Roles of Neuropeptide S Receptor and Orexin in Mouse Models of Anxiety Disorders and Trauma- and Stressor-Related Disorders

Thesis

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Summary

Title: Roles of Neuropeptide S Receptor and Orexin in Mouse Models of Anxiety Disorders and Trauma- and Stressor-Related Disorders

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Fear memory generalization and social anxiety are adaptive survival mechanisms that are highly conserved across species. Fear memory generalization promotes flexible fear responses to novel situations, whereas social anxiety supports cautious behavior towards unfamiliar conspecifics in complex and constantly changing environments. However, prolonged or excessive fear/caution is maladaptive and may result in one of the anxiety disorders and trauma- and stressor-related disorders (AD-TSRD). These disorders are a tremendous social and economic burden in urgent need for further research to identify novel therapeutic targets. Many clinical and animal studies have linked the neuropeptide systems of the brain, such as neuropeptide S (NPS) and orexin, with an increased risk of developing AD-TSRD and related endophenotypes. Several studies have also implicated dysfunctions of the hypothalamic-pituitary-adrenal axis to the genesis and prognosis of AD-TSRD. Therefore, understanding these cross regulations in the development of AD-TSRD-related behavioral endophenotypes, such as fear overgeneralization and dysregulation in social behavior, might be a promising strategy for novel treatments. Hence, in this thesis, I focused on understanding the complex interplay of AD-TSRD-related behavioral endophenotypes in mice with genetically altered NPS and orexin systems. Moreover, I studied how stress hormones may play a critical role in the development and progression of these endophenotypes. To this end, I designed two paradigms to induce fear memory generalization and social fear using modified fear conditioning. Both sexes of NPS receptor (NPSR)-deficient mice were tested in the paradigms for 1) the specificity and strength of the fear memory, 2) acquisition, expression, and extinction of the conditioned social fear, while orexin-deficient mice were tested only in the latter. Moreover, mice were subjected to a battery of additional behavioral paradigms (sociability test, acoustic startle response, and light-dark box) to have a comprehensive understanding of behavioral outcomes.

These experiments revealed that NPS and orexin systems are associated with behavioral endophenotypes of overgeneralization and dysregulation in social behavior. Specifically, I observed the generalization of fear memories only when NPSR-deficient mice were injected with a high dose of corticosterone (CORT, 5 mg/kg), administered during the memory consolidation stage. Moreover, the generalization was expressed only after a specific incubation period. I did not observe the fear generalization in either wild-type or heterozygous animals, at low doses of CORT, or 24 hours after injection. These highly specific results point towards the importance of a synergistic role of the NPS system, high levels of CORT, and the incubation period as the main component behind the development of generalization of fear memory. Further, I observed decreased startle responses (both sexes) and significantly less body weight gains (females) only in those animals exhibiting the generalization of fear memories. Notably, this generalization of fear memory did not affect anxiety-related behavior, suggesting the specificity of the fear generalization-related endophenotypes. Concerning dysregulation in social behavior, heterozygous NPSR-deficient mice exhibited reduced sociability behavior (only females) and impaired extinction of social fear (both sexes). However, the extinction of social fear was facilitated in homozygous NPSR-deficient mice, suggesting the role of compensatory mechanisms. Moreover, only female orexin-deficient mice showed reduced sociability and preference for social novelty behavior, suggesting sex-specific impairments. However, both sexes of orexin-deficient mice showed facilitation in the acquisition and expression of conditioned social fear and impaired extinction of this fear, suggesting the importance of orexin for proper memory encoding and retrieval in social fear paradigms.

In this work, considerable insights were obtained into the complex roles of the NPS and orexin systems in the regulation of AD-TSRD-related behavioral endophenotypes. Hence, this work contributes towards elucidating the mechanisms underlying the development of some of these disorders.

Zusammenfassung

Titel : Die Rollen von Neuropeptid S Rezeptor und Orexin in Mausmodellen für Angststörungen und Trauma- und stressorbezogene Störungen

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Die Generalisierung des Furchtgedächtnisses und soziale Angst sind adaptive Überlebensmechanismen, die über die Arten hinweg hoch konserviert sind. Die Generalisierung des Furchtgedächtnisses fördert flexible Furchtreaktionen auf neue Situationen, während soziale Angst vorsichtiges Verhalten gegenüber unbekannten Artgenossen in komplexen und sich ständig verändernden Umgebungen unterstützt. Anhaltende oder übermäßige Furcht/Vorsicht ist jedoch maladaptiv und kann zu einer der Angststörungen und Trauma- und stressorbezogenen Störungen (AD-TSRD) führen. Diese Störungen stellen eine enorme soziale und ökonomische Belastung dar und bedürfen dringend weiterer Forschung, um neue therapeutische Ziele zu identifizieren. Viele klinische und tierexperimentelle Studien haben die Neuropeptidsysteme des Gehirns, wie Neuropeptid S (NPS) und Orexin, mit einem erhöhten Risiko für die Entwicklung von AD-TSRD und verwandten Endophänotypen in Verbindung gebracht. Mehrere Studien haben auch Dysfunktionen der Hypothalamus-Hypophysen-Nebennierenrinden-Achse mit der Genese und Prognose von AD-TSRD assoziiert. Daher könnte das Verständnis dieser Kreuzregulationen in der Entstehung von AD-TSRD-bezogenen Verhaltensendophänotypen, wie übergeneralisierte Furcht und dysreguliertes Sozialverhalten, eine vielversprechende Strategie für neue Behandlungen sein. Daher habe ich mich in dieser Arbeit darauf konzentriert, das komplexe Zusammenspiel von AD-TSRD-bezogenen Verhaltensendophänotypen in genetisch veränderten NPS- und Orexin-Systemen zu verstehen und wie Stresshormone eine kritische Rolle bei der Entwicklung und Progression dieser Endophänotypen spielen können. Zu diesem Zweck entwarf ich zwei Paradigmen zur Induktion von Furchtgedächtnis-Generalisierung und sozialer Furcht mittels modifizierter Furchtkonditionierung. Weibliche und männliche NPS Rezeptor (NPSR)-defiziente Mäuse wurden in diesen Paradigmen auf 1) Spezifität und Stärke des Furchtgedächtnisses, 2) Erwerb, Ausprägung und Extinktion von sozialer Furcht getestet, während Orexin-defiziente Mäuse nur in letzterem getestet wurden. Außerdem wurden die Mäuse einer Reihe von zusätzlichen Verhaltensparadigmen unterzogen (Soziabilitätstest, akustische Schreckreaktion und Hell-Dunkel-Box), um ein umfassendes Verständnis der Verhaltensergebnisse zu erhalten.

Diese Experimente zeigten, dass die NPS- und Orexin-Systeme mit Verhaltensendophänotypen von Übergeneralisierung und Dysregulation im sozialen (Furcht-) Verhalten assoziiert sind. Insbesondere beobachtete ich die Generalisierung des Furchtgedächtnisses nur, wenn NPSR-defizienten Mäusen eine hohe Dosis Corticosteron (CORT, 5 mg/kg) während der Phase der Gedächtniskonsolidierung verabreicht wurde. Außerdem wurde die Generalisierung nur nach einer bestimmten Inkubationszeit gezeigt. Die Furchtgeneralisierung konnte weder bei Wildtyp- noch bei heterozygoten Tieren, noch bei niedrigen CORT-Dosen oder 24 Stunden nach der Injektion beobachtet werden. Diese hochspezifischen Ergebnisse weisen auf die Bedeutung einer synergistischen Rolle des NPS-Systems, hoher CORT-Spiegel und der Inkubationszeit als Hauptbestandteil für die Entwicklung des generalisierten Furchtgedächtnisses hin. Darüber hinaus beobachtete ich verminderte Schreckreaktionen (beide Geschlechter) und signifikant geringere Körpergewichtszunahmen (Weibchen) nur bei jenen Tieren, die eine Generalisierung des Furchtgedächtnisses zeigten. Bemerkenswerterweise hatte diese Generalisierung des Furchtgedächtnisses keinen Einfluss auf angstbezogenes Verhalten, was auf die Spezifität der mit der Furchtgeneralisierung verbundenen Endophänotypen hindeutet. Heterozygote NPSR-defiziente Mäuse zeigten ein reduziertes Soziabilitätsverhalten (nur Weibchen) und eine gestörte Extinktion von sozialer Furcht (beide Geschlechter). Bei homozygoten NPSR-defizienten Mäusen war die Extinktion von sozialer Furcht jedoch verstärkt, was auf kompensatorische Mechanismen hinweist. Darüber hinaus zeigten nur weibliche Orexin-defiziente Mäuse eine reduzierte Soziabilität, was auf geschlechtsspezifische Beeinträchtigungen hindeutet. Allerdings zeigten Orexin-defiziente Mäuse

beider Geschlechter eine Verstärkung beim Erwerb und der Ausprägung von sozialer Furcht, sowie eine beeinträchtigte Extinktion dieser Furcht, was auf die Bedeutung von Orexin für Gedächtniskodierung und -abruf in sozialen Furchtparadigmen hinweist.

In dieser Arbeit wurden beträchtliche Einblicke in die komplexe Rolle des NPS- und Orexin-Systems bei der Regulation von AD-TSRD-bezogenen Verhaltensendophänotypen gewonnen. Sie tragen somit zur Aufklärung der Mechanismen bei, die der Entwicklung einiger dieser Störungen zugrunde liegen.

1 Introduction

1.1 Anxiety disorders and trauma- and stressor-related disorders (AD-TSRD)

Anxiety disorders (AD) and trauma- and stressor-related disorders (TSRD), together abbreviated as AD-TSRD, constitute the largest group of mental disorders in most western societies. They lead to a variety of functional impairments and a strong decrease in the quality of life. AD are the most prevalent psychiatric disorders with a current worldwide prevalence of 7.3% of the population (for review see: Thibaut 2017). In 2010, >60 million Europeans had an anxiety disorder (Haro et al. 2014), resulting in a total cost of >74 billion Euros (Wittchen et al. 2011). Additionally, 8.7% of the population of the United State is estimated to be at risk of post-traumatic stress disorder (PTSD), which is the most common prevalent form of TSRD. Collectively, AD-TSRD makes a significant mental health challenge of the 21st century.

AD-TSRD show a high degree of connectivity, even to the extent that TSRD were grouped under AD until 2013. Later on, the American Psychiatric Association released DSM-5 (the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders) and reclassified them as a separate class of mental disorders. Re-classification was done to highlight the causal role of stress and trauma in the formation of TSRD as compared to only a predisposing role in AD. The diagnostic criteria for both groups of diseases are generally similar, such as excessive and enduring fear, anxiety, or avoidance of perceived threats. However, TSRD require the presence of additional environmental risk factors and a history of stress and/or trauma exposure.

Table 1 summarizes the classification of different AD-TSRD

Anxiety disorders	Trauma- and stressor-related disorders
Separation anxiety disorder	Reactive attachment disorder
Selective mutism	Disinhibited social engagement disorder
Specific phobia	Post-traumatic stress disorder (PTSD)
Social anxiety disorder (SAD, Social phobia)	Acute stress disorder
Panic disorder	Adjustment disorders
Panic attack specifier	Other specified trauma- and stressor-related disorder
Agoraphobia	Unspecified trauma- and stressor-related disorder
Generalized anxiety disorder	
Substance/medication-induced anxiety disorder	
Anxiety disorder due to another medical condition	
Other specified anxiety disorder	
Unspecified anxiety disorder	

AD-TSRD often remain underdiagnosed and undertreated in primary health care, even in economically advanced countries despite its great public health significance. Disappointingly, since 2005 there have been no indications of improvement in the care and treatment of these mental disorders (Wittchen et al. 2011). The currently available treatments of these two groups of diseases usually consist of the combination of psychotherapy, where the gold standard is cognitive-

behavioral therapy (CBT), and pharmacotherapy. Pharmacological treatment involves selective serotonin reuptake inhibitors (SSRIs), selective serotonin-norepinephrine reuptake inhibitors (SNRIs), and symptomatic treatments with benzodiazepines (BZDs). However, the established pharmacological anxiolytic treatment is not very optimal due to limited efficacy and side-effects. The prolonged use of BZDs leads to undesirable side-effects such as the development of tolerance, increased risk for dependence, and higher suicide risk (e.g., Buffett-Jerrott and Stewart 2002; Rosenberg and Chiu 1985).

The exact neuropathological mechanisms underlying AD-TSRD have not been identified so far. However, several studies have implicated dysfunctions of multiple mechanisms related to the brain's fear circuitry, the hypothalamic-pituitary-adrenal (HPA) axis, and stress hormones. Moreover, overgeneralization of fear along with various genetic factors affecting the neuropeptides system of the brain, such as the neuropeptide S (NPS) and orexin systems have also been linked to the genesis and prognosis of AD-TSRD. Additionally, the interplay of these factors has never been systematically studied. Studying endophenotypes in animal models with predisposing genetic factors can be a very valuable research tool to elucidate the important molecular interplay that might be common between animal models and AD-TSRD patients. Hence, during my Ph.D., I focused on measuring the behavioral endophenotypes in the genetically altered NPS and orexin systems along with the interplay of stress hormones.

1.1.1 Role of stress and dysregulation of the HPA axis in AD-TSRD

Adaptation to stress is a major priority for all organisms which involves an acquired response by the organism in reducing the impact of a stressor. Upon stress, stressor-related information from all major sensory systems is delivered to the brain. That recruits neural and neuroendocrine systems to make the necessary physiological and metabolic changes required for an adequate stress response to minimize the cost of stress and maintain the physiological balance even in the most demanding of circumstances. The HPA axis, the organism’s major neuroendocrine stress response system, is the primary system for the maintenance or reinstatement of homeostasis during stress (for review see: Carrasco and Van de Kar 2003).

Stress systems activation is essential for preparing our body for difficult, maybe even dangerous, circumstances. However, chronic or unpredictable stress can lead to dysregulations of stress-responsive physiological systems, causing the development of AD-TSRD. A considerable number of patients suffering from AD-TSRD such as panic disorder, generalized anxiety disorder, or PTSD, exhibit alterations of the HPA axis (for review see: Abelson et al. 2007; Risbrough and Stein 2006; Tafet and Nemeroff 2020). Interestingly, hyperactivity, hypoactivity, and no change in the activity of the HPA axis have been found in the various subgroups of AD-TSRD patients (e.g., Tafet et al. 2001). Therefore, it is currently unclear whether changes in the cortisol are consistent across AD-TSRD (e.g., Daskalakis et al. 2016; Zorn et al. 2017). The reasons for these disparities are yet to be fully understood but, it seems to be due to various factors, e.g., differences in the developmental time course of the diseases, the degree of developmental advancements of the disorders, the chronicity of trauma exposure, age differences and genetic predisposition of each individual.

Moreover, the currently available pharmacological treatments of AD-TSRD consist of medications that, in addition to their well-known pharmacological effects, have also effects on various components of the HPA axis, including glucocorticoid receptors (GRs) and corticotropin-releasing

factor (CRF). Briefly, they have a normalizing effect on the HPA axis, which is considered to be related to their therapeutic effect. It has been shown that BZDs reduce the activity of CRF neurons in the hypothalamus and decrease the cortisol levels in healthy volunteers in a dose-dependent manner (Calogero et al. 1988; Gram and Christensen 1986). Chronic treatment with tricyclic antidepressants (TCAs) also resulted in a significant reduction in the HPA axis activity by decreasing CRF messenger RNA (mRNA) expression in the hypothalamus (Brady et al. 1991; Heydendael and Jacobson 2008; Melia et al. 1992; Michelson et al. 1997). Additionally, in vitro data have shown that in cell cultures derived from the hypothalamus or amygdala (AMY), incubation in the presence of TCAs, increased GRs mRNA expression (Pepin et al. 1989; Pepin et al. 1992). Similarly, long-term treatment with SSRIs inhibits CRF release in the central nucleus of the AMY while increasing GRs density in the hypothalamus and hippocampus both in vitro and in vivo (Lai et al. 2003; Lee et al. 2016; Yau et al. 2004). Collectively, there is a complex interplay between dysregulation of the HPA axis and fear circuitry of the brain which plays a crucial role in the development of AD-TSRD. Moreover, the pharmacological treatments for AD-TSRD seem to target the dysregulation of the HPA axis that in part contributes to the observed clinical improvement (Lenze et al. 2011). However, with biological complexity and pronounced heterogeneity, it has been difficult to associate biological, often contradictory, symptoms to any single gene. Therefore, in this thesis, I used the endophenotype concept on the animal model, which simplifies the relation between cognitive and emotional symptoms, triggered by abnormalities in the HPA axis activity, with the molecular and genetic mechanisms.

1.1.2 Fear overgeneralization as a hallmark of many AD-TSRD

One phenomenon associated with the fear circuits of the human brain is the generalization of fear memory (for review see: Asok et al. 2018; Hermans et al. 2013). This is an adaptive mechanism that promotes flexible responses to novel situations in complex and changing environments. Fearful experiences are never identical. Therefore, when confronted with the potential threat, we generalize fear based on previous experiences, weighing cues, and contextual information that may help to predict safety or danger and select an appropriate defensive response. Though fear and generalization of fear are conserved across species and are important survival mechanisms, the overgeneralization of fear to inappropriate situations can also produce maladaptive behaviors and contributes to the development of AD-TSRD such as PTSD, panic disorder, or general anxiety disorder (Kaczkurkin et al. 2017; Lissek et al. 2014; Lissek et al. 2010). However, such overgeneralization hasn't been demonstrated consistently (Tinoco-González et al. 2015) and not for all AD-TSRD, e.g., social phobia (Ahrens et al. 2016). Therefore, although fear overgeneralization is a hallmark of many AD-TSRD, it is unclear how important role it can play as a pathogenic marker for these two groups of diseases. It seems appropriate to take into consideration that the generalization of fear can have a multi-dimensional mechanism.

The extreme form of fear generalization (overgeneralization) is the main feature of PTSD (Kaczkurkin et al. 2017; Lissek et al. 2010). Fear overgeneralization in PTSD patients is characterized by impairment of the specificity and strength of the fear memory, which decrease their capacity to identify the correct predictors of the threat and restrict fear to the correct place and/or to the correct cues (Elzinga and Bremner 2002; Layton and Krikorian 2002). Patients who are suffering from trauma, experience symptoms when they are confronted with traumatic reminders or trauma-related stimuli and re-experience the fear response even in safe situations. This

form of fear overgeneralization leads to avoidance behaviors thereby resulting in impairments of global and social functioning. PTSD patients tend to avoid activities, places, or people that arouse recollections of the trauma, which leads to decreased quality of life. Interestingly, PTSD patients show the clinical symptoms after an incubation time of a minimum of one month after the traumatic event suggesting an important role of time to set in the pathological changes underlying the disorder.

Although clinical observations have suggested the relevance of overgeneralization of fear memory in many AD-TSRD, human experimental studies on this aspect investigated for almost a century (e.g., Bass and Hull 1934; Hovland 1937) have still left us with many open questions to answer. A key question is why some individuals develop fear overgeneralization in case of PTSD following trauma exposure while others are resilient. Several genetic variants have been linked to the predisposed individuals that develop the maladaptive form of fear generalization, however, little is known about the causal genetic risk variants. Also, the mechanisms underlying the crucial role of incubation time in the development of PTSD are also largely unknown.

Therefore, it is essential to keep expanding translational research with the use of animal models to answer clinically relevant questions linked with the generalization of fear memory, crucial genetic factors, and the incubation time.

1.1.2.1 Role of stress hormones and incubation time in cognition and fear generalization

There is strong evidence for an important role of stress and stress hormones in the modulation of cognitive processes, i.e., learning, memory, and attention. In healthy human subjects submitted to acute administration of GCs, the cortisol seems to influence cognitive processes by enhancing memory consolidation (for review see: Buchanan 2007; Het et al. 2005; Wolf 2009). Moreover, acute stress may have mixed-effects on memory, chronic stress is likely to impair it (McEwen 2004; Sapolsky 1999).

The timing of the effect of stress exposure and GCs secretion on memory consolidation follows an inverted-U-shape dose-response relationship: intermediate doses enhance memory, however, low and high levels may impair consolidation (Conrad et al. 1996; Luine et al. 1994; Luine et al. 1993). Mild stress levels and arousal have been found to have a positive effect on the acquisition and memory consolidation of newly acquired information. Acute systemic injections of corticosterone (CORT), dexamethasone, or other GRs (type II) agonists administered immediately after the aversive experience has been identified to improve memory of aversive motivated tasks, e.g., contextual fear conditioning (CFC), inhibitory avoidance, or the Morris water maze (e.g., Hui et al. 2004; Roozendaal and McGaugh 1996; Sandi et al. 1997; Sandi and Pinelo-Nava 2007). This memory consolidation enhancement by GCs is time-dependent, i.e., they only improve memory when given in a narrow time window, before or immediately following training (Hui et al. 2004; Thompson et al. 2004). In contrast, it has been shown that the removal of the adrenal glands or administration of a specific GRs antagonist influence memory consolidation in the water maze task (Oitzl and de Kloet 1992; Roozendaal et al. 1996). Interestingly, the post-training injection of dexamethasone was able to reverse this effect (Roozendaal et al. 1996). These observations indicate that glucocorticoids can modulate a wide variety of emotionally influenced memories and can enhance memory consolidation for such an emotional experience (Cordero and Sandi 1998; Hui et al. 2004; Liao et al. 2013)

In terms of fear generalization, several studies have implicated glucocorticoid-dependent signaling (Donley et al. 2005; Kaouane et al. 2012). For example, GRs in the ventral hippocampus or basolateral amygdala (BLA) are important for contextual fear, and infusion of the CORT into the hippocampus after fear conditioning prevents mice from discriminating between correct and incorrect predictors of threat (Donley et al. 2005; Kaouane et al. 2012). However, due to many key variables such as conditioning parameters, the corticosterone regimen, species, and strain differences, there is a significant variation in the existing data (Bueno et al. 2017). Additionally, the correct interpretation of the data is further confounded by the fact that the effects might be either due to the disruption of memory consolidation or due to impaired memory retrieval.

Given this diverse and contentious background of alterations in the activity of the HPA axis and its critical role in the development and treatment of AD-TSRD, there is a need for a better functional understanding of the HPA axis activation in both physiological and pathological paradigms of stress. It is important to note that the HPA axis is not an isolated system in the regulation of the stress response. Therefore, studying the complex interplay between different systems involved in fear circuitry can be crucial in finding the mechanisms underlying the development of AD-TSRD and might pave the way for the development of novel therapeutic strategies.

1.1.2.2 Methodological approaches to study fear generalization in mice model

There is considerable variation in methodology across behavioral studies in rodents that investigate the development of generalization of fear memory. Most of the studies have focused on measuring the specificity of contextual or cued fear and the strength of fear memory influenced by incubation time. To measure the specificity and the strength of the fear memory, various modifications of the classical fear conditioning paradigm are used. This paradigm is the prevailing model to elucidate the processes underlying the development of AD-TSRD (Mineka and Zinbarg 2006), and perhaps the most well established translational model of the acquisition of clinically relevant fear and anxiety.

In context generalization experiments, during the acquisition phase, a rodent is typically exposed to foot shock(s), which serves as the aversive unconditioned stimulus (US), presented in a particular context, which previously was a neutral environment. This context is called the conditioning context, and it becomes the conditioned stimulus (CS). Subsequent re-exposure of the animal to the conditioning context (CTX+) without delivery of the US induces a species-specific defensive reaction generally accepted as a proxy for fear such as freezing behavior, i.e., the cessation of all movement except for respiration (Blanchard and Blanchard, 1969). To measure the specificity of contextual fear generalization conditioned animals are subsequently tested for their freezing behavior to a new, neutral environment, different than conditioning context. This neutral context was not paired with foot shock(s) before (CTX−) (e.g., Rohrbaugh and Riccio 1968; Ruediger et al. 2011). In some context generalization experiments, several different contexts are used to measure the extent of specificity of contextual fear memory (e.g., Germer et al. 2019; Sauerhofer et al. 2012). The degree to which the CTX+ and CTX− contexts share similarities (e.g., odor, lighting, and chamber shape) can vary between different studies, and can significantly influence the results of the experiment.

In the acquisition phase of cue generalization experiments, a rodent is typically exposed to foot shocks (US) paired with a stimulus presented by one of the sensory modality such as a neutral tone

or odor (CS). Subsequent re-exposure of the animal to a cue that resembles the CS without delivery of the US induces a species-specific defensive response. The strength of the defensive response, i.e., freezing duration, varies depending on the degree to which the new CS approximates the original CS.

After conditioning, the defensive response will be elicited by the CS but will also generalize to non-threatening stimuli related to the CS. In both types of generalization experiments, behavior can be modulated by several external factors, e.g., intensity and duration of the US, the similarity between the CS and stimuli used to test generalization. Moreover, these parameters can interact with one another. Additionally, freezing in the CTX+ (context generalization experiments) or during CS exposure (cues generalization experiments) in the absence of a US can be measured at various time points after the conditioning. Measurements after 24 h correspond to long-term associative memory while measured after few weeks corresponds to remote associative memory.

To get as close as possible to the clinical symptoms related to AD-TSRD**,** external factors that can contribute to fear generalization should be considered during designing the experiments. Two perhaps the most important factors are the incubation time after exposure to the US and the influence of stress hormones. PTSD patients show the clinical symptoms after an incubation time of a minimum of one month after the traumatic event. By measuring freezing at various time points one can assess the influence of incubation time on strength of fear memory and developing fear generalization [(Balogh et al. 2002; Pollack et al. 2018; Poulos et al. 2016; Wiltgen and Silva 2007) but see (Biedenkapp and Rudy 2007; Vanvossen et al. 2017)]. The seminal experiment that shows the influence of incubation time on developing fear generalization was designed by Sauerhofer et al (Sauerhofer et al. 2012). Briefly, on day 0, animals were placed in the context where foot shock was given. Animals were divided into two groups. One group of animals was tested for their freezing behavior in the same context and similar context after 5 days. The same was done with another set of animals after 31 days. After 5 days, animals could differentiate between two different contexts meaning that they freeze more in the conditioned context and freeze less in another context. However, after 31 days, they seem to lose the ability to differentiate between the same and similar contexts showing decreased specificity of contextual fear generalization. In addition, more studies are beginning to identify the influence of stress hormones on developing fear generalization [(Donley et al. 2005; Kaouane et al. 2012) but see (Bueno et al. 2017)]. Another pioneering work by Kaouane et al. shows the influence of CORT on developing fear generalization (Kaouane et al. 2012). Briefly, foot shocks were given in a particular context along with the systemic injection of either the stress hormone CORT or saline. Interestingly, upon re-exposure of animals to the same context, animals infused with the CORT show less freezing compared to saline-injected mice suggesting memory impairment. The authors concluded that infusion of the CORT after fear conditioning can induce generalization of fear memory.

Despite methodological differences across studies, stress hormones and long incubation times, are likely a product of alterations in information processing within fear circuits contributing to maladaptive fear generalization.

1.1.3 Dysregulation of social behavior as a hallmark of many AD-TSRD

Social anxiety (SA) is an essential part of social behavior that serves a crucial function by making us alert to potential threats in the environment from conspecifics (for review see: Green and Phillips

2004; Karasewich and Kuhlmeier 2020). SA is an adaptive social response that makes individuals cautious when encountering unfamiliar members of their species because sick or aggressive conspecifics may jeopardize survival and well-being. Although the response of the body in the form of SA is an unpleasant experience. Evolutionary psychologists consider it an adaptation of animals and humans to evade possible harm and avoid the social threat to prevent conflict or limit its damage. Consequently, trait SA is widely considered as a valuable defense mechanism that is developed to help individuals preserve their relations, reputations, and maintain their position in the social hierarchy (for review see: Leary and Jongman-Sereno 2014). However, prolonged or excessive caution or generalized anxiety to familiar conspecifics, i.e., fear related to social situations, is maladaptive and can be a symptom of one of the psychiatric and neurological illnesses. Several, perhaps most of them, are characterized by prominent impairments in social functioning (for review see: Kennedy and Adolphs 2012).

