the manuscript