Difficulties in social functioning, e.g., social withdrawal and fear of social situations, are symptoms frequently observed in many AD-TSRD, even in those that are not necessarily associated with social impairments. In a large German study on SA and social phobia of young people (aged up to 24 years), SA preceded the additional anxiety diagnosis in 64.4% of people (Wittchen et al. 1999). Data collected by the Mental Health Supplement to the Ontario Health Survey from 8,116 Canadian respondents demonstrated that 52% of respondents with lifetime social anxiety disorder (SAD) reported at least one other lifetime mental disorder, and 27% reported three or more lifetime psychiatric disorders. Maladaptive SA is strongly comorbid with AD-TSRD (Chartier et al. 2003). Moreover, four out of five adults with a primary diagnosis of SAD experience at least one other psychiatric disorder at some time during their life (Magee et al. 1996).

Although maladaptive SA is a hallmark of many AD-TSRD, the primary concerns are not about fear of negative evaluation by others. For example, individuals with panic disorder have a primary fear, that the escape from social situations may be complicated in the event of a panic attack. Whereas individuals with specific phobias may fear humiliation or embarrassment, but it will be their secondary concern to their primary phobia. One of AD-TSRD, where SA is the primary concern, is SAD with an estimated lifetime prevalence of 12.1% (Kessler et al. 2005a). SAD usually originates in childhood or adolescence with young age characteristic traits (Ollendick and Hirshfeld-Becker 2002). Interestingly, a link between inhibited temperament in childhood and SA in adolescence has been suggested. According to the study by Schwartz et al., 44% of girls and 22% of boys labeled as “inhibited” at age of 2 years met the criteria for SAD in adolescence (Schwartz et al. 1999). SAD in adults is characterized by experiencing excessive fear and avoidance of most social situations, e.g., initiating conversations, attending social events, and even going to the doctor. SAD patients tend to be self-conscious in front of other people and experience excessive fear of being judged by others. Interestingly, due to intense fear during social interactions, SAD patients may have difficulty identifying the primary emotions, such as sadness, fear, the anger of the social partner, which makes the already challenging social interaction even more difficult (Kashdan and Farmer 2014). Moreover, they tend to experience some somatic symptoms such as rapid heartbeat, muscle tension, dizziness, and stomach trouble, etc. Consequently, SAD patients show impairments across various life domains including education, family, romantic relationships, and friendships. Furthermore, they experience higher rates of unemployment, more missed hours of work, and reduced work performance leading to a decreased quality of life (e.g., Alden et al. 2018; Dryman et al. 2016; Watanabe et al. 2010; Wittchen et al. 2000; Wong et al. 2012).

The current SAD treatment consists of CBT (Fedoroff and Taylor 2001; Ito et al. 2008) and medications such as antidepressants, BZDs, Gabapentin, or the neuroleptic Olanzapine (Baldwin et al. 2016). However, the efficacy of these treatments is far from being satisfactory (for review see: Jorstad-Stein and Heimberg 2009) and most of them are designed to address and reduce only symptoms of the disorder, not the source (Heimberg et al. 2014). 40–50% of patients, have been reported to be either resistant or not responding sufficiently to current treatments (Blanco et al. 2013). Moreover, individuals with severe SAD may be less likely to seek treatment due to avoidance of the social interaction inherent in therapy. Collectively, more efficient treatments are urgently needed to improve treatment outcomes for SAD patients.

Although SAD is the third most prevalent psychiatric disorder and has a chronic course (Kessler et al. 2005a; Kessler et al. 2005b), its etiology and neuropathology are still poorly understood. It should be noted that genetic effects play a significant role in developing maladaptive SA not only in AD-TSRD but also in other psychiatric and neurological illnesses. There is now a plethora of genes implicated in human social behavior and SAD as well as in animal models of SAD. However, with biological complexity and pronounced heterogeneity, it has been difficult to associate complex biological symptoms with any single gene. Therefore, in this thesis, I used the endophenotype concept on the genetically altered animal models, which simplifies the relationship between social withdrawal and social fear as observed by patients, with the potential underlying genetic mechanisms. Further research is required to better understand the neurobiological mechanisms underlying social fear that could pave the path to novel therapies.

1.1.3.1 Methodological approaches to study social behavior in mice model

SA and avoidance of social situations represent the behavioral hallmark of many AD-TSRD with social interaction as a primary fear in SAD. Different paradigms are used to study social interactions in rodents based on the foundation that rats and mice are social animals, i.e., they prefer to spend time with another conspecific and explore social stimuli rather than non-social stimuli (e.g., Berton et al. 2006; Insel 2010; Lukas et al. 2011). In laboratory conditions, they display several types of behaviors that can be described as social interaction, e.g., crawling under each other, allogrooming, following, and sniffing (for review see: Barnett 1963; Crawley 2007). Moreover, they can recognize individual conspecifics and choose appropriate social strategies depending on the identity of the social partners (Ferguson et al. 2000; Thor and Holloway 1982).

To study the alterations in social behavior and their correct interpretation, it is crucial to understand the natural social behavior of animals and design the experiments appropriately. Most studies quantify the amount of time an animal spends investigating a social partner as a measure of social behavior.

Two groups of paradigms are used to study social behavior in rodents, i.e., the paradigms that only evaluate social behavior and paradigms that also induce social avoidance and social fear. An increase in the amount of time animal spent investigating a social partner can be used as a measure of the increase in social interaction (hence decrease in social avoidance and social fear) while a decrease in the amount of time animal spent investigating a social partner reflect social avoidance and, consequently, might reflect social fear. Several paradigms have been used to evaluate social fear and avoidance in rodents. They include social interaction (e.g., File and Hyde 1978), social preference-avoidance (e.g., Berton et al. 2006), social approach-avoidance (e.g., Haller and Bakos

2002), partition (e.g., Kudryavtseva 1994), the modified Y-maze test (e.g., Lai and Johnston 2002) and the three-chamber test (e.g., Crawley 2004).

The three-chamber test known as Crawley's sociability test is the most widely used standard test for examining social interaction (for review see: Crawley 2004). This paradigm has been successfully employed in several inbred and mutant mouse lines (e.g., Clapcote et al. 2007; Labrie et al. 2008; Moy et al. 2004), and later has been modified to work in rats as well (Wee et al. 1995). It allows the evaluation of two critical but distinct aspects of social behavior. The first aspect is sociability that is described as a tendency to engage in nonviolent social interaction with a conspecific. The second aspect is social novelty behavior, which is described as a tendency to spend more time with unfamiliar conspecifics rather than with a familiar one. The main principle of this test is based on the free choice of the tested mouse to spend time in any of three box's compartments during three experimental phases: habituation phase, sociability phase, and social novelty phase. The second and the third phase include indirect contact with one or two social partners enclosed in wire mesh cages, respectively. To evaluate sociability and social novelty, the time that the animal spent in each of the three compartments is measured.

A broad range of paradigms, largely known as social stress paradigms, have been shown to induce social fear and avoidance in rodents and might be used to model human interaction anxiety. These paradigms make use of social stressors or a combination of social and non-social stressors, i.e., physical stressors such as foot shocks, social isolation, or restraint stress, and represent promising translational approaches. The social stress paradigms include social isolation (e.g., Hermes et al. 2011), social instability (e.g., Saavedra-Rodríguez and Feig 2013), social defeat (Lukas et al. 2011), conditioned defeat (e.g., Huhman et al. 2003), social defeat/overcrowding (e.g., Slattery et al. 2012) and chronic subordinate colony (CSC) housing (e.g., Slattery et al. 2012). The paradigms that combine social and non-social stressors include chronic mild stress (e.g., Venzala et al. 2013) and maternal separation (Franklin et al. 2011). These paradigms differ in the specificity and the severity of the social fear that they induce. Most of these paradigms also induce several other behavioral alterations such as increased general anxiety, depressive-like behavior, novelty anxiety, and motivation-related alterations such as anhedonia and can reflect the human clinical situation to a large extent. However, due to these confounding behavioral alterations that might contribute to the observed social avoidance, these paradigms might not be the method of choice to understand the mechanisms underlying the specific contribution of social fear required to study the etiology and role of pure social fear in the development of SAD.

Social fear conditioning (SFC) is also a social stress paradigm, however, induces specific social fear (e.g., Toth et al. 2012), which is not accompanied by alterations mentioned above. That makes SFC a promising paradigm for animal models to study mechanisms underlying the etiology and pathophysiology of social anxiety. Moreover, SFC induces both short- and long-term (for at least two weeks) specific social fear of unfamiliar conspecifics that can be reversed by acute diazepam treatment or chronic paroxetine treatment, respectively (Toth et al. 2012). Additionally, social fear extinction evoked by repeated exposure to social stimuli gradually reverses social fear in conditioned mice appropriately reflecting the outcome during CBT in SAD patients. SFC paradigm is based on instrumental conditioning. It is a type of associative learning process where an animal's voluntary behavior is associate with positive or negative (in this case, negative) consequences. Naïve animals are punished by mild electric foot shock when they explore social partners enclosed

in a wire mesh cage, which results in social fear and avoidance of social stimuli. Social fear is expressed as a decreased exploration of social stimuli and intense aversive responses toward them. In contrast, a gradual increase in a social investigation time and a decrease in the aversive responses indicates the extinction of social fear, which is invoked by repeated exposure to social stimuli.

Moreover, it is crucial to take sex differences of tested animals, the influence of housing conditions, and the impact of age-, weight-, sex-, and strain-matched social partner into account to minimize unspecific social avoidance. Additionally, different channels of communication during social interactions should be kept in mind, i.e., ultrasonic vocalizations, odor, and visual cues. Hence, a cautiously designed SFC paradigm can be an ideal paradigm to induce and then investigate social fear and social avoidance.

1.2 Neuropeptides and their role in fear generalization and dysregulation of social behavior

Neuropeptides are key regulators of multiple aspects of physiological as well as anxiety-related, social behavior. Neuropeptides are short chains of 3-100 amino acids produced by neurons that are involved in a wide range of physiological brain functions, including social behaviors, reward, food intake, metabolism, analgesia, reproduction, learning, and memory (for review see: Russo 2017; Salio et al. 2006). Although they play a variety of roles in many physiological processes, classically they have been described as the communication system of the brain under stress/challenge/disease due to their release under high neuronal stimulation (for review see: Hökfelt 1991; Hökfelt et al. 2000). It has been suggested that "peptidergic communication" may be a language of the diseased brain that makes them attractive therapeutic targets for drug development.

Neuropeptides are synthesized by a very selective and specific set of neurons and released via dense-core vesicles in low concentration with a slow-onset but long-lasting modulation of synaptic transmission. Generally, neuropeptides can be characterized into at least three groups based on their availability and mode of action (for review see: Hökfelt et al. 2000). Type 1 peptides are present at high levels under physiological conditions, which indicates that they are functionally available at any time such as substance P (SP) and calcitonin gene-related peptide (CGRP) in primary sensory neurons, galanin (GAL) in hypothalamic neurons, vasoactive intestinal peptide (VIP), and neuropeptide Y (NPY) in cortical neurons. Type 2 are peptides normally expressed at low or undetectable levels. However, their expression is up-regulated following specific stimuli in certain conditions, for example after nerve injury such is the case for VIP or NPY in sensory neurons. In type 3 mode, peptides are transiently expressed during development, often only prenatally, and they are then downregulated postnatally, such as in the case of somatostatin in central neurons. Over the past few years, there has been a significant increase in the number of identified neuropeptides. So far, there have been more than 100 known neuropeptides being reported and potentially many more yet to be identified from the over 1000 predicted peptides encoded by the genome.

Many neuropeptides play a crucial role in the neurodevelopment of SAD, (for review see: Kennedy and Adolphs 2012). In particular, oxytocin (OXT) and vasopressin (AVP) peptide systems have been identified as critical mediators of complex social behaviors, including attachment and social recognition, however, with opposite roles of OXT in regulating social preference, and AVP in increasing avoidance of social stimuli (for review see: Heinrichs and Domes 2008). It has been shown that OXT decreased behavioral and neuroendocrine responses to social stress by empowering animals to overcome avoidance of proximity and to inhibit their defensive behavior.

Moreover, OXT knockout mice showed social amnesia, i.e., they fail to recognize familiar conspecifics without any deficit in spatial, olfactory, or other forms of memory; and excessive social interaction characteristic of novel social investigations. Social recognition was restored by the central OXT administration into the AMY (for review see: Insel and Young 2001; Winslow and Insel 2002). Also, by central infusion of OXT into the dorsolateral septum, or intracerebroventricular (icv) administration of NPY, but not AVP, before social fear extinction training reduced expression of social fear (Kornhuber and Zoicas 2019; Zoicas et al. 2014) suggesting an important role of these neuropeptides in regulation of social behavior, social fear, and anxiety.

Moreover, NPY has also shown decreased social anxiety by infusion of NPY into the BLA (Sajdyk et al. 1999) or reverse nicotine-induced social anxiety-like behavior by systemic and daily injections of an NPY Y_2 receptor antagonist (Aydin et al. 2011). NPY has been found highly expressed in limbic areas of the brain, where it regulates fear- and anxiety-related behavior (Tasan et al. 2016). It has been also shown to influence multiple fear-related behaviors including the acquisition, incubation, expression, and extinction of conditioned fear. Specifically, mice lacking NPY or one of its receptors (Y_2) exhibit heightened generalization to auditory cues (Verma et al. 2012), and higher freezing levels were observed in Y_1 receptor knockout mice during fear conditioning (Fendt et al. 2009). There is substantial evidence that NPY plays a critical role in the maintenance of a healthy stress response system, especially adequate fear response, capable of adapting to challenging environments (Dumont and Quirion 2014; Reichmann et al. 2015; Sah et al. 2014).

Like other aspects of fear and anxiety, fear generalization and social anxiety are mediated, among other brain areas, by the central nucleus of the amygdala (CeA). In this region, the expression of various neuropeptides such as cholecystokinin (Micevych et al. 1988), NPY (Gustafson et al. 1986), CRF (Joseph and Knigge 1983), dynorphin (Weber and Barchas 1983), and enkephalin (Cassell et al. 1986) has been shown (Cassell et al. 1986). Many of these peptides have been shown to modulate threat-related behaviors, and they are considered to allow for comprehensive bidirectional control over a variety of defensive responses (Bowers et al. 2012; Davis et al. 2010) further suggesting a strong involvement of neuropeptides in social behavior and potentially in SAD.

A variety of neuropeptides and their receptor systems have gained increasing attention due to their potential involvement in SA and thereby engagement in AD-TSRD. It seems reasonable to predict that probably many more yet to be identified based on the fact that they share a high degree of homology between each other, coexist with each other, and modulate each other activities. On such lines, two additional neuropeptides systems such as the NPS system or the orexin system have gained recent attention and may also influence the mentioned hallmarks of AD-TSRD**.** However, their role in contextual and social fear generalization is not yet well understood.

1.2.1 The NPS system within the brain

NPS is 20 amino acids long peptide (for review see: Okamura and Reinscheid 2007; Pape et al. 2010) identified in 2002 (Xu et al. 2004). The primary structure of the peptide is highly conserved amongst vertebrates, including humans (Reinscheid 2007), which highlights the physiological significance of NPS in the vertebrate system. The peptide got even named after its conserved N-terminus **s**erine residue (Xu et al. 2004). Moreover, the absence of NPS in lower vertebrates (Reinscheid 2007) suggests a delayed evolution of this system and its potential significance in more

complex biological organisms like humans. Interestingly, the structure of NPS is unique and does not show any structural homology with other known neuropeptides.

In mice, NPS-positive neurons have been found only in two brain stem nuclei, i.e., peri locus coeruleus (LC) region and the Kolliker-Fuse nucleus of the lateral parabrachial area. LC is a nucleus in the pons of the brainstem and highly involved in the physiological responses to stress and panic. Kölliker-Fuse nucleus is a part of the parabrachial region which is a cytoarchitecturally highly organized region that represents essential relay stations for visceral afferents from the brainstem to the mammalian forebrain for higher-order processing and emotional behavior, suggesting an important function of NPS in these behavioral processes. Furthermore, densitometry analysis and retrograde tracing revealed moderate to high densities of NPS-positive nerve endings in the majority of limbic brain regions projecting from the brainstem to the rat hypothalamic paraventricular nucleus (PVN) (Adori et al. 2015; Clark et al. 2011; Grund et al. 2017), as well as the AMY further suggesting NPS release within main regulatory centers of fear and anxiety.

The only identified receptor for NPS so far is the NPS receptor (NPSR) (for review, see: Reinscheid et al. 2005). The NPSR is a typical G protein-coupled receptor (GPCR) whose activation leads to intracellular Ca^{2+} mobilization, increased cyclic adenosine monophosphate (cAMP) accumulation, and mitogen-activated protein (MAP) kinase activation (Erdmann et al. 2015; Reinscheid et al. 2005; Xu et al. 2004) leading to enhanced neuronal excitability (Xu et al. 2004). Interestingly, NPSR-expressing neurons, in contrast to NPS-expressing neurons, have much wider expression throughout the rodent and human brain including the AMY, which plays a determinant role in the control of social behavior, fear, and anxiety (Clark et al. 2011; Xu et al. 2019). Although our knowledge regarding the distribution of NPSR in the rodent brain is entirely based on in situ hybridization studies because of the lack of sensitive and selective NPSR antibody (Slattery et al. 2015).

To summarize, the NPS system is localized in regions of critical importance for the integration of emotional behavior and may play a significant role in the development of AD-TSRD and other most prevalent psychiatric disorders. I will further discuss their role in greater detail in anxiety-related disorder, fear generalization, and in the development of AD-TSRD.

1.2.1.1 NPS system in modulating stress associated with AD-TSRD

A variety of clinical studies have linked NPSR gene polymorphisms, which changes receptor efficacy for NPS stimulation by tenfold (Reinscheid et al. 2005), with enhanced AMY responsiveness to aversive stimuli, increased risk of developing AD-TSRD, and related endophenotypes (e.g., Dannlowski et al. 2011; Raczka et al. 2010). Additionally, these polymorphisms have been linked to increasing the salivary cortisol stress responses as a consequence of the enhanced activity of the HPA axis (Kumsta et al. 2013; Streit et al. 2017; Streit et al. 2014).

These observations are consistent with the animal studies supporting a significant role of the NPS system in the formation, consolidation, and extinction of fear memories and their modulation by stress. Injections of NPS into the cerebral ventricle or the AMY, i.e., the central site of the brain fear circuitry, showed anxiolytic effects and reduced conditioned fear (Fendt et al. 2010; Jüngling et al. 2008; Meis et al. 2008; Ruzza et al. 2012). Furthermore, NPS injections in the lateral AMY

revealed long-lasting effects on the extinction of conditioned fear by reversing stress-induced fear extinction deficits to levels typical of non-stressed animals (Chauveau et al. 2012) and boosting the beneficial effects of D-cycloserine on fear extinction (Sartori et al. 2016). Icv administration of NPS resulted in the release of CRF, ACTH, and the CORT further supporting the human data of association between the NPS system and the HPA axis (Reinscheid 2008; Smith et al. 2006). Furthermore, CRF mediated activation of NPS neurons in the brainstem result in the release of NPS in brain areas related to fear circuitry such as the AMY (Jungling et al. 2012). Additionally, to the effects of synthetic NPS, the endogenous NPS modulated by environmental stimuli and endogenous cues is also involved in the regulation of stress- and anxiety-related behaviors. Interestingly, by increasing exposure to emotional stress, we can increase endogenous levels of NPS. Rats submitted to forced swim stress showed enhanced NPS levels revealed by intra-amygdaloid microdialysis (Ebner et al. 2011). Moreover, the first investigation of NPS precursor knockout mice found that these mice seem to be more anxious compare to wild-type ones (Liu et al. 2017).

All these data suggest an important role of the NPS system in regulating fear and anxiety. However, NPSR-deficient mice, when bred on a C57BL/6J background (but see: Duangdao et al. 2009), express an unexpectedly modest anxiogenic-like phenotype and attenuation of arousal in behavioral paradigms of anxiety, fear, and stress. (Fendt et al. 2011; Zhu et al. 2010). Further studies are required to fully understand the role of the NPS system in anxiety and fear-related paradigms and how stress hormones can modulate the fear memories.

1.2.1.2 NPS system in modulating social behavior and social fear associated with AD-TSRD

The ability to create healthy social connections is an essential element in human relationships. Abnormalities in social behavior are often linked to many AD-TSRD (for review see: Heinrichs and Domes 2008; Insel 2002). Several animal studies uncovered the role of the NPS system in social behaviors. Icv injections of NPS reduced social fear induced by either social defeat or SFC in a dose-dependent manner (Zoicas et al. 2016). Intriguingly, NPS injections, as well as intranasally applied NPS, did not alter the normal sociability test outcomes, i.e., when the animals had no previous aversive social experiences suggesting a specific interaction of NPS and social fear (Lukas and Neumann 2012; Zoicas et al. 2016). In the resident-intruder (RI) test, NPS injections had anti-aggressive effects on behavior in mice and rats depending on the high innate levels of anxiety-related behavior (Beiderbeck et al. 2014; Ruzza et al. 2015). Moreover, NPSR-deficient mice showed elevated levels of aggressive behavior (Ruzza et al. 2015). Additionally, the association between NPSR gene polymorphism and the cortisol responses to social stress in humans has been observed (Kumsta et al. 2013). The analysis showed that in response to acute psychosocial stress challenge, T/T-allele carriers showed higher emotional reactivity with simultaneous higher salivary cortisol release compare to A/T- and A/A-allele carriers that demonstrated intermediate and lower outcomes, respectively. Collectively, these data suggest the possibility that NPS might interact with the social fear circuitry by modulating the innate levels of stress-related hormones via modulating the activity of the HPA axis and thereby manifest its anti-aggressive effects.

The structure of the NPSR may also be indirectly linked to the association between the NPS system and social behavior. NPSR is a typical GPCR, also known as seven-transmembrane domain receptors, and shares the highest degree of homology with OXT and AVP receptors (Reinscheid and Xu 2005). Both are well-described neuropeptides known for their modulatory effects on

emotionality and social behaviors (Jurek and Neumann 2018). Moreover, neuropeptides often co-localize with other neuropeptides to mediate between each other actions (see review: Hökfelt et al. 2000).

Direct associations between NPSR gene polymorphisms and social anxiety have not been found so far and need further experiments to demonstrate the role of the NPS system in regulating social fear circuitry.

1.2.1.3 NPS system in modulating fear generalization associated with AD-TSRD

Previous animal-based and clinical studies on the NPS system are mainly limited to its potent anxiolytic-like and fear-attenuating effects. However, despite the interest, very little is known about the involvement of this system in the developing pathological fear including fear generalization, which is critical for the pathogenesis of AD-TSRD (Lissek et al. 2014; Lissek et al. 2010; Mahan and Ressler 2012). Moreover, an association between the over-interpretation of fearful stimuli and healthy normal volunteers that carry NPSR gene polymorphisms has been identified (Dannlowski et al. 2011; Raczka et al. 2010). It seems that polymorphism causes an indirect effect on AD-TSRD traits and potentially contributes to the pathogenesis of these disorders by shaping fear-related limbic activity.

Our previous attempts to understand the involvement of the NPS system in fear generalization has shed some light on the complex interplay of the NPS system in fear generalization (Germer et al. 2019) Although, we could not observe a genotype effect on incubation-induced fear generalization in a one-trial CFC paradigm with only one retention test session. However, we observed a significant increase in the plasma CORT levels of the NPSR-deficient mice at the end of the experiment. Since the CORT levels were measured only at the end of the experiment, it is still not known whether the baseline CORT levels, the levels during the memory consolidation, or even more importantly, the levels during the memory expression tests, were affected by the genotype. This study opened various questions and brought a need for more systematic investigation not only on the relation between the NPS system and fear generalization but importantly on the interplay between the CORT and the NPS system.

The NPS system seems to play an important role in the anxiety circuit and has strong potential as a novel target for the treatment of AD-TSRD. The compatibility of data from various sources is probably why in recent years, there has been growing interest in the investigation of this system. In 2013 Merck Laboratories have patented their classes of small molecule NPSR antagonists (imidazole and quinolinone) claiming that their invention can be effective in treating, preventing, and alleviating a variety of AD-TSRD.

1.2.2 Orexin system within the brain

The orexin system contains two excitatory peptides, produced from the same pre-pro-orexin precursor transcript. Pre-pro-orexin results into two highly structurally similar and highly conserved peptides, orexin A (33 amino acids) and orexin B (28 amino acids), also known as hypocretin 1 and 2, respectively (Sakurai et al. 1998). There are approximately 1100 orexinergic neurons in the rat brain (de Lecea et al. 1998) and roughly 10,000–20,000 in the human brain (Nishino et al. 2000). Orexin peptides are expressed exclusively in neurons of the perifornical area (PFA), the

dorsomedial hypothalamus (DMH), and the lateral hypothalamus (LH) (de Lecea et al. 1998; Peyron et al. 1998; Sakurai et al. 1998), a part of the hypothalamus associated with fight or flight responses (Johnson et al. 2012a).

The binding targets of the mentioned ligands are two GPCRs, the orexin 1, and orexin 2, receptors (OX1R and OX2R, respectively). OX2R binds both peptides with similar affinity, while OX1R binds orexin A with a significantly higher affinity than Orexin B (approximately 10–1000 times higher) (Ammoun et al. 2003; Gotter et al. 2012; Sakurai et al. 1998). Some brain regions preferentially express one receptor subtype, thereby providing some degree of selectivity (Marcus et al. 2001). Specifically, OX1R is selectively expressed in LC and the prefrontal cortex, whereas OX2R is preferentially expressed in the PVN, and the nucleus accumbens (NAc) (Marcus et al. 2001; Trivedi et al. 1998). However, in most brain regions, the expression of orexin receptor subtypes is partially overlapping (Marcus et al. 2001). Despite this restricted expression pattern, this relatively small population of neurons exerts its versatile functions via an extremely broad distribution of projections and terminals throughout the brain (Nambu et al. 1999; Peyron et al. 1998) including the hypothalamus, thalamus, cortex, brain stem, and spinal cord (Peyron et al. 1998; van den Pol 1999). Also, OX1R and OX2R showed a broad distribution pattern.

The orexin system has broad implications in regulating several physiological functions such as regulation of sleep, arousal, emotional behavior, feeding and food-seeking, locomotion, energy metabolism, reward, and addictive behavior, sexual behavior, vigilance, and behavioral and neuroendocrine responses to stress (e.g., Boutrel et al. 2013; Burdakov et al. 2005; Chemelli et al. 1999; Furlong et al. 2009; Sutcliffe and de Lecea 2002). I will further discuss the role of the orexin system in two important hallmarks of AD-TSRD, i.e., anxiety-related disorders and social behavior.

1.2.2.1 Orexin system in stress associated with AD-TSRD

Although orexins play an important role in many of the phenotypes associated with AD-TSRD, including various aspects of stress responses such as arousal, attention, anxiety- or panic-like states, as well as the HPA axis, cardiovascular and respiratory activation (e.g., Berridge et al. 2010; Carrive 2013; Johnson et al. 2012a; Kuwaki and Zhang 2010), only a few clinical studies to date have investigated orexin in AD-TSRD patients (Johnson et al. 2010; Strawn et al. 2010). A specific polymorphism in the OX2R, which hinders the dimerization of the receptor, is more frequent in panic patients compared to subjects without panic anxiety (Annerbrink et al. 2011). Furthermore, panic disorder patients showed increased OX levels in the cerebrospinal fluid (Johnson et al. 2010), which can be reduced by anti-panic drug sertraline (Salomon et al. 2003). Interestingly, patients with chronic PTSD showed low concentrations of orexin A in cerebrospinal fluid and plasma (Strawn et al. 2010) also highlighting differential molecular signatures of these AD-TSRD related disorders.

Central administration of orexins increased stress-related behaviors, including grooming, face washing, and burrowing (Ida et al. 2000). Moreover, orexins stimulation increased struggling behavior during restraint stress (Grafe et al. 2017b). In contrast, OXRs antagonists blocked stress-related behaviors (Duxon et al. 2001; Grafe et al. 2017b), which was also diminished in orexin-deficient mice (Kayaba et al. 2003). Furthermore, the OX1R receptor antagonist has been shown to reduce anxiety-like behaviors in several different behavioral paradigms. Particularly, pretreatment with this antagonist reduced sodium lactate-induced anxiety-like behavior (Johnson et al. 2010) and

cat odors-induced anxiety-like behaviors in mice (Vanderhaven et al. 2015). Furthermore, a spontaneously hypertensive rat known for its anxiety-like behaviors compared to other rat strains showed increased expression of orexin (Clifford et al. 2015).

Although the orexin system plays a critical role in stress and anxiety-related disorders, current evidence revealed a dichotomy in orexin function. Oral administration of OX1R and OX2R antagonists did not influence social interaction, novelty, or restraint stress-induced CORT release (Steiner et al. 2013). However, icv administration of OX2R antagonist attenuated swim stress-induced increases in ACTH release (Chang et al. 2007). Intra-PVT infusions of OX1R antagonist attenuated the ACTH response to restraint but only following repeated swim stress (Heydendael et al. 2011). Moreover, increased Fos-protein expression is observed in orexin neurons following exposure to acute foot shocks (Harris and Aston-Jones 2006), fear-associated contexts, and novel environments (Furlong et al. 2009). Interestingly, similar effects are not observed following acute immobilization stress (Furlong et al. 2009). These ambiguous effects on the HPA axis function might depend on several factors such as previous stress exposure, the kind of stressor, its intensity, and duration, or whether it is acute or chronic stress exposure. One interpretation is that only previous stress exposure and sufficiently intense or relevant stimuli recruit the orexin system, e.g., increased Fos-expression in orexin neurons was followed by systemic injections of the anxiogenic drug (Johnson et al. 2012b), caffeine (Johnson et al. 2012b), and intravenous administration of sodium lactate (Johnson et al. 2010). Additionally, systemic administration of OX1R antagonist demonstrated functional inhibition in stress-relevant brain regions such as the NAc, dorsal thalamus, AMY, and ventral hippocampus following the administration of yohimbine (Gozzi et al. 2013).

Interestingly, a dual OXR antagonist had been already approved for the treatment of insomnia by reducing the severity of anxiety, arousal, wakefulness, and lowering stress hormone levels (for review see: Citrome 2014). Moreover, the case study during which a renal dialysis patient with insomnia, major depressive, and generalized anxiety disorders was submitted showed that the latter two were treated effectively. However, at the same time, the severity of depression of the patient worsened with the treatment (Petrous and Furmaga 2017) suggesting a wider involvement of the orexin system brain disorders.

Collectively, the clinical data, as well as data from animal research, indicate that enhanced orexin signaling contributes to increasing the variety of stress-like behavior, including fear responses, and promoting anxiety-like behaviors, which are the vital phenotypes in many AD-TSRD. Further, studies need to be done to clarify the specific role of the orexin system in anxiety-related disorders and decode the interplay between the orexin system and the HPA axis.

1.2.2.2 Orexin in modulating social behavior and social fear associated with AD-TSRD

Several lines of research point to the role of the orexin system in anxiety-related phenotypes, however, much less work has been done to understand the specific regulations of orexin in mediating social behaviors and their impairments. As orexin neurons are reciprocally connected to several limbic areas (Nambu et al. 1999; Sakurai et al. 2005; Yoshida et al. 2006), it is reasonable to predict that the orexin system may also influence social behaviors. Indeed, patients suffering from the sleep disorder narcolepsy, which is caused by the loss of orexins in the brain (Peyron et al. 2000; Thannickal et al. 2000), frequently express symptoms of social phobia, which may be a

direct consequence of the orexin deficiency (Fortuyn et al. 2010). Moreover, human orexin A levels in the AMY are maximal during positive emotions and social interactions (Blouin et al. 2013). In contrast, depressive, suicidal patients exhibit a reduced level of orexin A in cerebrospinal fluid (Brundin et al. 2007).

Furthermore, oral treatment with dual orexin receptor antagonists did not influence social interaction, social novelty, or restrain stress-induced corticosterone release in rats (Steiner et al. 2013). Blocking orexin receptors especially in the PVT regions showed an anxiolytic effect in the social interaction paradigm (Dong et al., 2015). Moreover, under non-aversive conditions, OX1R-deficient mice express reduced sociability and altered depressive behavior (Abbas et al. 2015). Mice, whose orexin neurons degenerate at the age of three months, display impairments in social memory, which can be ameliorated by nasal application of orexin A (Yang et al. 2013). Moreover, chemogenetic inhibition (DREADDs) of orexin neurons during social defeat increased social interaction time and decreased depressive behavior in vulnerable individuals (Grafe et al. 2018) specifically suggesting that lowering orexins promoted resilience to social defeat.

Most studies investigating the influence of orexin on social behaviors were based on social stress models, generally revealing a close relationship to the stress circuitry and indicating that orexin plays a critical role in coping with social situations in general and social threats in particular (e.g., Eacret et al. 2019; Grafe et al. 2018; Ji et al. 2019; Staton et al. 2018).

In order to understand the complex role of the NPS and orexin systems in anxiety, fear memory generalization, social behavior, and social fear, I designed experiments with the following aims.

2 Aims

1. What is the role of the NPS system in the generalization of conditioned fear, using NPSR-deficient mice? What is the role of the stress hormone CORT in this process?

2. What is the role of the NPSR receptor in social behavior and social fear conditioning?

3. What is the role of the orexin system in social behavior and social fear conditioning?

3 Publications

frontiers in Neuroscience

ORIGINAL RESEARCH
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Corticosterone Treatment and Incubation Time After Contextual Fear Conditioning Synergistically Induce Fear Memory Generalization in Neuropeptide S Receptor-Deficient Mice

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Fear memory generalization is a learning mechanism that promotes flexible fear responses to novel situations. While fear generalization has adaptive value, overgeneralization of fear memory is a characteristic feature of the pathology of anxiety disorders. The neuropeptide S (NPS) receptor (NPSR) has been shown to be associated with anxiety disorders and has recently been identified as a promising target for treating anxiety disorders. Moreover, stress hormones play a role in regulating both physiological and pathological fear memories and might therefore also be involved in anxiety disorders. However, little is known about the interplay between stress hormone and the NPS system in the development of overgeneralized fear. Here, we hypothesize that NPSR-deficient mice with high corticosterone (CORT) levels during the fear memories consolidation are more prone to develop generalized fear. To address this hypothesis, NPSR-deficient mice were submitted to a contextual fear conditioning procedure. Immediately after conditioning, mice received CORT injections (2.5 or 5 mg/kg). One day and 1 month later, the mice were tested for the specificity and strength of their fear memory, their anxiety level, and their startle response. Moreover, CORT blood levels were monitored throughout the experiment. Using this protocol, a specific contextual fear memory was observed in all experimental groups, despite the 5-mg/kg CORT-treated NPSR-deficient mice. This group of mice showed a generalization of contextual fear memory and a decreased startle response, and the females of this group had significantly less body weight gain. These findings indicate that interplay between CORT and the NPS system during the consolidation of fear memories is critical for the generalization of contextual fear.

Keywords: neuropeptide S, memory generalization, contextual fear, corticosterone, post-traumatic stress disorder

INTRODUCTION

Dysfunctions of the brain fear circuitry can lead to anxiety or trauma stress-related disorders such as panic disorder, generalized anxiety disorder, phobias, acute stress disorder, and post-traumatic stress disorder (PTSD). One phenomenon associated with the human brain fear circuitry is the generalization of fear memory, which is an adaptive mechanism. It promotes flexible fear responses to novel situations since the fear of a past experience is generalized to be better prepared for future similar aversive situations. However, whereas generalization is adaptive, overgeneralization of fear memory is maladaptive. It can lead to a pathological state since it may induce fear behavior in inappropriate situations. Indeed, fear overgeneralization is a major feature of PTSD (Lissek et al., 2010; Kaczkurkin et al., 2017). Overgeneralization in PTSD patients is characterized by disturbances of the peritraumatic memory, which is ultimately an impairment of the specificity and strength of the fear memory. Notably, according to the diagnostic and statistical manual of mental disorders (DSM-V), PTSD patients show the clinical symptoms generally after a certain incubation time of the traumatic event. The seminal experimental approaches to model PTSD-like memory in mice focus on the generalization of fear memories that are believed to mirror the mental state in anxiety patients (Kaouane et al., 2012; Sauerhofer et al., 2012). However, the mechanisms underlying the overgeneralization of fear memory are only partly understood so far.

The neuropeptide S (NPS)/neuropeptide S receptor (NPSR) system is a neuropeptide system in the brain that may play an important role in the overgeneralization of fear memories. NPS is a 20-amino-acid peptide identified in 2002 (for review, see: Okamura and Reinscheid, 2007; Pape et al., 2010) whose structure is unique and highly conserved in the mammalian system (Reinscheid, 2007). In mice, NPS mRNA is only found in about 500 glutamatergic neurons, localized in the pericoerulear area and the Kölliker-Fuse nucleus, which both project to several forebrain areas including the limbic system (Clark et al., 2011). In these projection areas, NPSR, the only identified receptor for NPS so far (Xu et al., 2004), is widely expressed and mediates excitatory signals (for review, see: Reinscheid and Xu, 2005).

There is a growing body of literature that emphasizes the role of the NPS system in modulating arousal, stress, emotions, and cognitive functions. Various clinical studies have identified an association between polymorphisms in the NPSR gene and increased sensitivity to aversive stimuli, a higher incidence of anxiety disorders, and related behavioral endophenotypes (e.g., Raczka et al., 2010; Dannlowski et al., 2011). This concurs well with data from mice models supporting an important role of the NPS system in regulating fear and anxiety. Injections of NPS into the cerebral ventricle or into the amygdala, the central site of the brain fear circuitry, reduce conditioned fear (e.g., Jüngling et al., 2008; Fendt et al., 2010), in line with the idea that NPS-deficient mice seem to be more anxious than wild-type mice (Liu et al., 2017). Moreover, NPS injections rescue stress-induced fear extinction deficits (Chauveau et al., 2012) and boost the beneficial effects of D-cycloserine on fear extinction (Sartori et al., 2016). Interestingly, exposure to forced swim stress induces an increase of amygdaloid NPS levels (Ebner et al., 2011). All these data suggest an important role of the NPS system in the formation, consolidation, and extinction of fear memories and their modulation by stress.

Interestingly, NPSR-deficient mice, when bred on a C57BL/6J background (but see: Duangdao et al., 2009), express only a modest anxiogenic-like phenotype in behavioral paradigms of anxiety, fear, and stress (Zhu et al., 2010; Fendt et al., 2011). However, little is known about their behavioral phenotype in paradigms of pathological fear including fear generalization. A recent study from our group (Germer et al., 2019) showed no genotype effect on incubation-induced fear generalization in a one-trial contextual fear conditioning paradigm with only one retention test session. Interestingly, a significant increase in plasma CORT levels of the NPSR-deficient mice was observed at the end of the experiment. However, since the CORT levels were only measured at the end of the experiment, it is still not known whether the baseline CORT levels, the levels during the memory consolidation, or even more importantly the levels during the memory expression tests were affected by the genotype.

Several studies have shown that the release of stress hormones during and immediately after the stressful situation might influence the encoding of both physiological and pathological fear memories and thereby contribute to the development of anxiety disorders. This is supported by the observation that the hypothalamic–pituitary–adrenal (HPA) axis, responsible for regulating stress hormones, is often dysregulated in PTSD (e.g., de Kloet et al., 2005; Chrousos, 2009). Moreover, a number of studies have implicated glucocorticoid-dependent signaling underlying fear generalization (Donley et al., 2005; Kaouane et al., 2012). Interestingly, few clinical studies have identified an association between polymorphisms in the NPSR gene and increased salivary cortisol stress responses (Kumsta et al., 2013; Streit et al., 2014, 2017). This concurs well with data from mice models as the intracerebroventricular administration of NPS results in the release of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and CORT (Smith et al., 2006; Reinscheid, 2008). Furthermore, NPS neurons in the brainstem can be activated by CRH and thereby result in the release of NPS in brain areas related to fear circuitry such as the amygdala (Jungling et al., 2012).

The aim of the present study was to investigate whether there is an interplay between CORT and the NPS system in the development of the generalization of fear memory. We hypothesized that NPSR-deficient mice with high corticosterone (CORT) levels during the fear memories consolidation might be more prone to develop a generalization of conditioned fear. To address this hypothesis, we submitted heterozygous and homozygous NPSR-deficient mice and their wild-type littermates to a fear conditioning paradigm. In this paradigm, intense foot shocks were used and the mice received systemic injections of CORT after conditioning. Then, specificity and strength of the fear memory were examined in recent and remote retention tests in the same groups of animals to evaluate the potential development of fear memory generalization. Importantly, intense foot shocks, CORT injections, and 1-month

incubation time are all factors known to induce a generalization of fear memory (Kaouane et al., 2012; Sauerhofer et al., 2012), whereas the recent memory test is able to reduce or prevent fear memory generalization (De Oliveira Alvares et al., 2013; Bueno et al., 2017). In addition to the fear memory, the startle response, anxiety-like behavior in the light–dark box, and the response to a stimulus that was presented explicitly unpaired during fear conditioning were tested. Moreover, CORT plasma levels were monitored throughout the experiment. This study shows that an interplay between CORT, NPSR deficiency, and incubation time is important in the development of generalization of fear memories.

MATERIALS AND METHODS

Animals

Two- to three-month-old male ($n = 90$) and female ($n = 83$) wild-type ($n = 65$), heterozygous ($n = 62$), and homozygous NPSR-deficient mice ($n = 46$) from our own breeding colony (C57BL/6J background) were used and genotyped by using adequate primers (Fendt et al., 2011). Mice were housed in groups with food and water available *ad libitum*, under a 12-h light/dark cycle, and body weight was measured in the beginning and at the end of the experiment. All experiments took place during the light phase. All procedures were performed in line with the European regulations for animal experiments (2010/63/EU) and approved by the local authorities (Az. 42505-2-1172 and 1309, UniMD).

Behavioral Studies—Apparatus and Procedure

All behavioral tests (see below) were performed with the same animals, according to the order and timelines shown in **Figure 1**.

Fear Conditioning Setup

A computerized fear conditioning system (TSE Systems, Bad Homburg, Germany) consisting of four boxes placed inside infrared sensor frames was used. Each box (46 cm × 46 cm × 32 cm) was placed in a sound-attenuating chamber provided with a loudspeaker and light sources. During the experiment (for timelines, see **Figure 1A**), three types of chambers were used that served to create different contexts. Context A was a square, transparent box with the floor consisting of steel grids, which were connected to a shock unit and able to deliver foot shocks of defined duration and intensity (**Figure 1B**). Illumination was 12 lux, the background noise was 58 dB SPL (sound pressure level), and the box was cleaned with 70% ethanol before placing the animals into it. Context A was used for fear conditioning. In addition to context A, two further contexts were used to test the specificity of the contextual fear memory. One of them [context A′ (similar)] was similar to context A and the other one [context B (different)] was clearly different. Context A′ was the same box as context A, but a diagonal divider was inserted, resulting in a triangular, transparent box. Furthermore, a lower illumination (0.7 lux) and a lower background noise (45 dB SPL) than in context A as well as 1% acetic acid as a cleaning agent were used. Context B was a square, black box with a regular floor, the brightness of 7 lux, the background noise of 55 dB SPL, and was cleaned with soapy water before each experiment. In the retention tests of the present study, the fear responses to all three chambers were tested.

Movements of animals were detected by the infrared sensors (distance: 14 mm). The freezing behavior (defined as no beam crosses for more than 1 s) was automatically recorded during all phases of the experiment. Automatically measured freezing in the TSE system is highly correlated with manual scoring (Misane et al., 2005; Endres et al., 2007).

Fear conditioning procedure

The timeline of the fear conditioning procedure is shown in **Figure 1C**. Before the experiment, animals were handled by the experimenter for 5 min for three consecutive days. On day one, all animals were individually placed into context B for 5 min (habituation). One day later, animals were fear-conditioned in context A with an unpaired cue-shock protocol (modified from Kaouane et al., 2012). Briefly, mice received the first foot shock (1.2 mA, 2 s) after 180 s, followed by an unpaired tone (T, 15 kHz, 66.5 dB SPL, 30 s) after a delay of 30 s. After an additional delay of 60 s, the same tone was presented for a second time, followed by another 45 s delay and a second foot shock. After further 30 s, mice were removed from the chamber, injected either with vehicle or one of two doses of CORT (see below) and put back in the home cages. The tones, which were presented explicitly unpaired with the foot shocks, were later used to check whether the mice erroneously associate them with the foot shocks.

Retention tests for contextual fear generalization and tone response

Animals were individually subjected to the retention test 24/48 h (recent retention test) and 1 month (remote retention test) after fear conditioning using the same experimental conditions. In both cases, animals were tested for their freezing behavior in context B (d3 and d33; see **Figure 1A**) followed by context A′ and 3 h later context A (both on d4 and d34).

In each of the contexts, the mice were placed into the box for 180 s (**Figure 1D**). Then, they were exposed to six acoustic stimuli (duration: 30 s) with inter-stimulus intervals of 30 s. The order of the stimuli presentation was the following: first two stimuli of white noise (N, i.e., different from the cue presented during fear conditioning) followed by two 10 kHz tones (T′, 70 dB SPL; i.e., similar to the tone used during fear conditioning) and further two presentations of tone T (tone used during fear conditioning). After a further 30 s, the mice were placed back in the home cages.

Systemic Injection of CORT

Immediately after fear conditioning, either vehicle (2% ethanol in saline), 2.5 mg/kg, or 5 mg/kg of CORT (Sigma-Aldrich, Taufkirchen, Germany) was injected intraperitoneally at a volume of 10 ml/kg.

CORT Plasma Levels

Repeated blood collection from the tail vein

At several time points during the experiment (see **Figure 1A**), blood was collected to measure plasma CORT levels. All samples

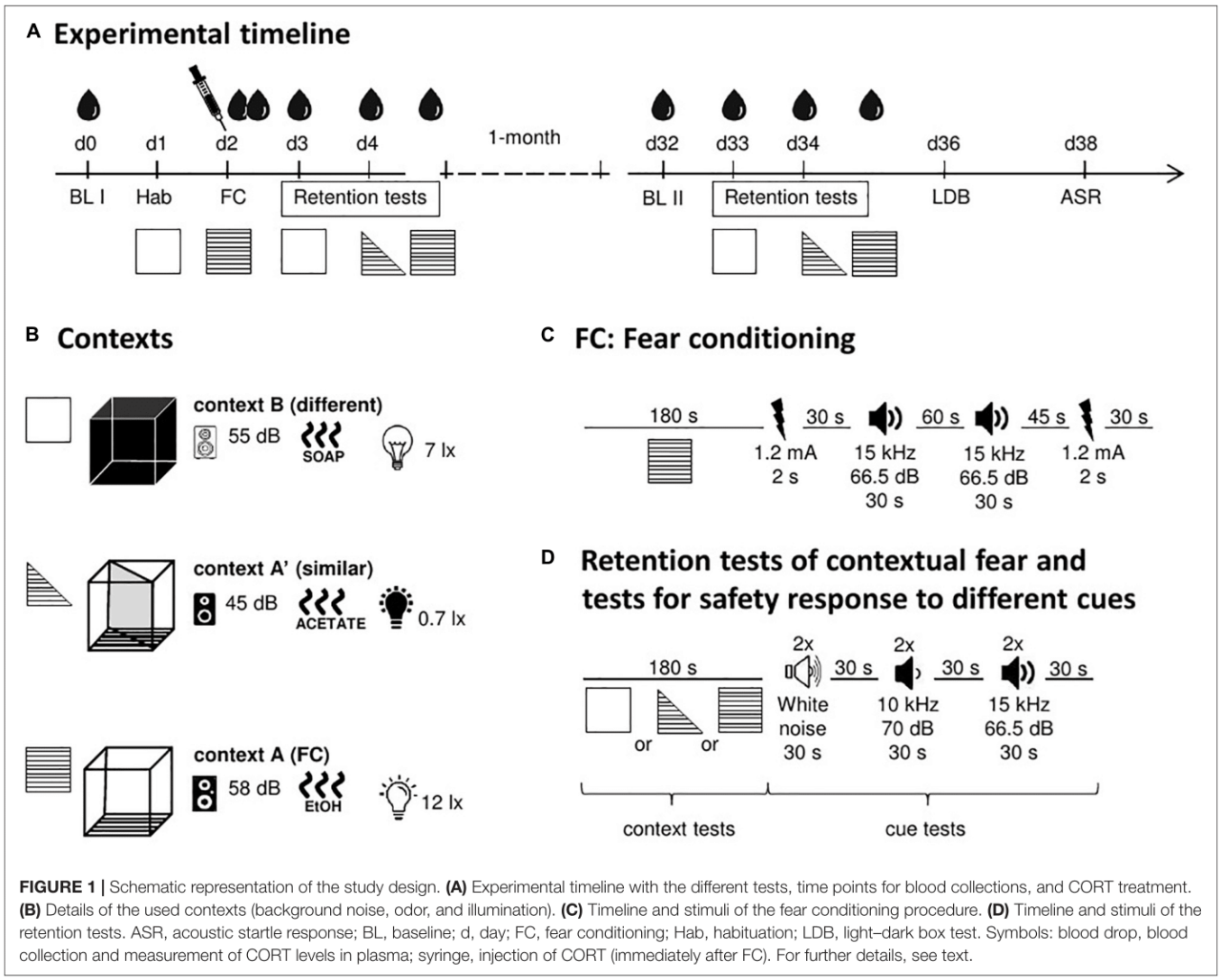

FIGURE 1 | Schematic representation of the study design. **(A)** Experimental timeline with the different tests, time points for blood collections, and CORT treatment. **(B)** Details of the used contexts (background noise, odor, and illumination). **(C)** Timeline and stimuli of the fear conditioning procedure. **(D)** Timeline and stimuli of the retention tests. ASR, acoustic startle response; BL, baseline; d, day; FC, fear conditioning; Hab, habituation; LDB, light–dark box test. Symbols: blood drop, blood collection and measurement of CORT levels in plasma; syringe, injection of CORT (immediately after FC). For further details, see text.

were collected in the morning hours (8.00–12:00 am). The first baseline (BL) samples were collected before the habituation phase (BL I, d0). The second baseline samples were collected 1 day before the remote retention test (BL II, d32). All other samples were collected 30 min after exposing the animals to the different phases of the experiment. For blood collection, the mice were put into a Plexiglas restrainer, to which they were habituated before the experiment (5 min, 3 days). Then, the lateral tail vein was cut with a scalpel at a position 2–3 cm away from the tip of the tail (Durschlag et al., 1996). The blood was collected into EDTA-coated tubes (Microvettes, Sarstedt, Germany) and placed immediately on ice. Then, the blood samples were centrifuged (2000 rpm, 4°C for 10 min followed by collection and storage (−80°C) of plasma.

CORT assay

CORT levels were quantified in 100 times diluted plasma samples by an ELISA kit (Enzo Life Sciences, Lörrach, Germany). This conventional competitive ELISA was performed according to the manufacturer's guidelines.

Light–dark box test

We used a system consisting of four identical boxes (49.5 cm × 49.5 cm × 41.5 cm). Each of the boxes was placed inside a frame with infrared sensors (TSE Systems, Bad Homburg, Germany). Boxes were separated into two compartments of the same size that were connected by an opening (8 cm × 6 cm). One of the compartments was dark (0.2–1.5 lux) while the other one was bright (410–570 lux).

On day 36 of the experiment (**Figure 1A**), mice were placed into the dark compartment and could freely explore both compartments for 10 min. Localization of the mice was measured via the infrared sensors and further processed by the TSE Phenomaster software. Between different trials, boxes were cleaned with water.

Acoustic startle response test

The startle response system consists of eight sound-attenuating chambers (SR-LAB, San Diego Instruments, United States). Each of the chambers (35 cm × 35 cm × 38 cm) was equipped with a loudspeaker for delivering acoustic stimuli. During the test,

animals were placed into Plexiglas cylinders (4 cm × 10 cm) fixed onto a plate with a motion sensor underneath. Mice movements were detected by the sensor and were further analyzed by the SR-LAB software. The mean amplitude of the motion sensor output signal within 10 to 30 ms after the acoustic startle stimulus onset was used as the startle magnitude and is expressed in arbitrary units.

The acoustic startle response was measured on day 38 of the experiment (**Figure 1A**). After an acclimation time of 5 min, three blocks of acoustic startle stimuli (40 ms white noise) of eight different intensities (78, 84, 90, 96, 102, 108, 114, and 120 dB SPL) were presented (background noise: 60 dB SPL). Within these blocks, the order of the stimuli was pseudo-randomized. An inter-stimulus interval of 20 s was used.

Data Analysis

To estimate the specificity and strength of the fear memory, we used the percent freezing duration (%FreD) to calculate two different indices, called context discrimination index and incubation time index. The context discrimination index was used as a measure of the specificity of contextual fear. It is defined as the ratio between the difference of freezing during exposure to two different contexts (X and Y) and the sum of the freezing in these two contexts:

$$\text{CONTEXT DISCRIMINATION INDEX} = \frac{(\%\text{FreD, context X}) - (\%\text{FreD, context Y})}{(\%\text{FreD, context X}) + (\%\text{FreD, context Y})}$$

A positive index means that animals express more freezing in context X than in context Y; i.e., the memory is relatively specific to context X. When the index is close to zero (means that the freezing response is equal in the two contexts) and/or have a negative value (means that animals froze more in context Y than in context X) indicates that the fear memory is not specific to a context and thereby generalized.

The incubation time index was used as a measure for the influence of incubation time (here 1 month) on the freezing in a particular context, indicating the strength of the fear memory. It is defined as the ratio between the difference of freezing during two different exposures (1st and 2nd time point) to a particular context (X) and the sum of the freezing during both exposures:

$$\text{INCUBATION TIME INDEX} = \frac{(\%\text{FreD, context X, 2nd time point}) - (\%\text{FreD, context X, 1st time point})}{(\%\text{FreD, context X, 2nd time point}) + (\%\text{FreD, context X, 1st time point})}$$

A positive index indicates an increase, zero indicates no change, and a negative value indicates a decrease of the fear memory to a particular context. Therefore, when the index is significantly more positive than zero or has a significantly more negative value, it indicates that the fear memory changed over time and thereby generalized.

We realized that the tones in the retention test decreased the freezing response of the mice, which we interpreted as a safety response. The intensity of this safety response was calculated by the following formula:

$$\text{SAFETY RESPONSE to X (stimuli T and T}' \text{ or stimulus N, respectively)} = (\%\text{ FreD during presentation of X}) - (\%\text{ FreD during pauses before presentation of X})$$

For statistical analysis, Prism 6.0 (GraphPad Software Inc., La Jolla, United States) and SYSTAT 12.0 (SPSS Inc., San Jose, United States) were used. The normal distribution of the data was checked with the D'Agostino-Pearson normality test. According to the respective experimental design, multifactorial analyses of variance (ANOVA) followed by separated one-factor or two-factor ANOVAs, if appropriate with repeated measures, were used followed by *post hoc* Holm-Sidak's comparisons.

RESULTS

In all of the experiments, we tested male and female mice. In all measures except startle response (3.6.) and body weight (3.7.), a multifactorial ANOVA revealed no main effects of sex and no interactions of sex with other factors. For the measures without sex effects, we pooled sexes for further analyses and showed the pooled data in the figures.

5 mg/kg CORT Impaired Discrimination Between Context A and Context A′ in NPSR−/− Mice After the Incubation Time

Our first question was whether genotype or/and treatment of the mice affected the fear responses expressed in the original fear conditioning context A, the similar context A′, and the different context B. To evaluate whether the fear responses were similar or different in these contexts, we calculated the context discrimination indices (section "Data Analysis") for each context and each phase of the experiment. These indices were analyzed by a multifactorial ANOVA using treatment and genotype as between-subject factors and time as a within-subject factor. For the discrimination indices for contexts A and A′ (**Figure 2**, left panel; for original freezing data see **Supplementary Figure S1**), this ANOVA revealed main effects of genotype ($F_{2,164}$ = 3.24, p = 0.04), treatment ($F_{2,164}$ = 4.05, p = 0.02), and time ($F_{1,164}$ = 4.38, p = 0.04), as well as an interaction between these three factors ($F_{4,164}$ = 2.74, p = 0.03). All other interactions did not reach statistical significance. To identify the source of this interaction, we then performed ANOVAs for each treatment separately. In vehicle-treated mice, neither time nor genotype nor their interaction affected context discrimination (Fs < 1.31, n.s.; **Figure 2A**, left panel). After 2.5 mg/kg CORT treatment (**Figure 2B**, left panel), there was an effect of genotype ($F_{2,67}$ = 3.40, p = 0.04) but not of time and no interaction between time and genotype (Fs < 2.56, n.s.). Notably, after treatment with 5 mg/kg CORT (**Figure 2C**, left panel), there was a significant interaction between genotype and time ($F_{2,164}$ = 4.66, p = 0.01). *Post hoc* tests revealed that incubation

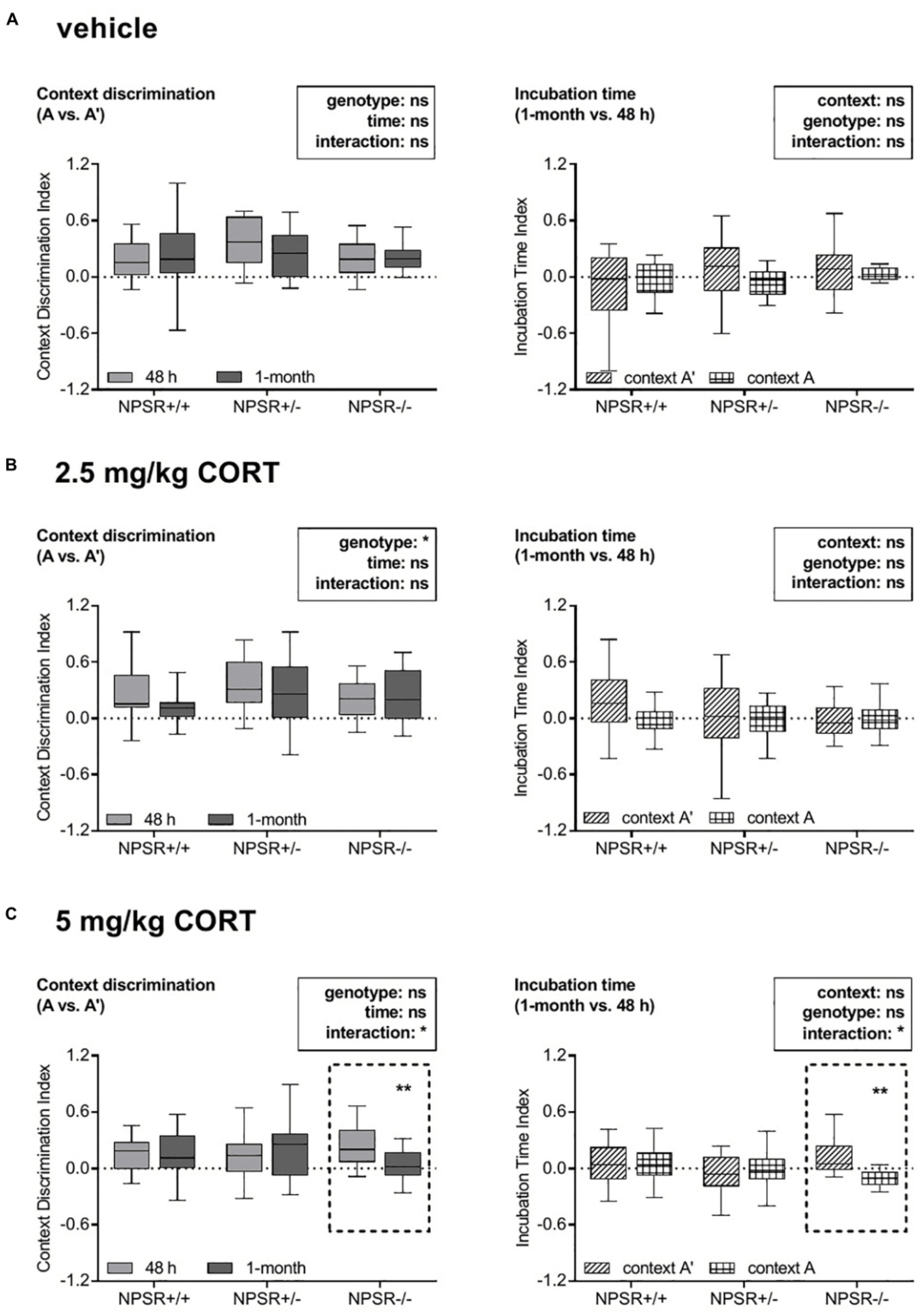

FIGURE 2 | Effects of corticosterone treatment on specificity and strength of contextual fear memory in NPSR-deficient mice. The left panel depicts the context discrimination index (A vs. A′) and the right panel depicts the incubation time index (1 month vs. 48 h). Injections of **(A)** vehicle and **(B)** 2.5 mg/kg CORT did not affect specificity or strength of the contextual memory. However, injections of **(C)** 5 mg/kg CORT significantly decreased both specificity and strength of the contextual memory in NPSR−/− mice (dashed frames), suggesting a generalization of the fear memory, but not in NPSR+/+ and NPSR+/− mice. Group sizes: NPSR+/+: $n = 65$; NPSR+/−: $n = 62$; NPSR−/−: $n = 46$. Data are illustrated with box plots (median, quartiles) and Tukey's whiskers. $^{*}p < 0.05$; $^{**}p < 0.01$; *post hoc* Holm-Sidak's comparisons after ANOVA.

time did not affect context discrimination in NPSR+/+ and NPSR+/− mice ($ts < 0.14$, n.s.); however, context discrimination was significantly decreased in NPSR−/− mice after 1 month ($t = 3.24$, $p = 0.006$).

Taken together, these data indicate that all treatment and genotype groups were able to discriminate between context A′ and A in the two retention tests, except the 5 mg/kg CORT-treated NPSR−/− mice, which had impaired discrimination after 1 month. The latter indicates a less specific memory, i.e., a generalization of contextual fear memory. Context A and B could always be well discriminated and no effects of time, genotype, or treatment were found (**Supplementary Figure S2**, left panel; for original freezing data see **Supplementary Figure S3**).

5 mg/kg CORT Impaired Contextual Fear Memory to Context A and Increased Fear to Context A′ After an Incubation Time

Next, we analyzed whether genotype or/and treatment of the mice affected the influence of incubation time on freezing behavior in contexts A, A′, and B. To address this question, we calculated the incubation time indices (section "Data Analysis") for all contexts, treatments, and genotypes (**Figure 2**, right panel; for original freezing data see **Supplementary Figure S1**). The indices were analyzed by a multifactorial ANOVA using treatment and genotype as between-subject factors and context as a within-subject factor. This ANOVA revealed no main effects of genotype ($F_{2,164} = 0.84$, $p = 0.43$), treatment ($F_{2,164} = 0.68$, $p = 0.51$), and context ($F_{1,164} = 1.94$, $p = 0.17$), but a significant interaction between these three factors ($F_{4,164} = 3.34$, $p = 0.01$). Other interactions did not reach statistical significance ($Fs < 2.37$, n.s.). Subsequently, we performed separated ANOVAs for each treatment to determine the source of this interaction. After treatment with vehicle and 2.5 mg/kg CORT, neither context nor genotype had an influence on the incubation time indices and these two factors did also not interact ($Fs < 3.05$, n.s.; **Figures 2A,B**, right panel). However, there was a significant interaction between context and genotype in mice treated with 5 mg/kg CORT ($F_{2,53} = 4.97$, $p = 0.01$; **Figure 2C**, right panel). *Post hoc* comparisons revealed no influence of incubation time on the freezing behavior of NPSR+/+ and NPSR+/− mice in context A and A′ ($ts < 0.92$, n.s.). In contrast, the incubation time had significantly fewer effects on the freezing behavior of NPSR−/− mice in context A than in context A′ ($t = 3.19$, $p = 0.007$).

In sum, the effect of incubation time on freezing in context A and A′ was not affected by treatment and genotype, except in 5 mg/kg CORT-treated NPSR−/− mice. In this group, the incubation time index for context A was significantly lower than for A′, indicating an impairment of contextual fear memory after the incubation time for the original fear conditioning context and/or an increase of contextual fear in the similar context A′. This means that there is a disbalance of contextual fear to context A and A′, which suggests a contextual fear memory generalization. We also compared the incubation time index of context B with those of context A. We found no overall main effects of context, genotype, or treatment, but an effect of genotype in the group of 5 mg/kg CORT-treated mice (**Supplementary Figure S2**, right panel; for original freezing data see **Supplementary Figure S3**).

Plasma CORT Levels

Plasma CORT Levels Were Enhanced After Fear Conditioning and Further Increased by Systemic CORT Injections

We were wondering how plasma CORT levels change after fear conditioning and after systemic injection of CORT. **Figure 3A** depicts the mean plasma CORT levels in the different groups, 30 min and 60 min after fear conditioning. For analysis, a multifactorial ANOVA using treatment and genotype as between-subject factors and time as a within-subject factor was performed. There were significant main effects of treatment ($F_{2,6} = 12.63$, $p = 0.01$) and time ($F_{2,12} = 20.56$, $p < 0.0001$), as well as an interaction between these two factors ($F_{4,12} = 12.08$, $p < 0.0001$). There were neither main effects of genotype ($F_{2,6} = 0.05$, $p = 0.96$) nor significant interactions with genotype ($Fs < 0.06$, n.s.). For further analysis, we performed ANOVAs for each treatment separately. In all three treatment groups, strong effects of time were found ($Fs < 23.91$, $ps < 0.0001$), whereas the factor genotype or the interaction time and genotype did not reach significance ($Fs < 0.24$, n.s.). Subsequent *post hoc* tests revealed a significant increase in CORT levels in all three genotypes 30 min and 60 min after fear conditioning ($Fs < 41.44$, $ps < 0.001$) in relation to the first baseline CORT levels (BL I). In 2.5 mg/kg and 5 mg/kg CORT-treated mice, CORT levels declined again after 60 min (compared with 30 min; $Fs < 26.86$, $ps < 0.001$). This decline was not found in vehicle-treated animals ($F = 0.16$, $p = 0.69$).

In conclusion, CORT levels were significantly increased 30 min after conditioning in all experimental groups. The intraperitoneal CORT injections immediately after conditioning strongly further enhanced CORT levels. Sixty minutes after conditioning, CORT levels were already decreased in 2.5 and 5 mg/kg CORT-treated animals. The NPSR genotype had no effects on these plasma CORT level changes and also not on baseline CORT levels (BL I) (**Figure 3B**).

CORT Treatment Increased CORT Levels After Exposure to Context A Compared With B (and A′) in the Recent Retention Test

The CORT levels after the recent and remote retention tests for contextual fear are depicted in **Figures 3C,D**. Separated ANOVAs with genotype as between-subject factor and context as a within-subject factor were calculated for each treatment. After the recent retention test, in vehicle-injected animals, neither context nor genotype had main effects on the CORT levels and there was no interaction ($Fs < 0.85$, n.s.; **Figure 3C**, left panel). However, there were significant context effects after injection of 2.5 mg/kg CORT ($F_{2,44} = 3.54$, $p = 0.0002$; **Figure 3C**, middle panel) and 5 mg/kg CORT ($F_{2,64} = 22.50$, $p < 0.0001$; **Figure 3C**, right panel). *Post hoc* comparisons revealed significantly increased CORT levels after exposure to context A compared to context B in 2.5 mg/kg CORT-treated NPSR+/− ($t = 4.09$, $p = 0.0004$) and NPSR−/− mice ($t = 3.47$, $p = 0.002$). Additionally, there were

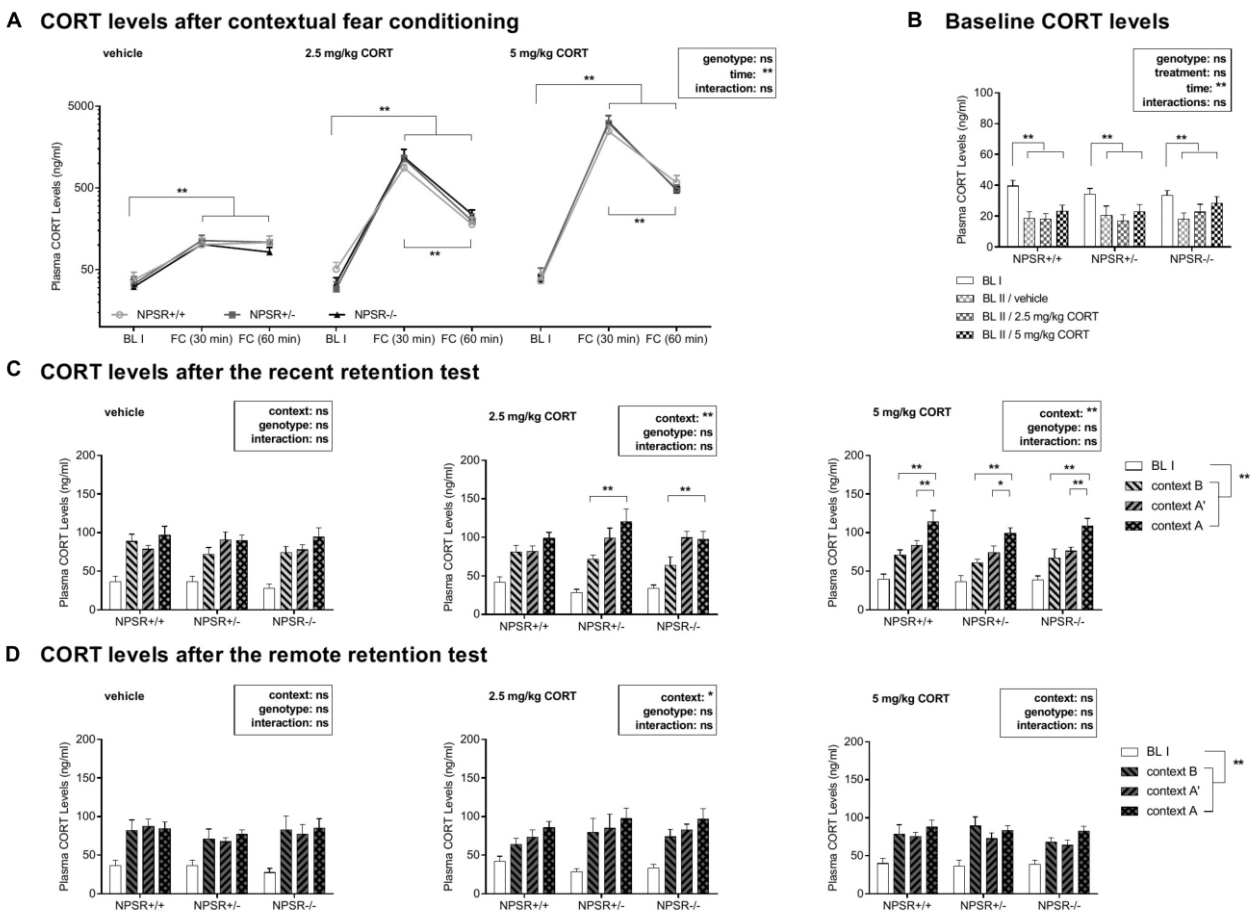

FIGURE 3 | Plasma CORT levels during the study. **(A)** CORT levels are enhanced after fear conditioning and CORT injections strongly further increased these levels. **(B)** The NPSR genotype did not affect baseline CORT levels before fear conditioning. After 1 month incubation time, baseline CORT levels were decreased in all genotypes. **(C,D)** Exposure to the different contexts in the retention tests induced an increase in CORT levels. **(C)** In the recent retention tests, this increase was more pronounced in the CORT-treated NPSR+/− and NPSR−/− mice. **(D)** However, this effect was not observed in the remote retention tests. Group sizes: **(A)** NPSR+/+: n = 12 to 35; NPSR+/−: n = 15 to 41; NPSR−/−: n = 14 to 34. **(B)** NPSR+/+: n = 33 to 35; NPSR+/−: n = 40 to 41; NPSR−/−: n = 33 to 34. **(C)** NPSR+/+: n = 27; NPSR+/−: n = 27; NPSR−/−: n = 31. **(D)** NPSR +/+ : n = 27; NPSR+/−: n = 28; NPSR−/−: n = 28. Line and bar diagrams show the means + SEMs. *$p < 0.05$; **$p < 0.01$; *post hoc* Holm-Sidak's comparisons after ANOVA.

increased CORT levels after exposure to context A compared to contexts B and A′ in all genotypes treated with 5 mg/kg CORT ($ts < 3.45$, $ps < 0.03$).

Taken together, 2.5 and 5 mg/kg CORT-treated mice showed increased CORT levels after exposure to context A compared with B (and A′) in the recent retention test.

After Incubation Time, Baseline CORT Levels Were Generally Decreased

After incubation time, baseline CORT levels (BL II) were measured again before submitting the mice to the remote retention test (**Figure 3B**). These levels were compared with baseline CORT levels of day 0 (BL I) and the influence of treatment and genotype as between-subject factors and time as a within-subject factor on the baseline CORT levels were examined by a multifactorial ANOVA. This analysis showed a main effect of time ($F_{1,95} = 45.71$, $p < 0.0001$) but no main effects of genotype ($F_{2,95} = 0.34$, $p = 0.71$), treatment ($F_{2,95} = 1.83$, $p = 0.17$), or interactions between these factors ($Fs < 0.38$, n.s.). Separated ANOVAs for each genotype were performed after. In all genotypes, CORT levels were significantly lower after incubation time (BL II) than before fear conditioning (NPSR+/+: $F_{1,30} = 22.04$, $p < 0.0001$; NPSR+/−: $F_{1,37} = 10.39$, $p = 0.003$; NPSR−/−: $F_{1,28} = 18.30$, $p < 0.0001$). Treatment did not influence baseline CORT levels in NPSR+/+ and NPSR+/− ($Fs < 0.19$, n.s.); however, there was a trend for increased CORT levels (BL II) in NPSR−/− mice ($F_{2,28} = 2.57$, $p = 0.09$). Then, when the mice were exposed to different contexts in the remote retention test (**Figure 3D**), the context did only have a weak effect on CORT levels. There was only a significant main effect of context in 2.5 mg/kg CORT-treated mice ($F_{2,46} = 3.54$, $p = 0.04$).

Overall, baseline CORT levels were decreased after an incubation time of 1 month in all three genotypes. No significant difference between different genotypes or treatments was observed. In addition, the different contexts in the remote retention test did not differently affect CORT levels.

Treatment With 2.5 and 5 mg/kg CORT Decreased Fear Inhibition by Previously Unpaired Stimuli in NPSR+/+ Mice but Not in NPSR+/− and NPSR−/− Mice

During fear conditioning, also two tones T were presented explicitly unpaired to the electric foot shocks. These tones were presented to test whether such unpaired stimuli would maybe (erroneously) be associated with the aversive foot shocks and thereby later be able to induce fear. Surprisingly, presentations of this tone T and also a similar tone T′ during exposures to contexts A and A′ robustly and significantly inhibited freezing in both recent and remote retention tests (one-sample *t* tests, comparison with zero, i.e., no change in freezing: *t*s > 2.13, *p*s < 0.05). Notably, tone T had no effects on freezing during the conditioning procedure (**Figure 4A**). Moreover, presentations of the novel stimulus N during exposures to contexts A and A′ had also no effects in freezing inhibition (**Supplementary Figure S4**). This suggests that tone T was learned as a safety signal and that this memory was generalized to the similar tone T′. Importantly, all three stimuli (T, T′, and N) had no robust effects in the different context B, in which also less freezing was expressed (cf. **Supplementary Figure S3**). Hence, only the mean inhibition of freezing by the tones T and T′ during exposures to contexts A and A′ are depicted in **Figure 4B**. To analyze the safety effects of the tones, we calculated the mean difference between freezing during the tone presentations and freezing in the minute before tone presentation. Separated ANOVAs for each genotype revealed a significant treatment effect in NPSR+/+ mice ($F_{2,56}$ = 5.63, p = 0.006; **Figure 4B**) but no effects of time or an interaction of time and treatment (*F*s < 1.54, n.s.). *Post hoc* comparisons with vehicle treatment showed decreased safety responses in 2.5 and 5 mg/kg CORT-treated NPSR+/+ mice in the recent retention tests (*t*s > 2.11, *p*s < 0.04) and in 5 mg/kg CORT-treated NPSR+/+ mice in the remote retention test after 1 month (t = 2.55, p = 0.02). In NPSR+/− and NPSR−/− mice, neither treatment nor time affected the difference scores, and there was no interaction (*F*s < 2.06, n.s.; **Figure 4B**).

Taken together, treatment with 2.5 mg/kg and 5 mg/kg CORT decreased fear inhibition by stimuli T and T′ in NPSR+/+ but not in NPSR+/− and NPSR−/− mice.

Anxiety-Like Behavior in the Light–Dark Box Test Was Not Affected

The light–dark box test was used to evaluate potential changes in anxiety-like behavior. **Figure 5** (middle panel) depicts the percent time the animals spent in the bright compartment. Additionally, we analyzed the distance traveled, latency, and number of entries to the bright compartment during the test (**Supplementary Figure S5**). A multifactorial ANOVA using treatment, genotype, and sex as between-subject factors was used to analyze the data. The time the animals spent in the bright compartment was not affected by any of these factors. Additionally, no interactions (*F*s < 0.84, n.s.) were found, despite a significant interaction between all these three factors ($F_{4,128}$ = 2.51, p = 0.045). Separated ANOVAs by treatment were used to identify the cause of this

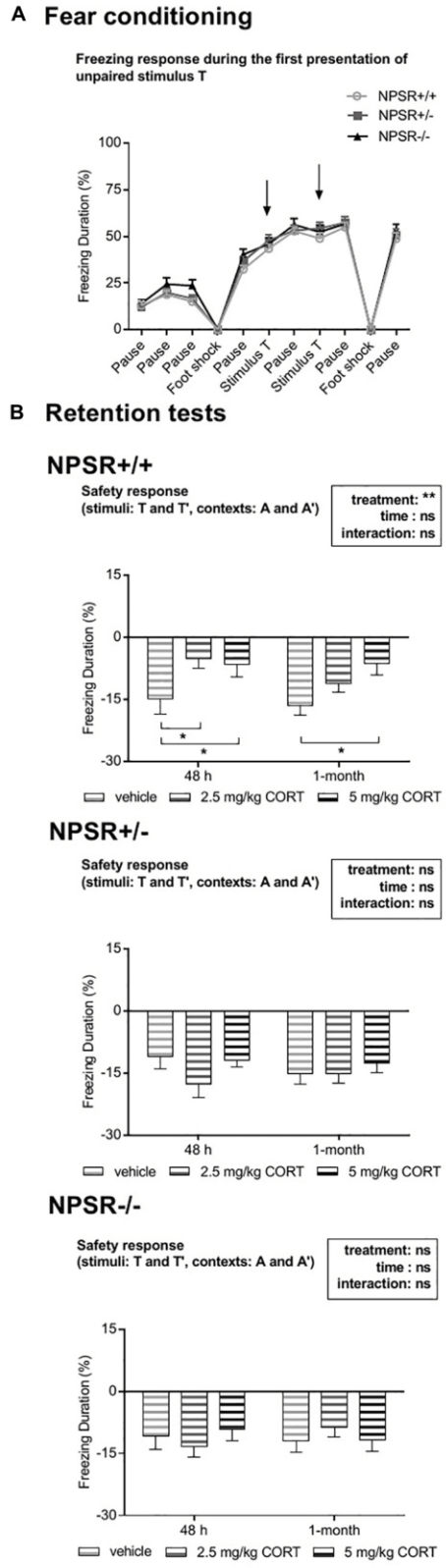

FIGURE 4 | Effects of explicitly unpaired tone stimuli on contextual fear. **(A)** During fear conditioning, presentations of the tone T (arrows) did not affect *(Continued)*

FIGURE 4 | Continued
freezing behavior. **(B)** Presentations of T and T′ during the different retention tests reduced contextual fear, indicating fear inhibition (i.e., a safety effect). **(B)** In CORT-treated NPSR+/+ mice, this inhibition was significantly reduced. Group sizes: NPSR +/+ : $n = 65$; NPSR+/−: $n = 62$; NPSR−/−: $n = 46$. Line and bar diagrams show the means + SEMs. *$p < 0.05$; *post hoc* Holm-Sidak's comparisons after ANOVA.

interaction, however, no further effects were found (Fs < 2.41, n.s.). Analysis of the other behavioral readouts provided very similar results (**Supplementary Figure S5**).

Overall, anxiety-like behavior was not affected in the present study.

5 mg/kg CORT-Treated NPSR−/− Mice Showed Decreased Startle Responses After the Incubation Time

The acoustic startle response paradigm was chosen to evaluate potential changes in stimulus reactivity and/or arousal (**Figure 5**, left panel). A multifactorial ANOVA using treatment, sex, and genotype as between-subject factors and startle stimulus intensity as a within-subject factor was performed. In general, male mice had higher startle magnitudes than female mice ($F_{1,126} = 4.67$, $p = 0.03$) but no interactions between sex and other factors were found (Fs < 1.70, n.s.). The ANOVA further revealed a main effect of startle stimulus intensity ($F_{7,945} = 141.57$, $p < 0.0001$) but not of genotype ($F_{2,135} = 0.80$, $p = 0.45$) or treatment ($F_{2,135} = 0.87$, $p = 0.42$), and there was no interaction between these factors ($F_{4,135} = 0.51$, $p = 0.73$). This analysis was mainly confirmed by separated ANOVAs for each treatment. Startle magnitude was affected by startle (stimulus) intensity (Fs > 14.80, ps < 0.0001) but not by genotype (Fs < 2.61, n.s.). However, a significant interaction between stimulus intensity and genotype was found in 5 mg/kg CORT-treated mice ($F_{14,259} = 1.79$, $p = 0.04$; **Figure 5C**, left panel) but not after vehicle or 2.5 mg/kg CORT treatment (Fs < 0.41, n.s.; **Figures 5A,B**, left panel). *Post hoc* tests revealed that 5 mg/kg CORT-treated NPSR−/− mice had significantly decreased startle magnitudes after stimulus intensities of 114 and 120 dB SPL.

In conclusion, after the incubation time, only 5 mg/kg CORT-treated NPSR−/− mice expressed decreased startle magnitudes at higher startle stimulus intensities.

5 mg/kg CORT-Treated NPSR−/− Female Mice Showed No Significant Body Weight Gain With the Incubation Time

We further tested whether the body weight of the mice was affected by sex, genotype, treatment, or time (from d0 to d39; **Figure 5**, right panel). We performed a multifactorial ANOVA with treatment, sex, and genotype as between-subject factors and time as a within-subject factor. There were no main effects of genotype or treatment (Fs < 1.06, n.s.). However, there were main effects of sex ($F_{1,124} = 256.06$, $p < 0.0001$) and time ($F_{1,124} = 298.06$, $p < 0.0001$) as well as a significant interaction between these two factors ($F_{1,124} = 24.37$, $p < 0.0001$). Moreover, we found interactions of genotype and treatment ($F_{4,124} = 2.84$, $p = 0.03$), time, genotype, and treatment ($F_{4,124} = 2.61$, $p = 0.04$), and time, genotype, sex, and treatment ($F_{4,124} = 3.11$, $p = 0.02$). All other interactions did not reach statistical significance. To identify the source of these interactions, we then performed ANOVAs for each sex and treatment separately. Separated ANOVAs revealed that body weight was affected by time (Fs > 17.63, ps < 0.0005) but not by genotype (Fs < 2.13, n.s.) in all experimental groups. Additionally, a trend for an interaction between time and genotype was found in 5 mg/kg CORT-treated female mice ($F_{2,18} = 2.50$, $p = 0.11$; **Figure 5C**, right panel) but not after vehicle or 2.5 mg/kg CORT treatment (Fs < 0.56, n.s.). *Post hoc* tests revealed a significant body weight gain for males and females, independent of the genotype, in all three treatment groups (ts > 2.84, ps < 0.01) but not in NPSR−/− female mice after 5 mg/kg CORT treatment ($t = 0.53$, $p = 0.60$).

Taken together, male mice generally had a higher body weight gain than females. Notably, 5 mg/kg CORT-treated NPSR−/− female mice expressed no body weight gain during this experiment.

DISCUSSION

The main goal of this study was to investigate the hypothesis that NPSR-deficient mice with high CORT levels during fear memory consolidation are more prone to develop generalized fear. In order to study the development of fear memory generalization, we used contextual fear conditioning and systemically injected CORT during the consolidation of fear memories. Fear memory generalization was evaluated by measuring two parameters: the strength of the fear memory and the specificity of the fear memory. To investigate the strength of the fear memory, we submitted the same group of mice to both a recent and a remote memory test, which usually improves the fear memories in the long term, i.e., it can prevent the generalization of fear memory. This allowed in some experimental groups a more specific investigation of the possible interplay between CORT and the NPS system in the development of generalized fear memory. To investigate the specificity of fear memory, we exposed the mice to three different contexts. Two of them were very similar to each other (conditioning context A and context A′) and one of them was different (context B).

In our analyses, we first examined whether incubation time, treatment, genotype, and sex affected the specificity of fear memory in different contexts, i.e., generalization of fear memory. We observed that at both experimental time points (24–48 h and 1 month after fear conditioning), all treatment and genotype groups, regardless of the sex, showed a relatively specific fear memory to the conditioning context A (**Figure 2**, **Supplementary Figure S2**, left panels; for original freezing data see **Supplementary Figures S1, S3**), except the group of 5 mg/kg CORT-treated NPSR−/− mice. These animals expressed very similar fear responses in the two similar contexts (A and A′) after incubation time; i.e., they did not discriminate between these two contexts any more (**Figure 2C**, left panel; for original freezing

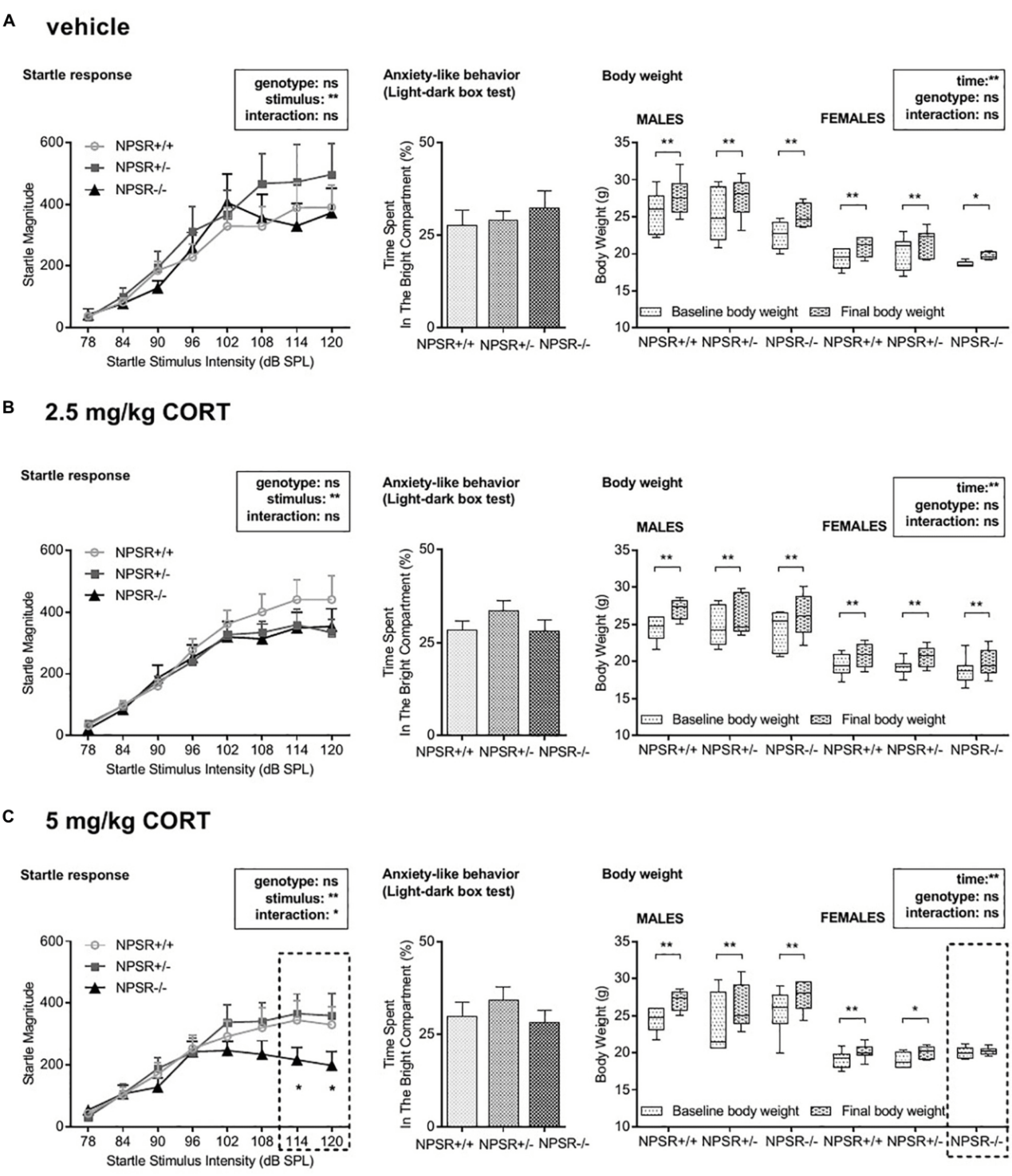

FIGURE 5 | Acoustic startle magnitudes (left panel), anxiety-like behavior in the light–dark box test (middle panel), and body weight gain (right panel). **(A)** The magnitude of the acoustic startle response was generally dependent on the startle stimulus intensity. Genotype and CORT treatment had no effects except in 5 mg/kg CORT-treated NPSR−/− mice, in which the startle response to high stimulus intensities was impaired. **(B)** Neither CORT treatment nor genotype affected anxiety-like behavior in the light–dark box. **(C)** Male mice had generally more body weight gain than females. During the experiment, body weight was increased independently of genotype and treatment except for 5 mg/kg CORT-treated NPSR −/− female mice. Group sizes: NPSR +/+ : $n = 54$; NPSR+/−: $n = 50$; NPSR−/−: $n = 40$. Data are illustrated with bar and line diagrams (means + SEMs; left and middle panel) or box plots (median, quartiles) and Tukey's whiskers (right panel). $^{*}p < 0.05$; $^{**}p < 0.01$; *post hoc* Holm-Sidak's comparisons after ANOVA.

data see **Supplementary Figure S1**). This impaired specificity of fear memory indicates that a synergistic interplay of CORT treatment and NPSR deficiency interferes with the consolidation of fear memories and induces fear memory generalization after incubation time. This generalization may mirror the maladaptive state that can be measured in, e.g., PTSD patients.

We further examined whether treatment, genotype, and sex affected the strength of the fear response in a particular context with incubation time (**Figure 2** and **Supplementary Figure S2**, right panels; for original freezing data see **Supplementary Figures S1, S3**). The incubation time index was not affected by treatment and genotype, regardless of the sex, except again in the group of NPSR−/− mice treated with 5 mg/kg CORT. Here, the incubation time index for context A was significantly lower than for A′, indicating an impairment of contextual fear memory after the incubation time for the original fear conditioning context and/or an increase of contextual fear in the similar context A′.

Both the decrease in the strength of fear memory after incubation time and the impaired specificity of the fear memory discussed above underlines our hypothesis. NPSR deficiency and high CORT levels during fear memory consolidation support the generalization of fear memory, here induced by incubation time. Previous studies have shown that CORT injections after fear conditioning training or incubation time alone lead to a generalization of fear memories in wild-type animals (e.g., Cordero and Sandi, 1998; Siegmund and Wotjak, 2007; Kaouane et al., 2012; Sauerhofer et al., 2012) (but see: Bueno et al., 2017). We could not observe such a generalization in our NPSR+/+ littermates and NPSR+/− mice. However, it has been shown that exposing animals to more than one retention test improves the specificity of fear memories in the long term, i.e., prevents the development of generalized fear memory (De Oliveira Alvares et al., 2013; Bueno et al., 2017). These findings explain the absence of fear generalization in our NPSR+/+ littermates and NPSR+/− mice after CORT treatment and incubation time. However, adding a further factor, i.e., NPSR deficiency, leads to fear memory generalization in our study. This clearly supports the idea that the NPS system is involved in fear memory generalization.

In addition to freezing behavior, we also measured the response of the mice to a tone stimulus (T) that was previously presented during the fear conditioning session, as well as to a similar tone T′ and a novel sound N (**Figure 1D**). Importantly, tone T had not predicted the unconditioned stimuli during fear conditioning but had been presented explicitly unpaired (**Figure 1C**). In fact, such explicitly unpaired stimuli can be learned as a safety stimulus (for review, see: Kong et al., 2014) but we did not expect such a learning, since only two tone stimuli were presented in our protocol, whereas in safety learning studies, typically much more of these explicit unpairings are presented (>12; e.g., Pollak et al., 2010a; Pollak et al., 2010b; Khalil and Fendt, 2017). Our original aim was to present these stimuli to test whether such explicitly unpaired stimuli would erroneously be associated with the unconditioned stimuli and thereby later be able to induce fear which would be a further sign of fear generalization. However, we observed robust inhibition of contextual freezing during the presentation of the tones T and T′ (**Figure 4B**) in the retention tests. Importantly, tone T did not affect freezing behavior during the conditioning session (**Figure 4A**), indicating that this fear inhibition during the retention tests was learned and most probably reflects a safety response. In NPSR+/+ mice, this effect of the tones T and T′ was reduced by CORT treatment. However, CORT treatment did not affect the tone effects in NPSR+/− and NPSR−/− mice. In human anxiety disorders, an impaired safety response has been repeatedly reported (e.g., Lohr et al., 2007; Jovanovic et al., 2009, 2012; Lissek et al., 2009; Norrholm et al., 2013; Sijbrandij et al., 2013). In the present study, CORT treatment only impaired the safety response in NPSR+/+ mice but not in NPSR+/− and NPSR−/− mice, despite the latter showing a generalization of fear memory. This indicates a beneficial effect of NPSR deficiency in learned safety (since CORT had no effects) and is in line with recent findings of our laboratory showing that NPSR-deficient mice have more pronounced safety learning (Kreutzmann et al., 2019). Of note, the observation that NPSR deficiency and CORT treatment differently interact in fear generalization and fear inhibition argues for dissociative mechanisms underlying these two phenomena.

Regarding CORT levels, dysregulations in HPA axis functioning and the connected changes in CORT levels were implicated in the development of anxiety disorders (for review, see: de Kloet et al., 2005; Chrousos, 2009). In our study, we systematically measured CORT levels throughout the different phases of the experiment with the aim to understand how changes in CORT plasma levels may be associated with the generalization of fear memories. As expected, we observed a significant increase in CORT levels 30 min after conditioning in all experimental groups (**Figure 3A**) showing that CORT levels robustly reflect the stress by fear conditioning. As intended, CORT injections after fear conditioning strongly enhanced the CORT levels. In the first/recent retention test, all mice injected with 5 mg/kg CORT had higher CORT levels after exposure to the conditioning context than to the similar or different context (**Figure 3C**, right panel). Considering 2.5 mg/kg CORT injections, this effect was only observed in NPSR+/− and NPSR−/− mice and not in the wild-type littermates. This suggests that the NPS system is involved in the modulation of CORT release during fear retention by previous CORT injections. Elevated plasma CORT levels during the consolidation of fear memories might be related to increased CORT levels in the stress response in early retention tests and NPSR deficiency seems to affect this process. Interestingly, this effect of CORT treatment was not observed in the remote retention test after 1 month of incubation (**Figure 3D**).

We further measured consistently lower levels of baseline CORT after 1-month incubation time (i.e., before the remote retention tests) in all genotype and treatment groups. Our observations regarding the CORT levels are in line with observations in PTSD patients showing lower baseline levels of cortisol and higher cortisol levels following exposure to trauma reminders (for review, see: Pan et al., 2018).

Several studies have shown a functional cross-regulation of the NPS system and the HPA axis. NPS is released in the basolateral amygdala upon stress (Ebner et al., 2011) and NPS injections into the ventricle increase CORT levels (Smith et al., 2006), suggesting a bidirectional interaction of the NPS system and the HPA axis. In humans, a functional variant of the NPSR1 gene has been associated with higher levels of cortisol (Streit et al., 2017). However, in the present study, there were no effects of the NPSR genotype on baseline CORT levels or on the levels after exposure to the different contexts. This is in line with previous reports showing no difference in CORT levels in NPSR−/− mice during baseline or after forced swim stress (Zhu et al., 2010) and suggests compensatory mechanisms in NPSR-deficient mice.

Moreover, we submitted animals to the light–dark box test to evaluate the innate fear of the mice (Bourin and Hascoet, 2003). We did not observe any changes in anxiety-like behavior in our experimental groups (**Figure 5**, middle panel; **Supplementary Figure S5**). Previous reports found that NPSR−/− showed more anxiety-like behavior in the light–dark box as compared to NPSR+/− mice and the wild-type littermates (e.g., Zhu et al., 2010; Germer et al., 2019). However, the present study has only investigated anxiety-like behavior at the very end of the experiment, i.e., after fear conditioning and a total of six different retention tests. Nevertheless, we were able to show that impairment of the specificity and strength of the fear memory in NPSR−/− mice did not affect their anxiety-related behavior.

In addition, we also tested the startle response of the mice. As previously reported by others (Zhu et al., 2010; Fendt et al., 2011; Germer et al., 2019), we observed higher startle magnitudes in male mice. While previous studies observed a decreased startle response in NPSR+/− and NPSR−/− mice, we did not observe such genotype effects within the vehicle or 2.5 mg/kg CORT-treated groups. Interestingly, we only observed such a decrease in the startle response in NPSR−/− mice treated with 5 mg/kg CORT (**Figure 5C**, left panel). In this group, startle magnitudes to higher startle stimulus intensities were significantly lower than in 5 mg/kg CORT-treated NPSR+/+ and NPSR+/− mice. Notably, abnormalities in the expression of the startle response are commonly observed in anxiety disorders (for review, see: Beck and Catuzzi, 2012). Whereas exaggerated startle magnitudes are often reported in PTSD, there are also many studies reporting blunted startle reactivity in PTSD patients. Such blunted startle reactivities are also observed in a number of animal studies using different forms of stress. Importantly, such a reduction in the startle reactivity could not be attributed to enhanced habituation to the startle stimuli (for review, see: Beck and Catuzzi, 2012). This again supports the idea that NPSR deficiency and high CORT levels during fear memory consolidation lead to behavioral changes that are similar to those of PTSD patients.

Disturbances in the body weight are often described in response to acute or chronic stress as well as to CORT treatment (for review, see: Harris, 2015; van der Valk et al., 2018). We observed a significant increase in the body weight of all mice but interestingly not in the group of NPSR−/− mice treated with 5 mg/kg CORT, i.e., the only group that expressed a generalized fear memory and decreased startle response. However, this effect was only observed in female mice. This suggests an NPS/CORT-related hormonal imbalance in the female mice of our experiment which seems to affect body weight gain.

In summary, the present study shows that a lack of NPSR gene and high CORT levels during fear memory consolidation make mice more prone to develop a fear memory generalization over time. Notably, this fear memory generalization did not affect the anxiety-related behavior of these mice but was associated with the reduced startle response magnitudes and in females with less body weight gain. Notably, NPSR+/− mice displayed the same behavioral phenotypes as the wild-type littermates indicating that one functional copy of NPSR is sufficient for normal behavior. In our view, these findings indicate a complex interplay between the NPS system, the HPA axis, and incubation time and constitute an initial step toward finding the mechanisms underlying the development of fear memory overgeneralization. Moreover, the present data may help to explain why human polymorphisms in the NPSR gene are associated with behavioral endophenotypes of overgeneralization ("catastrophizing"; Raczka et al., 2010) and thereby also with a higher probability to develop anxiety disorders (Domschke et al., 2011; Klauke et al., 2014).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Landesverwaltungsamt Sachsen-Anhalt.

AUTHOR CONTRIBUTIONS

Both authors designed the research study, wrote the manuscript, and analyzed the data. MK performed all the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00128/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

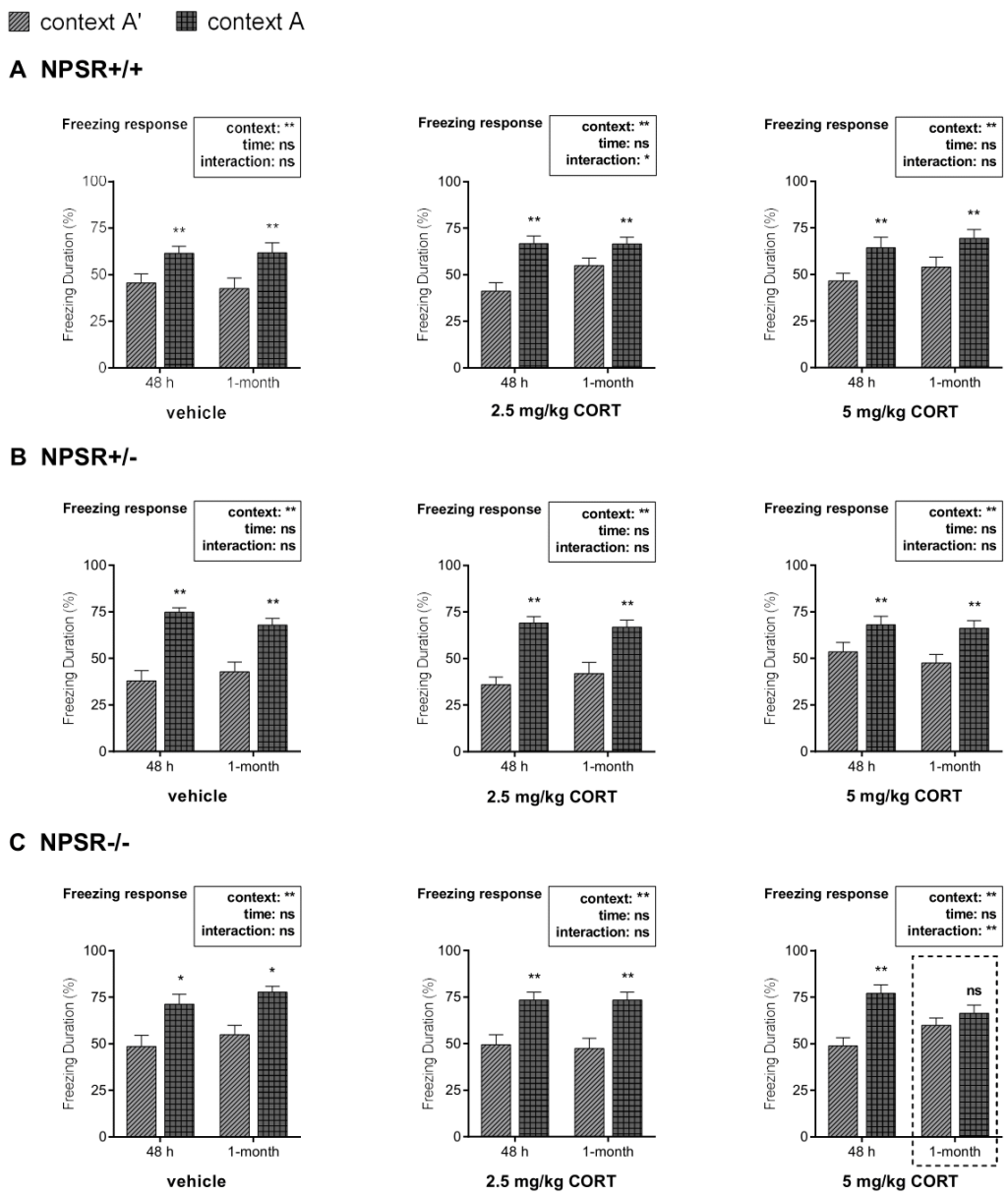

Figure S1. Effects of corticosterone CORT treatment on the freezing response during recent (48 h) and remote (1-month) retention tests in NPSR-deficient mice (context A' vs. A). Separated ANOVAs for each combination of different genotypes (**(A)** NPSR+/+, **(B)** NPSR+/- and **(C)** NPSR-/-) and treatments identified a main effect of context in all experimental groups (Fs < 10.33, ps < 0.0001) and an interaction between time and context in 2.5 mg/kg CORT-treated NPSR+/+ and 5mg/kg CORT-treated NPSR-/- mice (Fs < 7.38, ps < 0.0008). *Post hoc* tests revealed that all experimental groups showed significantly less freezing during exposure to context A' in comparison to context A, independent of the time point of the exposure (ts < 3.22, ps < 0.0001) with the exception of 5 mg/kg CORT-treated NPSR-/- mice. The freezing response of these mice in context A' and context A did not differ in the remote retention test (t = 1.71, p = 0.20; dashed frame) indicating generalized contextual fear. Group sizes: NPSR+/+: n = 65; NPSR+/-: n = 62; NPSR-/-: n = 46. Bar diagrams show the means + SEMs. *p < 0.05; **p < 0.01; *post hoc* Holm-Sidak's comparisons after ANOVA. For further details see text.

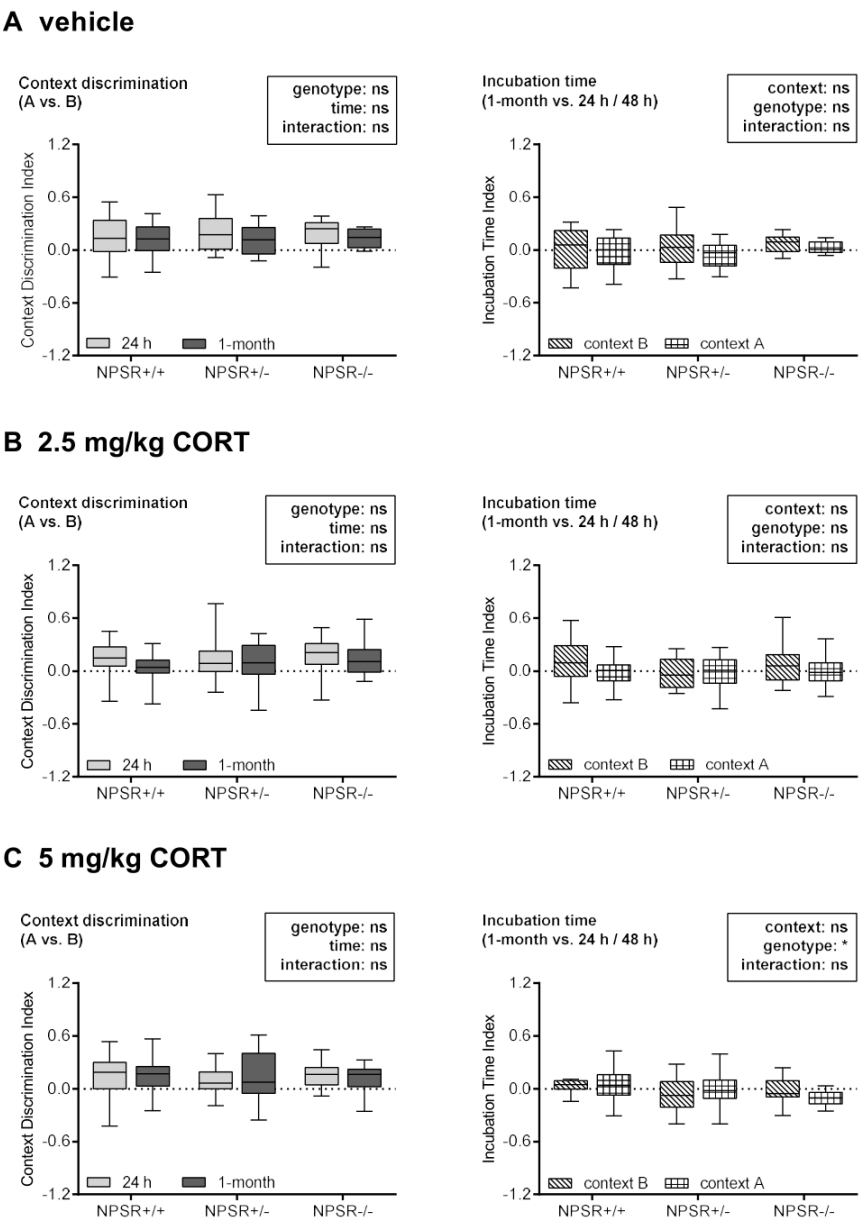

Figure S2. Effects of CORT treatment on specificity and strength of contextual fear memory (context A vs. B) in NPSR-deficient mice. The left panel depicts the context discrimination index. The higher the values of this index are, the more specific is the memory. The right panel depicts the incubation time index (1-month vs. 24 h / 48 h). Index values close to zero indicate no change in the strength of the memory, negative values less and positive values more freezing in the remote memory test vs. the recent memory test. We found no overall main effects of context, genotype, or treatment. That means that injections of **(A)** vehicle, **(B)** 2.5 mg/kg CORT and **(C)** 5 mg/kg CORT did neither affect specificity nor strength of the contextual memory. Group sizes: NPSR+/+: n = 65; NPSR+/-: n = 62; NPSR-/-: n = 46. Data were illustrated with box plots (median, quartiles) and Tukey's whiskers. For further details see text.

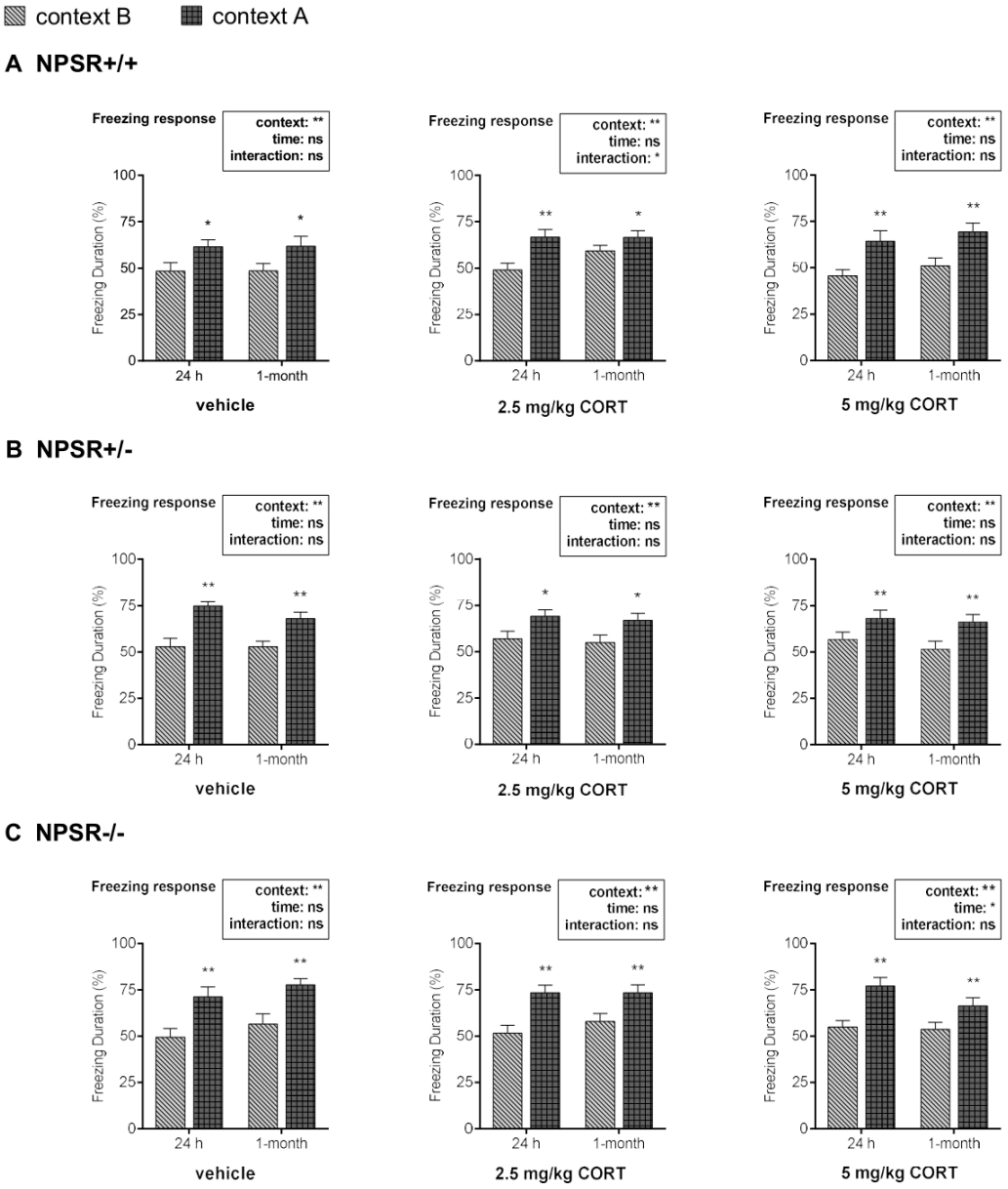

Figure S3. Effects of corticosterone treatment on the freezing response during recent and remote contextual retention tests in NPSR-deficient mice (context B vs. A). Separate ANOVAs for each combination of different genotypes (**(A)** NPSR+/+, **(B)** NPSR+/- and **(C)** NPSR-/-) and treatments identified a main effect of context in all experimental groups (Fs < 8.29, ps < 0.0001), an effect of time in 5 mg/kg CORT-treated NPSR-/- mice ($F_{1, 16} = 5.04$, p = 0.04, right panel) and an interaction between time and context in 2.5 mg/kg CORT-treated NPSR+/+ mice ($F_{1, 26} = 6.06$, p = 0.02, middle panel). *Post hoc* tests revealed that all experimental groups showed significantly less freezing during exposure to context B in comparison to context A despite the time point of the exposure (Fs < 2.40, ps < 0.0001), indicating that neither different genotypes nor treatment affected the contextual memory of the tested animals. Group sizes: NPSR+/+: n = 65; NPSR+/-: n = 62; NPSR-/-: n = 46. Bar diagrams show the means + SEMs. *p < 0.05; **p < 0.01; *post hoc* Holm-Sidak's comparisons after ANOVA. For further details see text.

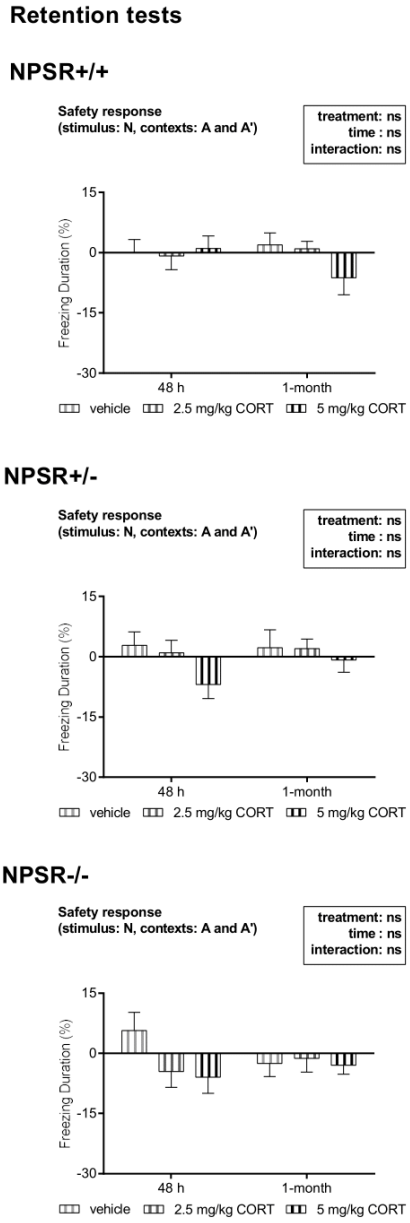

Figure S4. Effects of white noise stimulus on contextual fear. Presentations of white noise during the different retention tests did not influence contextual fear. Group sizes: NPSR+/+: n = 65; NPSR+/-: n = 62; NPSR-/-: n = 46. Bar diagrams show the means + SEMs.

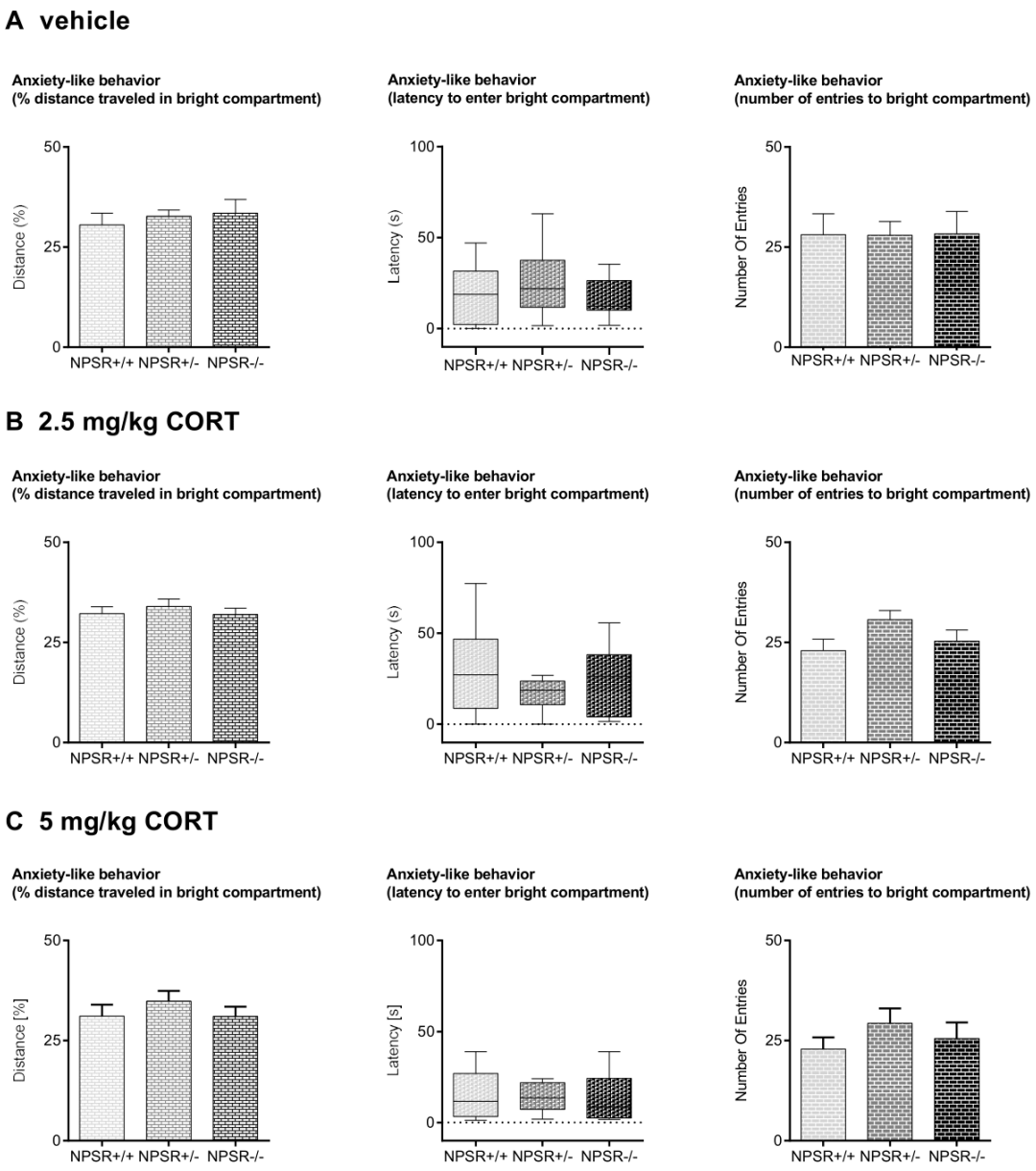

Figure S5. Anxiety-like behavior in several readouts of the light-dark box test. Percent distance traveled in the bright compartment (left panel), latency to enter the bright compartment (middle panel), and the number of entries to the bright compartment (right panel). Multifactorial ANOVAs neither identified main effects of genotype nor treatment (**(A)** vehicle, **(B)** 2.5 mg/kg CORT and **(C)** 5 mg/kg CORT) nor an interaction between these factors. Group sizes: NPSR+/+: n = 54; NPSR+/-: n = 50; NPSR-/-: n = 40. Data were illustrated with bar diagrams showing the means + SEMs (left and right panel) or with box plots (median, quartiles) and Tukey's whiskers (middle panel).

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Sociability and extinction of conditioned social fear is affected in neuropeptide S receptor-deficient mice

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ABSTRACT

Being cautious of unfamiliar conspecifics is adaptive because sick or aggressive conspecifics may jeopardize survival and well-being. However, prolonged or excessive caution, i.e. fear related to social situations, is maladaptive and may result in social anxiety disorder. Some anxiety disorders in humans are associated with polymorphisms of the neuropeptide S receptor (NPSR) gene. In line with this finding, animal studies showed an important role of NPS and NPSR in anxiety and fear. The present study investigated the role of NPSR deficiency in social behavior under non-aversive and aversive conditions. For this, female and male NPSR-deficient mice were tested for (1) sociability and social novelty and (2) acquisition, expression, and extinction of conditioned social fear. The present study revealed very particular effects of the NPSR genotype: Sociability was reduced in female heterozygous NPSR-deficient mice, but was unaffected in males and the other genotypes. Furthermore, the NPSR genotype did not affect the acquisition and expression of conditioned social fear, but its extinction was impaired in heterozygous and facilitated in homozygous NPSR-deficient mice. This indicates that the NPS system plays a role in social behavior under non-aversive and aversive conditions, partly in a sex-dependent manner. The present findings may help to explain social symptoms in anxiety disorders associated with the NPSR genotype.

1. Introduction

Being cautious when encountering unfamiliar members of the own species is an adaptive strategy of animals and humans to avoid potential harm. If the latter happens, it may be even adaptive to fear unfamiliar conspecifics. However, if such fear (i.e. social fear) is prolonged, excessive, or generalized to familiar conspecifics, it becomes maladaptive. In humans, this may result in social anxiety disorder (SAD), also known as social phobia. Although SAD is the third most prevalent psychiatric disorder and has a chronic course [1,2], its etiology and neuropathology are still poorly understood. SAD is characterized by experiencing excessive fear and avoidance of most social situations, e.g. initiating conversations, attending social events, and even going to the doctor when the concerned patient feels unwell. Consequently, SAD patients show impairments across various life domains including education, employment, family, romantic relationships, and friendships. Therefore, the quality of life among SAD patients before, but also during and after treatment is relatively low [e.g. 3–5]. The current SAD treatment consists of cognitive-behavioral therapy [6,7] and medications such as antidepressants, benzodiazepines, anticonvulsants, or the neuroleptic olanzapine [8]. However, since the efficacy of these treatments is far from being satisfactory [for review see: 9], more efficient treatments are needed to improve treatment outcomes for SAD patients. Hence, it is necessary to better understand the neurobiological mechanisms underlying the etiopathogenesis and the expression of social fear.

The neuropeptide S (NPS)/NPS receptor (NPSR) system is a neuropeptide system in the brain that may play an important role in social fear. NPS is a 20 amino acids long neuropeptide with a highly conserved primary structure among vertebrates, including humans [10]. While there are only around 500 NPS–positive neurons found in two brain stem nuclei [11], NPSR is widely expressed in the rodent brain including the amygdala, which plays a determinant role in the control of social behavior, fear, and anxiety, as well as in the prefrontal cortex,

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which has an essential role in social fear expression [12].

Interestingly, various clinical studies have identified an association between NPSR gene polymorphisms and the incidence of some anxiety disorders and related behavioral endophenotypes [e.g. 13,14]. Nonetheless, to the best of our knowledge, direct associations between NPSR gene polymorphisms and SAD have not been found so far. However, animal studies found a role of the NPS system in social behaviors. Intracerebroventricular (icv) NPS injections dose-dependently reduced social fear induced by either social fear conditioning (SFC) or social defeat [15]. Of note, NPS injections had no effects in a normal sociability test, i.e. when the animals had no aversive social experiences before [15,16]. In the resident-intruder test, NPS injections reduced aggressive behavior in rats and mice, whereas NPSR-deficient mice showed elevated levels of aggressive behavior [17,18]. Taken together, this led to our hypothesis that the NPS system is involved in the modulation of social behavior under aversive conditions, but not under non-aversive conditions.

The present study aimed to investigate this hypothesis in female and male NPSR-deficient mice. These mice were used in many studies focused on fear and anxiety, locomotor activity, and reward processing [e.g. 18–22], but are poorly characterized regarding social behavior. Here, we tested heterozygous and homozygous NPSR-deficient mice and their wild-type littermates in two behavioral paradigms. In the first experiment, the effects of NPSR deficiency on sociability and social novelty behavior were tested in a modified version of Crawley's sociability test [23]. In the next experiment, mice were submitted to an SFC protocol, in which approaches to an unfamiliar conspecific were punished [12,24]. Using sociability tests, the effects of NPSR deficiency on the acquisition, expression, and extinction of conditioned social fear were evaluated.

2. Material and methods

2.1. Animals

For all experiments, two- to four-month-old experimentally naive male and female mice from our breeding colony were used. Genotyping was performed using a standard end-point PCR (DreamTaqTM Green PCR, Thermo Fisher Scientific, Vilnius, Lithuania) with adequate primers [20]. Mice were housed in single-sex and mixed-genotype groups during the whole experiment (experiment 1) or only until the start of the experiment and then housed individually (experiments 2 and 3). For housing, standard cages in a temperature- and humidity-controlled room, with food and water available *ad libitum*, and a 12 h light/dark cycle (lights on at 06:00 h) were used. The experiments took place during the light phase. All animal care and behavioral tests were conducted in compliance with the European regulations for animal experiments (2010/63/EU) and were approved by the local authorities (Az. 42502-2-1351 & 42502-3-747 UniMD).

2.2. Behavioral tests - setups and procedures

2.2.1. Experiment 1: Sociability test

2.2.1.1. Setup. We used a system consisting of two separated sociability boxes (46cm × 23cm × 32 cm) with black opaque walls and a grey floor that allowed us to test two individual mice in parallel. Both boxes were placed inside a sound-attenuating chamber provided with mild illumination (22 − 32 lx) and a camera on the ceiling for recording the test. In two opposite corners of each of these boxes two wire mesh cages (diameter: 8.5 cm, height: 10 cm) were localized for enclosing social partners. On top of these cages, weighted bottles were placed to prevent the test mice to climb on top of them.

2.2.1.2. Sociability test procedure. Before the mice were submitted to the sociability test, they were handled by the experimenter for 2 × 5 min daily for two consecutive days. Additionally, the social partner mice were habituated to the wire mesh cages with the same regularity.

On the third day, the mice were tested for sociability. Our protocol consisted of three phases: habituation phase, sociability phase, and social novelty phase [23]. At the beginning of the habituation phase, the test mice were individually placed into the center of the sociability boxes with the empty wire mesh cages in opposite corners. The mice could freely explore the boxes for 10 min. Next, the mice were shortly removed from the sociability box and placed in a home cage-like container with bedding material on the floor. In the meantime, one of the empty wire mesh cages was replaced by another cage with an enclosed social partner (stranger 1). Immediately after that, the test mice were placed back into the center of the sociability box for the sociability phase. Again, the mice could freely explore the boxes for 10 min. Next, the test mice were again removed and the empty cage was replaced by another cage with an enclosed new social partner (stranger 2). Immediately after that, the test mice were again placed back into the center of the sociability boxes for the social novelty phase, in which the mice could again freely explore the boxes for 10 min. After finishing this phase, all mice were placed back to their home cages and the boxes were cleaned with water.

The locations of the wire mesh cages containing strangers 1 and 2 were pseudo-randomized throughout the experiment. Of note, the social partners always belonged to the same sex and had similar age and weight as the test animals. The partner mice were always wild-type mice from different breeding lines.

2.2.2. Experiments 2 & 3: Social fear conditioning (SFC) paradigm

2.2.2.1. Setup. For measuring sociability before and after SFC, we used the same system as described above. Only for the conditioning procedure, a modified setup was used that differed from the sociability setup regarding the following features: the box was placed inside another sound-attenuating chamber and had transparent walls. Additionally, the floor of this box consisted of steel grids (bars: 4 mm diameter, distance: 8.9 mm) and was connected to a shock unit that allowed the delivery of foot shocks of defined duration and intensity (Fear Conditioning System, TSE Systems, Bad Homburg, Germany). As for the sociability setup, two wire mesh cages were placed in opposite corners. The floors of these cages were made of plastic, i.e. the cages were electrically isolated from the floor grid.

2.2.2.2. Social fear conditioning procedure. Our SFC protocol was modified based on the paradigm introduced by Toth and colleagues [24]. The timeline of this protocol is shown in Fig. 1. One week before starting the experiment, all test animals were put in separate holding cages and remained single-housed until the end of the experiment. Two days before the experiment, all animals were handled by the experimenter for 2 × 5 min daily for two consecutive days. In parallel, the future social partner mice were habituated to the wire mesh cages with the same regularity. The experiment consisted of a pre-test (day 1), followed by the conditioning test (day 3) and six consecutive expression/extinction tests (days 6 and 7).

The pre-test on day 1 was used for assessing the test animals' baseline sociability. This test was identical to the first two phases (habituation and sociability) of the sociability test described above (see 2.2.1.2.), except that each phase lasted only 5 min.

On day 3, the conditioning was performed. Again, the procedure was almost identical to the sociability test, however, the test phases were also shorter (5 min) and were performed in a different context (see 2.2.2.1.). Importantly, in the sociability phase, the test mice were punished with mild foot shocks (0.4 mA, 1 s) when they investigated the cage containing the social partner, while an investigation of the empty cage was not punished. That means that the number of received foot shocks was depending on the animals' approach behavior (between 1–4 in the present study). The foot shocks were manually triggered by the experimenter who observed the mice via a monitor. After SFC, there was a break of two days without tests. The reason for this was that we

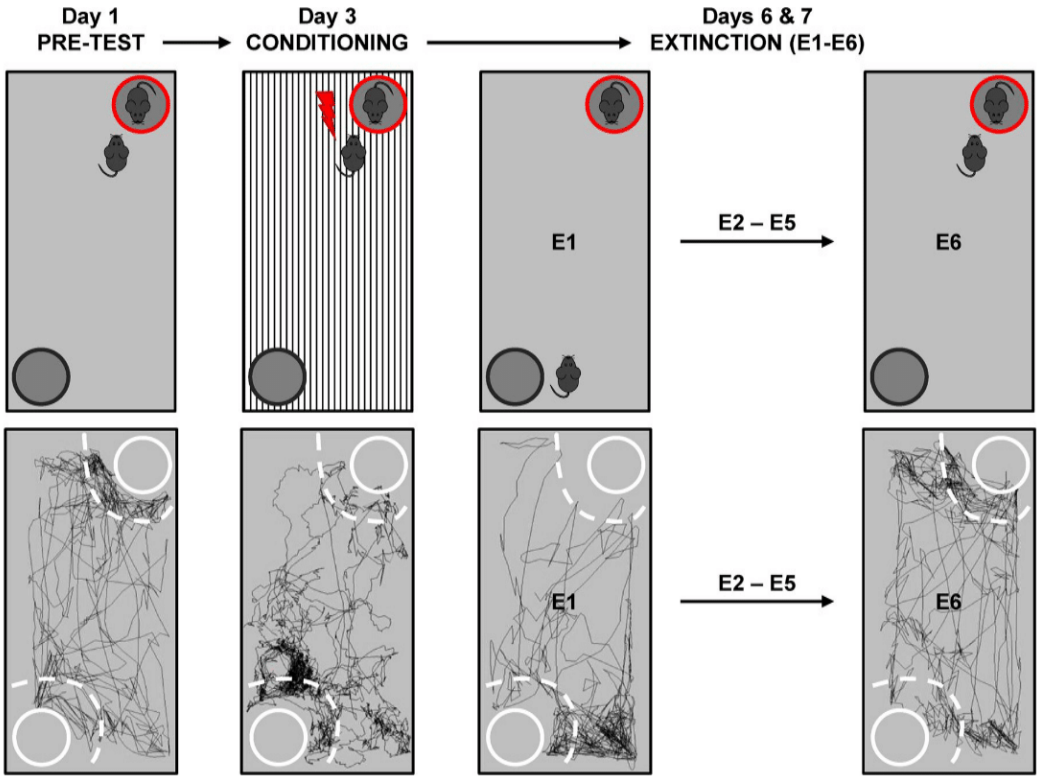

Fig. 1. Social fear conditioning paradigm. This protocol included a pre-test (day 1) for assessing the mice's baseline sociability, a conditioning test on day 3, during which mice were punished for social approach by mild foot shocks (0.4 mA, 1 s), and finally six consecutive extinction tests (E1-E6) on days 6 and 7. In the lower panel, tracking paths of one representative social fear conditioned wild-type mouse during the respective tests are depicted. White circles indicate the location of the wire mesh cages and dashed lines the area in which entries of the nose of the mice were considered as social or cage exploration.

observed generalized fear towards empty wire mesh cages when using only a 24 h break (pilot studies; data not shown).

Then, the mice were tested six times for their sociability to test for the expression and extinction of conditioned social fear. These six tests (E1-E6), identical to the pre-test, were performed on two consecutive days (days 6 and 7) with three tests daily and breaks of ca. 3 h between the tests.

Of note, we also included an unconditioned control group, which was tested in the same way except that these mice did not receive any foot shocks during conditioning. Importantly, in all eight tests of the SFC procedure, different and unfamiliar wild-type strangers of the same sex and similar age and weight as the test animals were used. Also, the location of cages containing the strangers was pseudo-randomized for each test mouse.

2.3. Analyses

2.3.1. Behavioral analyses

The recorded videos were analyzed with the EthoVision XT software (Version 11.0.928, Noldus Information Technology, Wageningen, The Netherlands) which was used to track the nose-points and the body center of the test mice. Of main interest were the investigation times of the cages within the sociability boxes. Cage investigation was defined as the presence of a test animal's noise-point in an area of ca. 3 cm around the cages. This readout was highly correlated with the investigation time manually scored by two observers (observer MHK: $R^2 = 0.95$, observer NF: $R^2 = 0.98$, observer correlation: $R^2 = 0.96$, ps < 0.0001). Furthermore, using the body center, general locomotor activity was measured.

2.3.2. Data analyses

For experiment 1, only the first 5 min of the different test phases were used for the descriptive and statistical analyses and the main figures. The reason for this was our observation that the investigation times during the first 5 min were much more robust and that preferences for particular cages were more pronounced. Based on this experience, experiments 2 and 3 were then planned with 5 min phase duration.

Using the investigation times, a performance index was calculated with the following formula (cage 1 is the cage with the first stranger, if applicable):

$$\textit{performance index} = \frac{(\textit{investigation time cage } 1) - (\textit{investigation time cage } 2)}{(\textit{investigation time cage } 1) + (\textit{investigation time cage } 2)}$$

This performance index was used as a measure of cage preference (habituation phase), of sociability (sociability phase) and preference for social novelty (social novelty phase) of the individual mice. A positive index indicated, dependent on the phase, preference for one cage, sociability towards the first social partner (stranger 1), or no preference for the second social partner (stranger 2), respectively. An index of zero indicated that individual mice showed the same investigation time close to both cages. A negative index indicated, dependent on the phase, preference for the other cage, no sociability towards the first social partner (stranger 1), or preference for the second social partner (stranger 2), respectively. Of note, in the SFC experiments, mice showing performance indices below 0.1 in the pre-test were excluded from further analyses.

Data are presented as the mean ± SEM. The normal distribution of

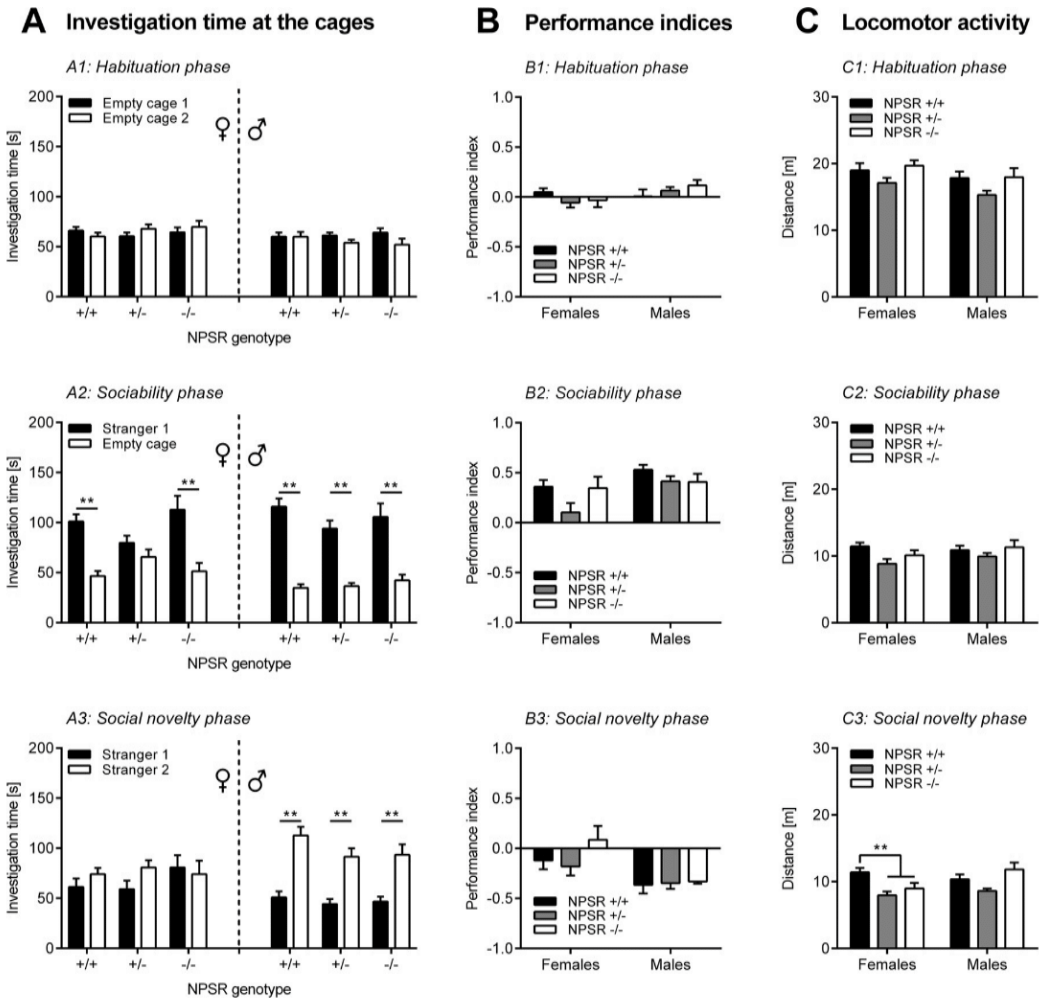

Fig. 2. Sociability and social novelty in NPSR-deficient mice. **A** The investigation times at the respective cages, **B** the performance indices, and **C** the distance traveled within the three test phases (habituation: **A1-C1**; sociability: **A2-C2**; social novelty: **A3-C3**) are depicted for female and male NPSR +/+, NPSR +/- and NPSR -/- mice. Female NPSR +/- mice displayed reduced sociability, and female mice generally did not show a preference for social novelty. There were no genotype effects in male mice. All data are presented as mean + SEM and for group sizes, see text. ** $p < 0.01$, comparisons as indicated.

the data was checked with the D'Agostino-Pearson normality test. Statistical analyses were performed with analyses of variance (ANOVAs), if appropriate with repeated measures, and *post hoc* Holm-Sidak's multiple comparison tests. Additionally, for the sociability test, *t* tests corrected for multiple comparisons (Holm-Sidak method) were used to evaluate the investigation times at the two cages within each experimental group. Proportions were compared using chi-squared tests. All analyses were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, USA) or SYSTAT 12.0 (SPSS Inc., San Jose, USA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Experiment 1: Sociability is reduced in female heterozygous NPSR-deficient mice

In the first experiment, the effect of NPSR deficiency on sociability and social novelty was investigated. Female and male NPSR +/+, NPSR +/- and NPSR -/- mice were tested (group sizes: NPSR +/+: females n = 14, males n = 13; NPSR +/-: females n = 15, males n = 15; NPSR -/-: females n = 10, males n = 9). In Fig. 2A, the investigation times for the respective test phases are depicted. We used *t* tests corrected for multiple comparisons to evaluate the potential influence of the NPSR genotype on the duration of investigation for female and male mice in each test phase separately [25]. In the habituation phase (Fig. 2A1), mice of all groups spent comparable amounts of time at the two empty cages (ts < 1.76, n.s.). In the sociability phase (Fig. 2A2), mice of all genotypes and sexes, except female NPSR +/- mice ($t = 1.38$, $p = 0.18$), displayed a clear preference for the cage containing the social partner (stranger 1) compared to the empty one (ts > 3.79, ps < 0.001). In the social novelty phase (Fig. 2A3), males of all three genotypes devoted significantly more time to explore the cage with the newly introduced social partner (stranger 2) compared to the cage with the already familiar one (stranger 1), indicating a clear preference for social novelty (ts > 4.04, ps < 0.001). Interestingly, these effects were absent in female mice of each genotype (ts < 1.96, n.s.), indicating that female mice did not show a preference for social novelty with the present test protocol.

In Fig. 2B, the performance indices (see 2.3.2.) are depicted. They were analyzed using a multifactorial ANOVA with genotype and sex as

between-subject factors and test phase as a within-subject factor. This analysis revealed a main effect of phase ($F_{2, 136}$ = 85.99, p < 0.0001), but no main effects of genotype or sex (Fs < 2.29, n.s.). Moreover, a significant interaction between phase and sex was found ($F_{2, 136}$ = 14.21, p < 0.0001), however, further interactions did not reach statistical significance (Fs < 2.29, n.s.). Next, two-way ANOVAs for each test phase were calculated separately to identify the sources of the interaction between sex and phase. In the habituation phase (Fig. 2B1), neither sex nor genotype influenced the performance indices and there was also no interaction between these factors (Fs < 3.01, n.s.). In the sociability phase (Fig. 2B2), male mice generally displayed higher sociability than female mice ($F_{1, 70}$ = 7.94, p = 0.006). Furthermore, the ANOVA showed a main effect of genotype ($F_{2, 70}$ = 3.35, p = 0.04), but no interaction ($F_{2, 70}$ = 1.26, p = 0.29). To reveal the source of the genotype effect, we calculated one-way ANOVAs for each sex separately. The NPSR genotype did not influence the sociability of male mice ($F_{2, 34}$ = 1.39, p = 0.26), but there was a tendency for reduced sociability in female mice ($F_{2, 36}$ = 2.73, p = 0.08). In the social novelty phase (Fig. 2B3), the performance indices of male mice were generally lower, indicating a stronger preference for social novelty, compared to those of female mice ($F_{1, 68}$ = 14.01, p = 0.0004). No effects of genotype and no interaction (Fs < 1.26, n.s.) were found.

Finally, we analyzed the animals' locomotor activity during the different test phases (Fig. 2C). For that, we analyzed the total distance traveled by separate ANOVAs for each test phase. In the habituation phase (Fig. 2C1), main effects of genotype ($F_{2, 70}$ = 4.84, p = 0.01) and sex ($F_{1, 70}$ = 4.1, p = 0.047) were found. *Post hoc* tests revealed that NPSR +/- and male mice were generally less active than the other respective groups (ts > 2.55, ps < 0.03). However, there was no interaction between genotype and sex ($F_{2, 70}$ = 0.08, p = 0.92). In the sociability phase (Fig. 2C2), the same effects of genotype were observed ($F_{2, 70}$ = 4.1, p = 0.02), i.e. NPSR +/- mice traveled shorter distances than NPSR +/+ mice (t = 2.78, p = 0.02), but no effects of sex and no interaction between the two factors (Fs < 1.11, n.s.) were found. In the social novelty phase (Fig. 2C3), there were again the same effects of genotype ($F_{2, 68}$ = 9.92, p = 0.0002) and no influence of sex ($F_{1, 68}$ = 2.21, p = 0.14). However, an interaction between genotype and sex ($F_{2, 68}$ = 3.8, p = 0.03) was revealed. *Post hoc* comparisons showed that female NPSR +/- (t = 4.04, p = 0.0003) and NPSR -/- mice (t = 2.55, p = 0.01) traveled significantly less than female NPSR +/+ mice, respectively, while these effects were not observed in male mice (ts < 1.95, n.s.).

In sum, we observed impaired sociability in female NPSR +/- mice, but not in all other groups. In general, female mice did not show a preference for social novelty in the present study, while this preference was observed in male mice. Furthermore, NPSR +/- mice were generally less active during all phases of the sociability test.

3.2. Experiment 2: Social fear conditioning induces conditioned social avoidance and its extinction in C57BL/6J mice

Fig. 1 outlines the SFC protocol and shows tracking paths of a wild-type mouse with robust SFC. We first evaluated our SFC paradigm in C57BL/6J mice (Fig. 3; females: n = 16–18 per group; males: n = 11–12 per group). For the statistical analyses, the performance indices (see 2.3.2.) for each test were calculated and analyzed by multifactorial ANOVAs using conditioning group (control, conditioned) and sex as between-subject factors and test (pre-test, E1-E6) as a within-subject factor. First, we tested whether the mice were social fear conditioned, i.e. the analysis included only the pre-test and the first expression test E1 (Fig. 3A). The ANOVA revealed main effects of group ($F_{1, 53}$ = 13.01, p = 0.001) and test ($F_{1, 53}$ = 85.18, p < 0.0001), as well as an interaction between these two factors ($F_{1, 53}$ = 21.78, p < 0.0001). This indicated successful conditioning in the conditioned but not in the control group. Furthermore, significant interactions between sex and test ($F_{1, 53}$ = 5.76, p = 0.02), and between all three factors ($F_{1, 53}$ = 5.06, p = 0.03) were found, while there were no main effects of sex and no interaction of sex and group (Fs < 0.93, n.s.). To identify the source of these interactions, *post hoc* Holm-Sidak's tests were calculated for each sex separately. As shown in Fig. 3A1, female and male mice displayed significantly lower performance indices in E1 compared to the pre-test (control mice: ts > 2.3, ps = 0.03; conditioned mice: ts > 5.11, ps < 0.0001). Between-group comparisons further revealed that the performance indices of conditioned mice of both sexes were significantly lower compared to controls in E1 (ts > 2.61, ps < 0.02), while they did not differ at basal levels (pre-test, ts < 0.8, n.s.).

Although these results show that our protocol generally induces conditioned social fear (expressed as social avoidance) in both female and male C57BL/6J mice, closer inspection of the data showed substantial variability in the data. In detail, approximately one fourth (27.6 %) of the conditioned animals did not express conditioned social avoidance (E1 index ≥ 0). This proportion of non-responders was not different in the two sexes (chi-squared test: χ^2 (1, n = 29) = 0.0009, p = 0.98). Since we assumed that the conditionability of the animals could be associated with their basal sociability, we further analyzed whether the sociability in the pre-test was correlated with the sociability after conditioning, i.e. social avoidance. We found that this was not the case (Fig. 3A2; R^2 = 0.03, p = 0.37).

Next, we were interested in the question of whether conditioned social fear is also extinguished after repeated exposure to new strangers (Fig. 3B). Since extinction can only be studied in mice which acquired conditioned social fear, we grouped the mice in those expressing social avoidance after conditioning ("responders"; E1 index < 0; females: n = 13; males: n = 8; cf. Fig. 3A2) and animals that did not express social avoidance ("non-responders"; E1 index ≥ 0; females: n = 5; males: n = 3). Importantly, the average number of received foot shocks did not differ between these two groups (t = 1.17, p = 0.25). Next, a multifactorial ANOVA with conditioning group (control, responders) and sex as between-subject factors and test (E1-E6) as a within-subject factor was performed. This analysis revealed main effects of group ($F_{1, 45}$ = 23.88, p < 0.0001) and test ($F_{5, 225}$ = 15.28, p < 0.0001), as well as an interaction between these two factors ($F_{5, 225}$ = 11.43, p < 0.0001). Furthermore, interactions between sex and test ($F_{5, 225}$ = 2.81, p = 0.02), and on trend level between all three factors ($F_{5, 225}$ = 2.16, p = 0.06) were found, while there were no main effects of sex and no interaction of sex and group (Fs < 0.21, n.s.). These results implied the presence of extinction. As shown in Fig. 3B1, *post hoc* Holm-Sidak's tests between groups demonstrated significantly lower sociability in responder mice of both sexes compared to controls in E1 and E2 (ts > 3.48, ps < 0.003), and in E3 in case of females (t = 2.55, p = 0.046), while the groups did not differ anymore in the remaining extinction tests (females E4-E6, males E3-E6: ts < 2.3, n.s.). Furthermore, within-group comparisons revealed that the performance indices of responder mice of both sexes steadily increased along the course of the extinction tests (comparison to E1: females: E2: t = 1.76, p = 0.08; E3-E6: ts > 3.68, ps < 0.0007; males: E2-E6: ts > 2.12, ps < 0.04), indicating the extinction of conditioned social fear, while control mice of both sexes did not change their sociability level throughout the tests (ts < 1.14, n.s.).

Finally, we found that there was a significant relationship between the conditioning effect (pre index – E1 index) and the extinction strength (E6 index – E1 index), i.e. the stronger the conditioning effect (social avoidance) was, the stronger the animals extinguished the social fear (Fig. 3B2; R^2 = 0.52, p = 0.0002).

In conclusion, our SFC paradigm induced conditioned social fear in C57BL/6J mice. However, approximately one fourth of the animals expressed no social avoidance after conditioning. In those mice which were conditioned (responders), we further observed the extinction of the conditioned social fear. In general, no sex differences were detected.

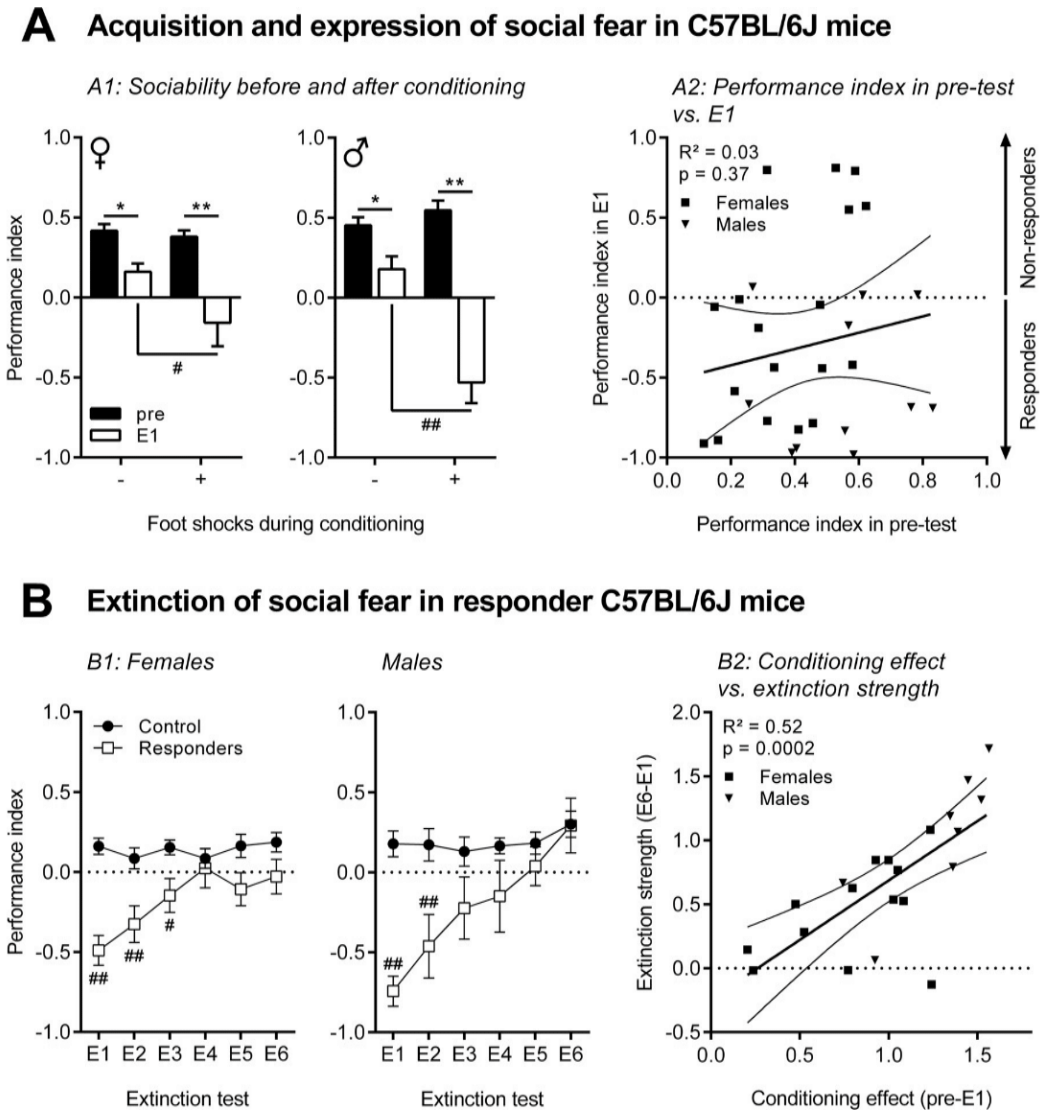

Fig. 3. Social fear conditioning in female and male C57BL/6J mice. **A** Acquisition and expression of social fear. **A1** Sociability was significantly reduced after conditioning (E1) compared to before (pre) in both groups. In both sexes, this reduction was significantly more pronounced in social fear conditioned mice (+) than in unconditioned controls (-). **A2** The sociability in the pre-test did not correlate with the sociability after conditioning (E1). Notably, approximately one fourth of the mice did not acquire social fear (non-responders, E1 index ≥ 0), while most of them did (responders, E1 index < 0). **B** Extinction of social fear. **B1** The performance indices of conditioned mice (responders) of both sexes steadily increased along the course of the extinction tests and eventually reached control level. **B2** The stronger the conditioning effect (pre index - E1 index) was, the better the animals extinguished the conditioned social fear (extinction strength, E6 index - E1 index). Data are presented as mean ± SEM (or individual values) and for group sizes, see text. * $p < 0.05$, ** $p < 0.01$, comparisons within groups. # $p < 0.05$, ## $p < 0.01$, comparisons between groups.

3.3. Experiment 3: NPSR deficiency does not affect the acquisition and expression of conditioned social fear, but has mild effects on its extinction

After establishing the SFC paradigm in C57BL/6J mice, we investigated the role of NPSR deficiency in the acquisition, expression, and extinction of conditioned social fear (Fig. 4). For that, female and male NPSR +/+, NPSR +/- and NPSR -/- mice were submitted to the SFC protocol (group sizes: NPSR +/+: females n = 10–13, males n = 12–13; NPSR +/-: females n = 10, males n = 12–13; NPSR -/-: females n = 8–10, males n = 8–9; per conditioning group, respectively).

Using the performance indices (see 2.3.2.), we first tested for the acquisition and expression of social fear in our NPSR-deficient mice (Fig. 4A). For that, a multifactorial ANOVA with conditioning group (control, conditioned), genotype, and sex as between-subject factors and test (pre-test, E1) as a within-subject factor was calculated. This analysis revealed main effects of group ($F_{1, 116} = 23.05$, $p < 0.0001$) and test ($F_{1, 116} = 142.27$, $p < 0.0001$), as well as an interaction between these two factors ($F_{1, 116} = 25.75$, $p < 0.0001$). No further main effects or interactions were found (Fs < 1.19, n.s.), especially regarding the factor sex, and that is why data of females and males were pooled. These results indicated successful conditioning, which was further supported by *post hoc* Holm-Sidak's tests calculated for each genotype separately. As shown in Fig. 4A1, mice of each genotype displayed significantly decreased sociability in E1 compared to the pre-test

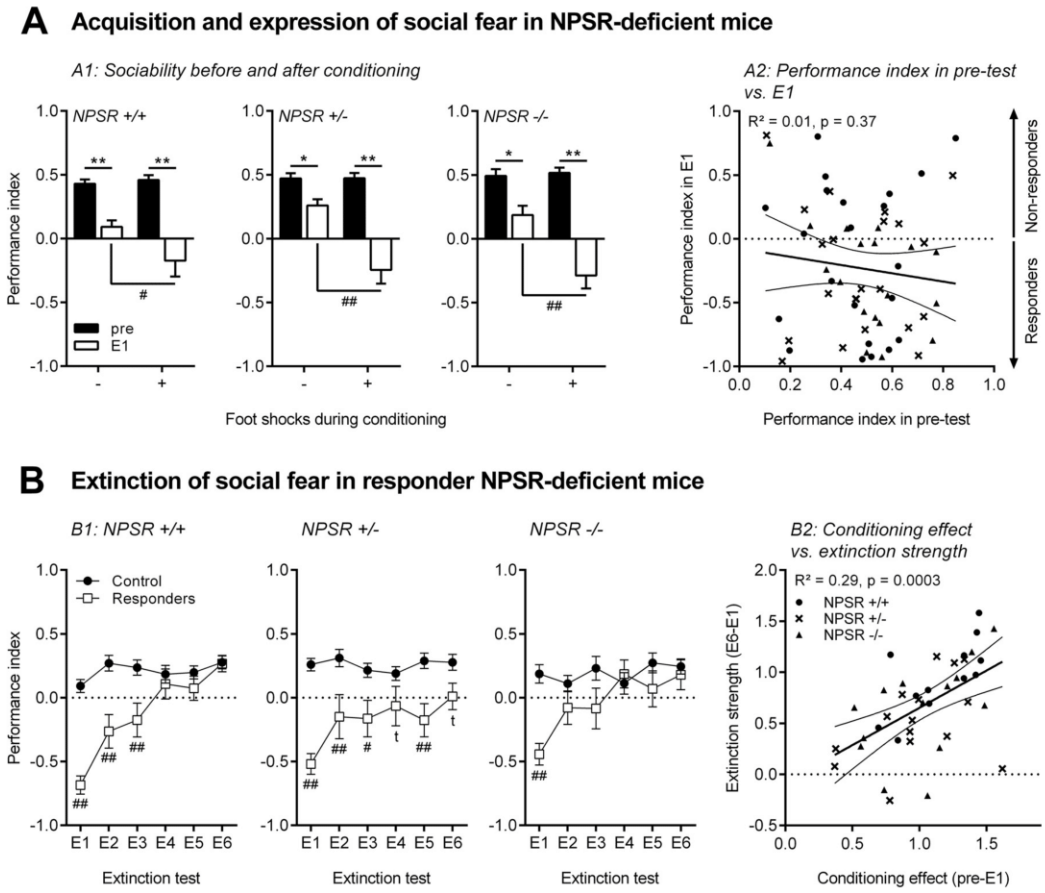

Fig. 4. Social fear conditioning in NPSR-deficient mice. **A** Acquisition and expression of social fear. **A1** Independent of the NPSR genotype, sociability was significantly decreased after conditioning (E1) compared to before (pre), but this decrease was significantly stronger in social fear conditioned mice (+) compared to unconditioned controls (-). **A2** No significant correlation between the sociability in the pre-test and E1 was found. Several mice of each genotype (see **B2** for symbol legends) did not acquire social fear (non-responders, E1 index ≥ 0), while most of them did (responders, E1 index < 0). **B** Extinction of social fear. **B1** In the responder mice, the low performance indices increased along the course of the extinction tests and eventually reached control level. This effect was less pronounced in NPSR +/- mice. **B2** The stronger the conditioning effect (pre index - E1 index) was, the better the animals extinguished the conditioned social fear (extinction strength, E6 index - E1 index). Data are presented as mean ± SEM (or individual values) and for group sizes, see text. * $p < 0.05$, ** $p < 0.01$, comparisons within groups. # $p < 0.05$, ## $p < 0.01$, t $p < 0.1$, comparisons between groups.

(control mice: ts > 2.33, ps < 0.03; conditioned mice: ts > 6.44, ps < 0.0001). Furthermore and accounting for all genotypes, comparisons between groups revealed that the performance indices of conditioned mice in E1 were significantly lower compared to the ones of control mice (ts > 2.66, ps < 0.02), while they were the same at basal levels (pre-test, ts < 0.3, n.s.).

These results implicated that our SFC paradigm induced conditioned social fear, independent of the NPSR genotype. However, we observed again that several animals (34.9 %) did not respond with social avoidance. This non-responder rate was neither affected by sex (chi-squared test: χ^2 (1, n = 63) = 0.61, p = 0.43) nor by genotype (χ^2 (2, n = 63) = 3.06, p = 0.22) and again, the level of sociability after conditioning was not correlated with the sociability in the pre-test (Fig. 4A2; R^2 = 0.01, p = 0.37).

Next, we analyzed whether the NPSR genotype influenced the extinction of social fear (Fig. 4B). As before, only responder mice (NPSR +/+: n = 12; NPSR +/-: n = 15; NPSR -/-: n = 14; cf. Fig. 4A2) were included in this analysis. A multifactorial ANOVA with conditioning group (control, responders), genotype, and sex as between-subject factors and test (E1-E6) as a within-subject factor was performed. This analysis revealed main effects of group ($F_{1, 94}$ = 31.42, p < 0.0001) and test ($F_{5, 470}$ = 23.62, p < 0.0001), as well as an interaction between these two factors ($F_{5, 470}$ = 18.11, p < 0.0001). Moreover, a trend for an interaction between test and genotype was shown ($F_{10, 470}$ = 1.62, p = 0.098), but no further main effects or interactions were found (Fs < 1.96, n.s.). These results implied the presence of extinction. As shown in Fig. 4B1, *post hoc* Holm-Sidak's tests between groups demonstrated significantly lower sociability in NPSR +/+ responder mice compared to controls in E1, E2, and E3 (ts > 3.71, ps < 0.001), while the groups did not differ anymore in E4, E5, and E6 (ts < 1.12, n.s.). For NPSR +/- mice, it was shown that responder mice displayed reduced sociability compared to controls throughout the extinction tests (E1, E2, E3, E5: ts > 2.91, ps < 0.02; E4, E6 on trend level: ts > 1.96, ps = 0.08). Responder and control NPSR -/- mice, on the other hand, displayed significantly different performance indices only in the first extinction test (E1: t = 4.46, p < 0.0001; E2-E6: ts < 2.22, n.s.). Also, within-group comparisons revealed that the performance indices of responder mice of each genotype increased along the course of the extinction tests (E1 vs. E2-E6: ts > 3.22, ps < 0.003), while control mice of each genotype did not change their sociability level throughout all tests (ts < 2.56, n.s.).

Finally, a correlation analysis of the individual data revealed a

significant correlation (R^2 = 0.29, p = 0.0003; Fig. 4B2) between the conditioning effect (pre index – E1 index) and the extinction strength (E6 index – E1 index), i.e. the stronger the conditioning effect (social avoidance) was, the better the animals extinguished the social fear.

In sum, our SFC paradigm induced conditioned social fear in mice independent of the NPSR genotype. In the conditioned mice (responders), we further observed the extinction of this fear. Although we did not find significant genotype effects, the extinction seemed to be weakened in NPSR +/- and facilitated in NPSR -/- mice considering the pairwise comparisons to unconditioned mice. In general, our data indicate that NPSR deficiency does not affect the acquisition and expression of conditioned social fear, but that it may influence its extinction.

4. Discussion

This study investigated the effects of NPSR deficiency on sociability under normal conditions and after SFC. Sociability was measured with a modified version of Crawley's sociability test [23,26]. SFC was induced using a protocol, in which the approach to an unfamiliar conspecific was punished [24]. The present study hypothesized that the NPS system plays a role in social behavior during aversive conditions, but not under non-aversive conditions. This hypothesis was only partly confirmed by the present findings since the data rather indicated that NPSR deficiency alters social interaction behavior under non-aversive conditions in a sex-dependent manner. Though, NPSR deficiency did not affect the acquisition and expression of conditioned social fear but had mild effects on its extinction.

In our first experiment, we investigated the effects of NPSR deficiency on social interaction under non-aversive conditions. Of note, we realized that preferences for social interactions were generally stronger during the first 5 min of the 10 min-phases, indicating that these behaviors may decrease with time. Therefore, this factor should be considered in future experiments. We observed that heterozygous NPSR deficiency, but not a complete lack of NPSR, reduced sociability in a sex-dependent manner. That means all groups showed a significant preference for the cage containing the social partner compared to the empty one, except for female NPSR +/- mice (Fig. 2A2 & B2). Of note, this is not the first finding demonstrating an altered phenotype in NPSR +/-, but not in NPSR -/- mice, since we previously found the same occurrence regarding the startle magnitude [21] [but see: 20,27], although this effect was driven by male mice [21]. Concerning these two behavioral phenotypes, this suggests compensatory mechanisms in NPSR -/- mice that do not occur in NPSR +/- mice, and that these mechanisms may be sex-specific, i.e. more sensitive regarding social behavior in females. Such compensatory mechanisms cannot be observed in spontaneous locomotor activity, in which NPSR -/- mice express a robust and NPSR +/- mice an intermediate phenotype [20]. No behavioral deficits were found in NPSR +/- (and NPSR -/- mice) regarding anxiety, conditioned fear, and prepulse inhibition [20,21,27]. However, NPSR +/- mice were unfortunately not tested in most other studies. The present data support the general advice to include heterozygous animals when transgenic animals are tested [28,29].

Although our study is the first testing sociability and social novelty behavior in NPSR-deficient mice, it has been shown that icv NPS injections did not affect social discrimination and social preference behavior of male wild-type rats [16]. Our data are in line with these findings since NPSR deficiency did not affect sociability and social novelty (i.e. social recognition) in male mice. We only detected a sociability deficit in female NPSR +/- mice. However, we were not able to evaluate whether also social recognition is affected in these mice since females generally did not express a preference for social novelty in the present study (Fig. 2A3 & B3). For the latter, we have no explanation. In the literature, normal social recognition is described in female mice [e.g. 26] and with the protocol we used here, robust social recognition of female wild-type mice of a different line with C57BL/6J background was observed in previous experiments (manuscript in preparation).

Moreover, we found that NPSR +/- mice of both sexes were less active during all three phases of the sociability test (Fig. 2C). Since male mice displayed normal social behavior, we are confident that the social impairments observed in female NPSR +/- mice were not caused by their reduced locomotor activity. It has been shown that NPSR-deficient mice display reduced levels of spontaneous locomotor activity [20] but normal reactive locomotor activity [20,22] [but see: 19]. However, there are indications that reactive locomotor activity is more reduced in NPSR-deficient mice the more aversive the test conditions are, e.g. not in the first but a second exposure to the elevated plus maze [20].

In the second part of our study, we asked whether NPSR deficiency affects social behavior under more aversive conditions. To address this question, we used an SFC paradigm. Toth and colleagues introduced an SFC paradigm for modeling SAD in animals [24]. This paradigm induces conditioned social fear, i.e. avoidance of stranger conspecifics, without additional behavioral alterations, e.g. neophobia or depressive-like behavior. Of note, not two small cages, as in Crawley's sociability test, but only one cage is used in this SFC paradigm. During the conditioning test, approaches to the cage with an enclosed social partner are punished by electrical stimuli via the floor grid [24]. In later tests, the mice avoid approaches to the cage with the enclosed stranger. While trying to establish this paradigm in our laboratory, we found that mice also expressed a robust avoidance of empty cages, i.e. they were not only conditioned to avoid social partners but also to the cages themselves. Consequently, we decided to modify the SFC paradigm (see 2.2.2.2. and Fig. 1). We included a pre-test for assessing the test animals' baseline sociability before the conditioning took place. This additional test provides valuable information about the strength of the conditioning effect concerning each test animal's sociability trait. This pre-test consisted of the first two phases (habituation and sociability) of the sociability test used in our experiment 1, i.e. two cages were always present in the setup. The two cages were also present during the SFC procedure and importantly, only approaches to the cage with the enclosed stranger, but not to the empty cage, were punished. Moreover, conditioned social fear and its extinction were evaluated in the same way by testing the mice's sociability for six times after conditioning. A very similar protocol was recently published by Xu and colleagues [12].

We first tested female and male C57BL/6J mice in our SFC protocol. We showed that mice were successfully social fear conditioned and that sociability after conditioning was not correlated with sociability in the pre-test (Fig. 3A). However, although the protocol worked well based on statistical analyses of the whole group of animals, we realized that there was a substantial portion of test mice (approximately one fourth), which did not show social avoidance after conditioning. This proportion was not different in the two sexes and was not affected by the number of received foot shocks during conditioning. Consequently, for analyzing the extinction of social fear, which can only be studied in mice that express social fear, we grouped all mice into responders and non-responders based on their performance in the first expression test E1. To date, we have no solid explanation for this relatively high proportion of non-responder mice. A potential explanation could be that non-responder mice simply did not respond with avoidance to SFC, but switched their behavioral response from avoidance to agonistic behavior [for review see: 30]. This conjecture should be addressed in future studies with more suitable conditions to investigate agonistic behavior, e.g. in paradigms of free social interaction. Moreover, the possibility that higher foot shock intensities [24] may increase the rate of responder mice should be also addressed.

However, in those C57BL/6J mice which avoided the social partner after SFC, the acquired social fear was extinguished by repeated exposure to different strangers, i.e. sociability steadily increased along the course of the extinction tests and ultimately reached control level (Fig. 3B1). Since the test animals were exposed to novel social partners in each of the experimental tests, our data also showed that this social

fear was neither acquired nor extinguished towards specific individual strangers [e.g. 31,32]. Of note, social avoidance after conditioning was strongly correlated with the extent of social fear extinction (Fig. 3B2). All these effects were observed in both females and males, indicating that the present SFC protocol can also be used to investigate sex-dependent mechanisms underlying social fear. However, a limitation of this protocol is that approximately one fourth of the animals expressed no social avoidance after conditioning (non-responders). Therefore, some optimization of the protocol parameters is required for future studies.

Using this modified SFC protocol, we examined in our third experiment the role of NPSR deficiency in the acquisition, expression, and extinction of conditioned social fear. NPSR deficiency did not affect the acquisition and expression of social fear since animals of each NPSR genotype displayed significantly lower sociability after conditioning compared to before (Fig. 4A1). Notably, in the pre-test of this experiment, i.e. before conditioning, we did not observe a sociability deficit in female NPSR +/- mice as it was observed in experiment 1. However, animals tested in experiment 1 were group-housed, while the mice in experiments 2 and 3 were housed individually before and during the SFC experiment to boost sociability during the different tests. As in C57BL/6J mice (experiment 2), we identified a proportion of animals, which did not show social avoidance after conditioning (approximately one third), but this proportion of non-responders was not affected by genotype or sex. Moreover, the NPSR genotype did not have main effects on the extinction of conditioned social fear (Fig. 4B1). However, considering the pairwise comparisons between the responder and control group within the different genotypes, mild differences in NPSR +/- and NPSR -/- responder mice were observed. While NPSR +/+ mice needed four extinction tests to come back to the sociability level of unconditioned mice, NPSR +/- mice seemed to require more exposures and NPSR -/- mice fewer. NPSR +/- mice showed a tendency for lower sociability in the fourth and sixth extinction test and had significantly lower sociability in the fifth. This indicated a less pronounced extinction of conditioned social fear in these mice. In contrast, extinction of conditioned social fear was faster in NPSR -/- mice than in wild-type animals, i.e. already in the second extinction test, sociability was not different from the unconditioned mice any more.

It has been shown that icv injections of NPS reduce learned avoidance of known conspecifics induced by social defeat or unknown conspecifics induced by SFC [15]. Since such injections did not affect normal sociability [16], it was suggested that NPS affects social investigation only under aversive conditions, i.e. in situations where usually impaired sociability is expressed. This suggestion is only partly supported by the present experiments using NPSR-deficient mice. In NPSR +/- mice, we found impaired sociability (in females) and weakened extinction of conditioned social fear after SFC (both sexes), whereas in NPSR -/- mice, sociability was not affected and extinction of social fear seemed to be rather improved. This indicates compensatory mechanisms in NPSR -/- mice that do not take place in NPSR +/- mice.

In conclusion, the present study extends our knowledge about the NPS system's role in social behavior under non-aversive and aversive conditions. In general, only subtle effects of NPSR deficiency were found. However, these effects on social behavior are more pronounced in NPSR +/- mice, while NPSR -/- mice seem to profit from the NPSR deficiency, most probably due to compensatory mechanisms. Since reduced social behavior and social avoidance are behavioral endophenotypes of several neuropsychiatric disorders, and NPSR polymorphisms play a role in some of them [13,14], the current data may have a translational impact.

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CRediT authorship contribution statement

Malgorzata H. Kolodziejczyk: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Nadine Faesel:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Michael Koch:** Resources, Writing - review & editing, Supervision. **Markus Fendt:** Conceptualization, Methodology, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research report

Orexin deficiency affects sociability and the acquisition, expression, and extinction of conditioned social fear

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ABSTRACT

Accumulating evidence indicates that the central orexin (hypocretin) system plays an important role in regulating emotional processes in both humans and rodents. Thus, the orexin system has been repeatedly implicated in the pathophysiology of several neuropsychiatric disorders, such as anxiety disorders. Among others, symptoms like social fear and social withdrawal are frequently observed in these disorders. Based on this, we investigated the role of orexin deficiency in social (fear) behavior. For that, female and male orexin-deficient mice were tested for (1) sociability and social novelty, and (2) acquisition, expression, and extinction of conditioned social fear. We found that female orexin-deficient mice displayed reduced sociability and decreased preference for social novelty compared to their wild-type littermates. These effects of orexin deficiency were not observed in males. Moreover, orexin deficiency facilitated the acquisition and/or expression of conditioned social fear and impaired the extinction of social fear in both sexes. Taken together, our results indicate an important, partly sex-dependent, regulatory role of the orexin system in social (fear) behavior. Our findings support the hypothesis of orexin being an integrator of motivation, affect, and emotion.

1. Introduction

Social withdrawal and fear of social situations are symptoms frequently observed in many neuropsychiatric disorders, such as social anxiety disorder (Dryman et al., 2016; Kennedy and Adolphs, 2012). These symptoms substantially impair the life quality of the patients. Therefore, research dedicated to better understand the neurobiological mechanisms underlying social withdrawal and social fear is needed in order to develop more effective and more specific treatments (Toth et al., 2012).

Accumulating evidence implicates that the central orexin (hypocretin) system, originally identified for its essential role in feeding behavior and sleep/wakefulness (Adamantidis et al., 2007; de Lecea et al., 1998; Sakurai et al., 1998), is critically involved in regulating further functions like fear and anxiety, stress, motivation, mood, and affect (for reviews see: Flores et al., 2015; Johnson et al., 2012; Nevárez and de Lecea, 2018; Sakurai, 2014). The orexin system comprises two peptides, orexin A and B (also known as hypocretin 1 and 2), which are produced from the same precursor transcript and exclusively expressed in neurons of the lateral and dorsomedial hypothalamus and perifornical area (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). Despite this restricted location, the orexin system exerts its versatile functions via extensive projections throughout the whole brain (Nambu et al., 1999; Peyron et al., 1998), which is also reflected in the broad distribution pattern of its G-protein coupled receptors, i.e., the orexin type 1 (OX1R) and type 2 receptors (OX2R) (Ch'ng and Lawrence, 2015; Hervieu et al., 2001; Marcus et al., 2001; Trivedi et al., 1998).

As orexin neurons are reciprocally connected to several limbic areas (Nambu et al., 1999; Sakurai et al., 2005; Yoshida et al., 2006), it is reasonable to predict that the orexin system may also influence social behaviors. Indeed, patients suffering from the sleep disorder narcolepsy, which is caused by a dramatic deficiency in orexin signaling (Peyron et al., 2000; Thannickal et al., 2000), frequently express symptoms of social phobia, which may be a direct consequence of the orexin deficiency (Fortuyn et al., 2010). Moreover, human orexin A levels in the amygdala, as measured with microdialysis, are maximal during positive emotions and social interactions (Blouin et al., 2013). Intriguingly, also animal research supports a role of orexin in social behaviors. For

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instance, OX1R-deficient mice express reduced sociability (Abbas et al., 2015), and mice, whose orexin neurons degenerate at the age of three months, display impairments in social memory, which can be ameliorated by nasal application of orexin A (Yang et al., 2013). However, most studies investigating the influence of orexin on social behaviors were based on social stress models, generally revealing a close relationship to the stress circuitry and indicating that orexin plays a critical role in coping with social situations in general and social threats in particular (e.g., Eacret et al., 2019; Grafe et al., 2018; Ji et al., 2019; Staton et al., 2018).

This study aimed to further extend the knowledge regarding orexin's role in social (fear) behavior by using female and male mice with life-long orexin deficiency. These mice were examined in several other studies regarding their narcolepsy-like phenotype (Chemelli et al., 1999; Leibiger and Fendt, 2014), spatial working memory (Dang et al., 2018), reward function (McGregor et al., 2011), fear and anxiety (Khalil and Fendt, 2017), and defensive responses (Kayaba et al., 2003), but are poorly characterized regarding social behavior. Here, we tested heterozygous (OX +/−) and homozygous (OX −/−) orexin-deficient mice and their wild-type littermates (OX +/+) in two behavioral paradigms. In the first experiment, the effects of orexin deficiency on sociability and social novelty were tested. In the second experiment, mice were submitted to a social fear conditioning (SFC) paradigm, in which approaches to an unfamiliar conspecific were punished (cf. Toth et al., 2013). Using sociability tests, the effects of orexin deficiency on the acquisition, expression, and extinction of conditioned social fear were evaluated.

2. Results

2.1. Experiment 1: Sociability and preference for social novelty are reduced in female orexin-deficient mice

We first investigated the effect of orexin deficiency on sociability and social novelty behavior (Fig. 1). For that, female and male OX +/+, OX +/− and OX −/− mice (n = 10–22/group) were tested in a modified

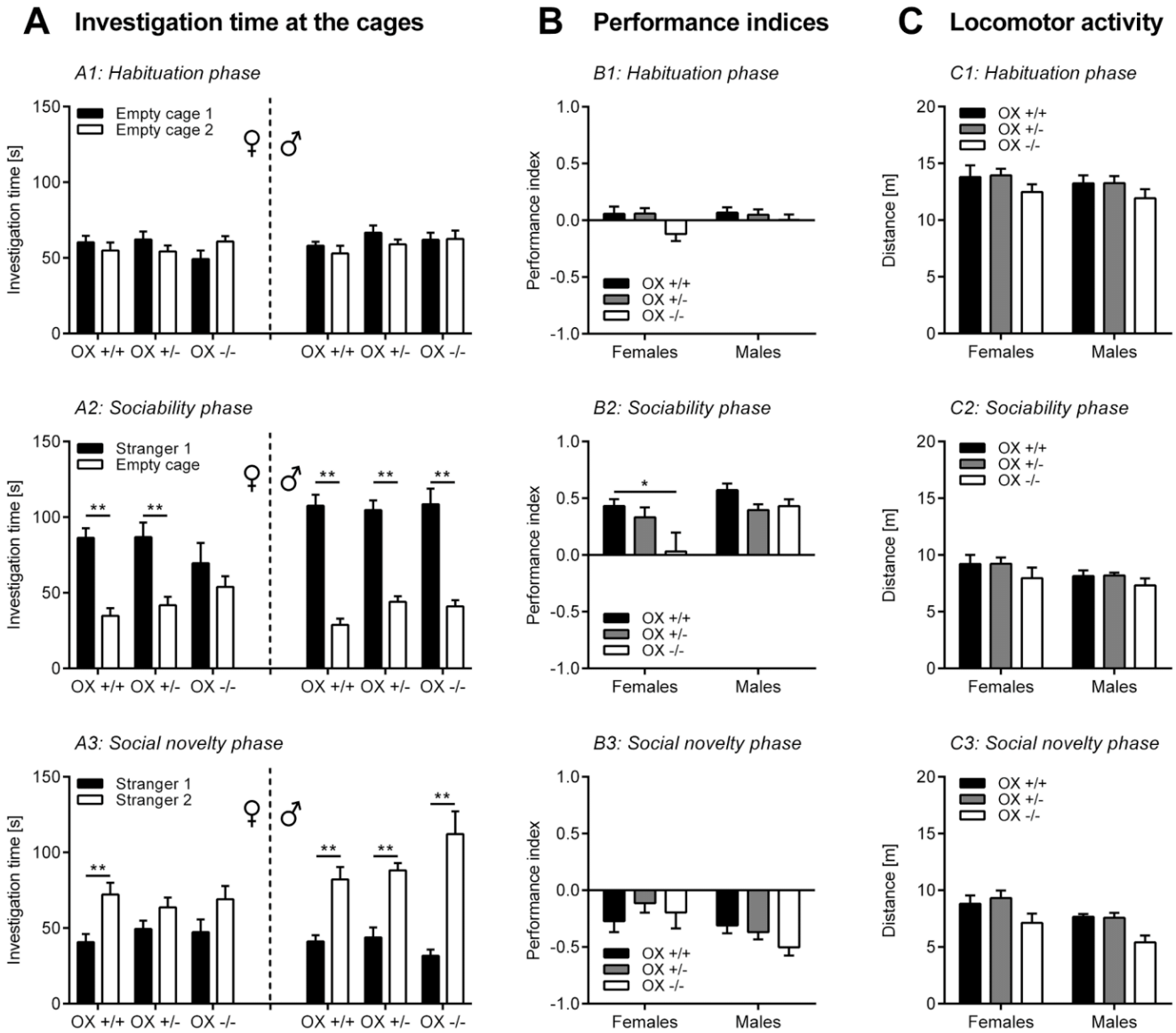

Fig. 1. Sociability and social novelty in orexin-deficient mice. A) The investigation times at the respective cages, **B)** the performance indices, and **C)** the distance traveled within the three test phases (habituation: **A1-C1**; sociability: **A2-C2**; social novelty: **A3-C3**) are depicted for female and male OX +/+, OX +/− and OX −/− mice. Female orexin-deficient mice displayed reduced sociability and decreased preference for social novelty, while these effects of orexin deficiency were absent in male mice. All data are shown as mean + SEM and for group sizes, see text. * $p < 0.05$, ** $p < 0.01$, comparisons as indicated.

version of Crawley's sociability test (Crawley, 2004). The test included a habituation phase with two empty wire mesh cages in opposite corners of the test box, followed by a sociability phase with one stranger mouse (stranger 1) in one of the cages, and then a social novelty phase with another stranger mouse (stranger 2) in the second cage. In Fig. 1A, the investigation times of the respective cages are depicted. To evaluate the potential influence of the orexin genotype, we performed *t* tests, corrected for multiple comparisons, for females and males in each test phase separately (cf. Yang et al., 2011). In the habituation phase (Fig. 1A1), all mice spent similar amounts of time at both empty cages (ts < 1.73, n.s.). In the sociability phase (Fig. 1A2), mice of all genotypes and sexes, except female OX −/− mice ($t = 1.03$, $p = 0.32$), clearly preferred the cage containing stranger 1 compared to the empty one (ts > 4.05, ps < 0.0005). In the social novelty phase (Fig. 1A3), mice of most groups spent significantly more time exploring the cage with stranger 2 compared to the cage with stranger 1, indicating a robust preference for social novelty (ts > 3.29, ps < 0.003). Notably, this preference was not observed in female OX −/− and OX +/− mice (ts < 1.8, n.s.).

In a second analytical approach, we calculated the performance indices for all groups (see 4.3.2.; Fig. 1B). A multifactorial ANOVA with genotype and sex as between-subject factors and test phase as a within-subject factor revealed main effects of genotype ($F_{2, 77} = 6.27$, $p = 0.003$) and phase ($F_{2, 154} = 116.97$, $p < 0.0001$), as well as an interaction between sex and phase ($F_{2, 154} = 11.0$, $p < 0.0001$). There were no main effects of sex and no further interactions (Fs < 1.69, n.s.). To identify the source of the genotype effect, two-way ANOVAs were calculated for each test phase separately. In the habituation phase (Fig. 1B1), neither sex nor genotype affected the performance indices, and there was also no interaction between the two factors (Fs < 2.87, n.s.). In the sociability phase (Fig. 1B2), orexin-deficient mice displayed reduced sociability ($F_{2, 77} = 5.43$, $p = 0.006$), and this reduction tended to be more pronounced in females (interaction: $F_{2, 77} = 2.35$, $p = 0.1$; sex: $F_{1, 77} = 9.68$, $p = 0.003$). These effects of genotype were further confirmed by separate ANOVAs for each sex. While orexin deficiency did only tend to affect sociability in males ($F_{2, 46} = 2.73$, $p = 0.08$), there was a significant effect in females ($F_{2, 31} = 3.62$, $p = 0.04$). *Post hoc* comparisons with OX +/+ mice showed that female OX −/− mice, but not OX +/− mice, displayed significantly reduced sociability ($t = 2.61$, $p = 0.03$, and $t = 0.69$, $p = 0.5$, respectively). In the social novelty phase (Fig. 1B3), the performance indices of males were generally more negative, indicating a stronger preference for social novelty, compared to those of females ($F_{1, 77} = 8.15$, $p = 0.006$). No main effects of genotype and no interaction (Fs < 1.35, n.s.) were revealed.

Last, we evaluated the animals' locomotor activity during the different test phases (Fig. 1C). For that, we analyzed the total distance traveled by separate ANOVAs for each test phase. In the habituation phase (Fig. 1C1), mice of all groups traveled similar distances (Fs < 1.93, n.s.). In the sociability phase (Fig. 1C2), males tended to travel less distances than females ($F_{1, 77} = 3.74$, $p = 0.06$), but no genotype effects and no interaction between the two factors were found (Fs < 2.02, n.s.). In the social novelty phase (Fig. 1C3), main effects of genotype ($F_{2, 77} = 7.92$, $p = 0.0007$) and sex ($F_{1, 77} = 10.32$, $p = 0.002$) were found. *Post hoc* tests revealed that OX −/− and male mice were generally less active than the other respective groups (ts > 3.37, ps < 0.002). However, no interaction between sex and genotype was shown ($F_{2, 77} = 0.16$, $p = 0.85$).

In sum, we observed reduced sociability and decreased preference for social novelty in female orexin-deficient mice, while orexin deficiency did not affect the social behavior of males.

2.2. Experiment 2: Orexin deficiency affects the acquisition, expression, and extinction of conditioned social fear

Next, we investigated the role of orexin deficiency in conditioned social fear and its extinction. For that, female and male OX +/+, OX +/− and OX −/− mice (n = 10–14/group) were first tested for their sociability behavior in a pre-test, followed by a SFC session two days later. In this session, approaches to the stranger mouse were punished with electric stimuli. After a further break of two days, six sociability tests were performed on two consecutive days (E1-E6; 3 tests/day) to evaluate the expression and extinction of conditioned social fear. Fig. 2 outlines this SFC protocol and shows representative tracking paths of an OX +/+ and an OX −/− mouse with robust SFC. Fig. 3 depicts the mean performance indices (see 4.3.2.) in the different tests of the protocol. For statistical analyses, the performance indices were analyzed by multifactorial ANOVAs with the between-subject factors conditioning group (control, conditioned), genotype, and sex, as well as the within-subject factor test (pre-test, E1-E6). To assess the acquisition and expression of conditioned social fear, we first compared the performance indices from the pre-test and the first expression test E1 (Fig. 3A). An ANOVA revealed main effects of conditioning group ($F_{1, 136} = 11.7$, $p = 0.001$) and test ($F_{1, 136} = 90.44$, $p < 0.0001$), as well as an interaction between these two factors ($F_{1, 136} = 11.1$, $p = 0.001$) indicating that the mice were successfully social fear conditioned. No further significant main effects or interactions were found (Fs < 2.6, n.s.), which is why we pooled data from females and males for the figures and subsequent analyses (*post hoc* Holm-Sidak's tests). As shown in Fig. 3A1, mice of all three genotypes displayed significantly reduced sociability in E1 compared to their pre-test level (control mice: ts > 2.15, ps < 0.04; conditioned mice: ts > 4.51, ps < 0.0001). Furthermore, comparisons with the respective control group (unconditioned mice) demonstrated that the performance indices of conditioned OX +/− and OX −/− mice in E1 were significantly lower (ts > 3.21, ps < 0.004), while they were the same in the pre-test (ts < 0.22, n.s.). No significant differences were observed in OX +/+ mice, neither in the pre-test nor in E1 (ts < 1.55, n.s.).

These results indicated that our SFC paradigm generally induced conditioned social fear (expressed as social avoidance), especially in the orexin-deficient mice. Closer inspection of the data demonstrated a strong variability and that approximately half (52.6 %) of the conditioned mice did not respond with social avoidance to the conditioning (E1 index ≥ 0). This non-responder rate was neither affected by sex (chi-squared test: χ^2 (1, n = 76) = 0.04, p = 0.85) nor by genotype (χ^2 (2, n = 76) = 0.11, p = 0.95). Since we assumed that the conditionability of the mice could be related to their basal sociability, we analyzed whether the sociability in the pre-test was correlated with the sociability (i.e., social avoidance) after conditioning in E1. We found that this was not the case (Fig. 3A2; $R^2 = 0.009$, $p = 0.42$).

Next, we examined whether conditioned social fear could be extinguished by repeated exposure to novel social partners and if orexin deficiency influenced this process (Fig. 3B). Since extinction can only be studied in mice which acquired and expressed conditioned social fear (cf. Kolodziejczyk et al., 2020), we grouped the mice in those displaying social avoidance after conditioning ("responders"; E1 index < 0; n = 11–13/group; Fig. 3A2), and mice that did not show social avoidance ("non-responders"; E1 index ≥ 0). Of note, the average number of received foot shocks during conditioning did not differ between these two groups ($t = 0.86$, $p = 0.39$). Next, we performed a multifactorial ANOVA with conditioning group (control, responders), genotype, and sex as between-subject factors and test (E1-E6) as a within-subject factor. This analysis revealed extinction of conditioned social fear in the responder mice (conditioning group: $F_{1, 96} = 80.65$, $p < 0.0001$; test: $F_{5, 480} = 25.64$, $p < 0.0001$; interaction: $F_{5, 480} = 15.01$, $p < 0.0001$). A significant interaction between genotype and conditioning group ($F_{2, 96} = 6.06$, $p = 0.003$) and – on trend level – between test × genotype × conditioning group ($F_{10, 480} = 1.72$, $p = 0.08$) suggested genotype differences in the extinction of conditioned social fear. The other factors or interactions did not reach significance level (Fs < 1.59, n.s.). As depicted in Fig. 3B1, subsequent *post hoc* Holm-Sidak's tests revealed significantly lower sociability in OX +/+ responder mice compared to their respective control group in E1, E2, and E3 (ts > 2.9, ps < 0.02), but not in E4,

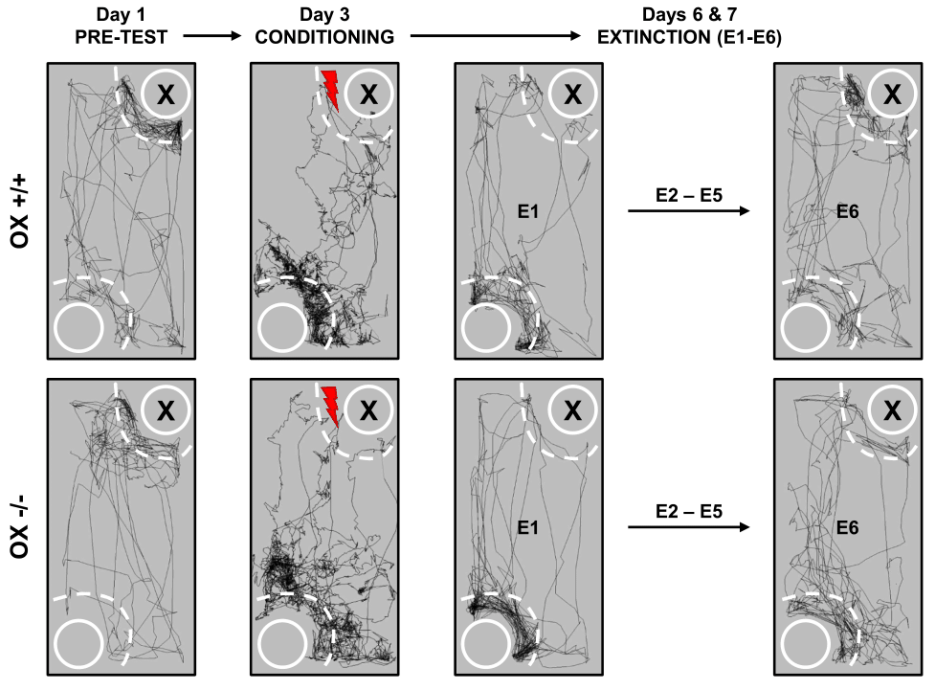

Fig. 2. Social fear conditioning paradigm. The protocol included a pre-test on day 1 for measuring the animals' baseline sociability, a conditioning test on day 3, during which mice were punished for social approach by mild foot shocks (0.4 mA, 1 s), followed by six consecutive extinction tests (E1-E6) on days 6 & 7. White circles indicate the location of the wire mesh cages, dashed lines the areas in which entries of the mice's nose were considered as social or cage exploration, and Xs depict the location of the unfamiliar social partners (strangers). Representative tracking paths of a social fear conditioned OX +/+ (upper panel) and OX −/− mouse (lower panel) during the respective tests are shown.

E5, and E6 (ts < 1.6, n.s.). Similarly, OX +/− responder mice displayed reduced sociability compared to controls from E1 to E4 (E1, E2, E4: ts > 2.78, ps < 0.02; trend in E3: t = 2.39, p = 0.052), but not in E5 and E6 (ts < 1.86, n.s.). In contrast, OX −/− responder mice showed significantly lower performance indices compared to controls throughout all extinction tests (E1-E6: ts > 2.42, ps < 0.02). This indicated a delayed extinction of conditioned social fear in OX −/− responder mice, which was further confirmed by within-group comparisons with E1 demonstrating significant reductions of social avoidance already in E2 in OX +/+ and OX +/−, but in E3 in OX −/− mice (ts > 2.97, ps < 0.003).

As reported earlier (Kolodziejczyk et al., 2020), a significant correlation between the conditioning effect (pre index – E1 index) and the extinction strength (E6 index – E1 index) was found in our animals. That means, the stronger the conditioning effect (social avoidance) was, the more the mice extinguished social fear ($R^2 = 0.25$, p = 0.002; Fig. 3B2).

To summarize, the SFC effect was more pronounced in orexin-deficient mice. In those mice which were well conditioned (responders), we further observed a weaker and/or delayed extinction of social fear in OX −/− mice. These findings indicate that orexin deficiency may result in facilitated and prolonged expression of social avoidance after SFC.

3. Discussion

This study investigated the effects of orexin deficiency on sociability and SFC using female and male orexin-deficient mice. Sociability was assessed with a modified version of Crawley's sociability test (Crawley, 2004), and SFC was performed using a protocol we recently published (Kolodziejczyk et al., 2020), in which the approach to an unfamiliar conspecific was punished (cf. Toth et al., 2013). We hypothesized that orexin plays a critical role in coping with social situations in general and social threats in particular. This hypothesis was confirmed by our findings. Orexin deficiency impaired sociability and social novelty behavior in a sex-dependent manner, and facilitated in both sexes the acquisition and/or expression of conditioned social fear, while its extinction was impaired and/or delayed.

In our first experiment, we evaluated the effects of orexin deficiency on sociability and social novelty. We found that a complete lack of orexins reduced sociability in female, but not in male OX −/− mice (Fig. 1A2 & B2). Furthermore, female OX +/− and OX −/− mice did not express a preference for social novelty, while males of each genotype did (Fig. 1A3 & B3). In general, locomotor activity in the sociability test was not affected by the genotype, except for the social novelty phase (Fig. 1C), during which OX −/− mice of both sexes were less active. However, since male mice displayed normal social behavior in this phase, we are confident that the impairments observed in female orexin-deficient mice were not caused by reduced locomotor activity.

The finding that the orexin system is involved in regulating sociability and social novelty is in line with previously published studies. For instance, mice with global OX1R deficiency and mice with a localized OX2R knockdown in the basolateral amygdala were found to express reduced sociability (Abbas et al., 2015; Arendt et al., 2014). Furthermore, nasal administration of orexin A and chemogenetic activation of the orexin system were shown to ameliorate induced social memory impairments in mice (Stanojlovic et al., 2019; Yang et al., 2013). Importantly, all these studies were conducted in male mice and were therefore not able to explore sex differences. We demonstrated that sociability and preference for social novelty were only affected in female, but not in male mice with a lifelong orexin deficiency. At this point, we cannot conclude whether these sex-dependent effects on sociability were directly caused by the orexin deficiency, by developmental compensatory mechanisms in these constitutive orexin knockout mice, or by a combination of both (see discussion in: Chowdhury et al., 2019). In general, compensatory mechanisms are important to consider when working with constitutive knockout animals (El-Brolosy and Stainier, 2017). Indeed, various reports have suggested that the orexin system plays a role in brain development (e.g., Stoyanova et al., 2010; van den Pol et al., 2001, 2002). Therefore, future studies should address whether acute (e.g., pharmacological) interventions aimed at the orexin system reveal similar effects as found in this study. Notably, sex-related effects of orexin deficiency were also observed in cognitive flexibility (Durairaja and Fendt, 2020), panic-like anxiety, and drinking behavior (unpublished observation). Moreover, sexually dimorphic changes of the orexin system have been reported on the mRNA, peptide and receptor levels at baseline conditions (Jöhren et al., 2002; Loewen et al., 2017; Taheri et al., 1999), but also with respect to stress adaptation (Grafe et al., 2017) and depression (Lu et al., 2017). Our findings, as well as these studies, strongly support sexual dimorphisms of the orexin system,

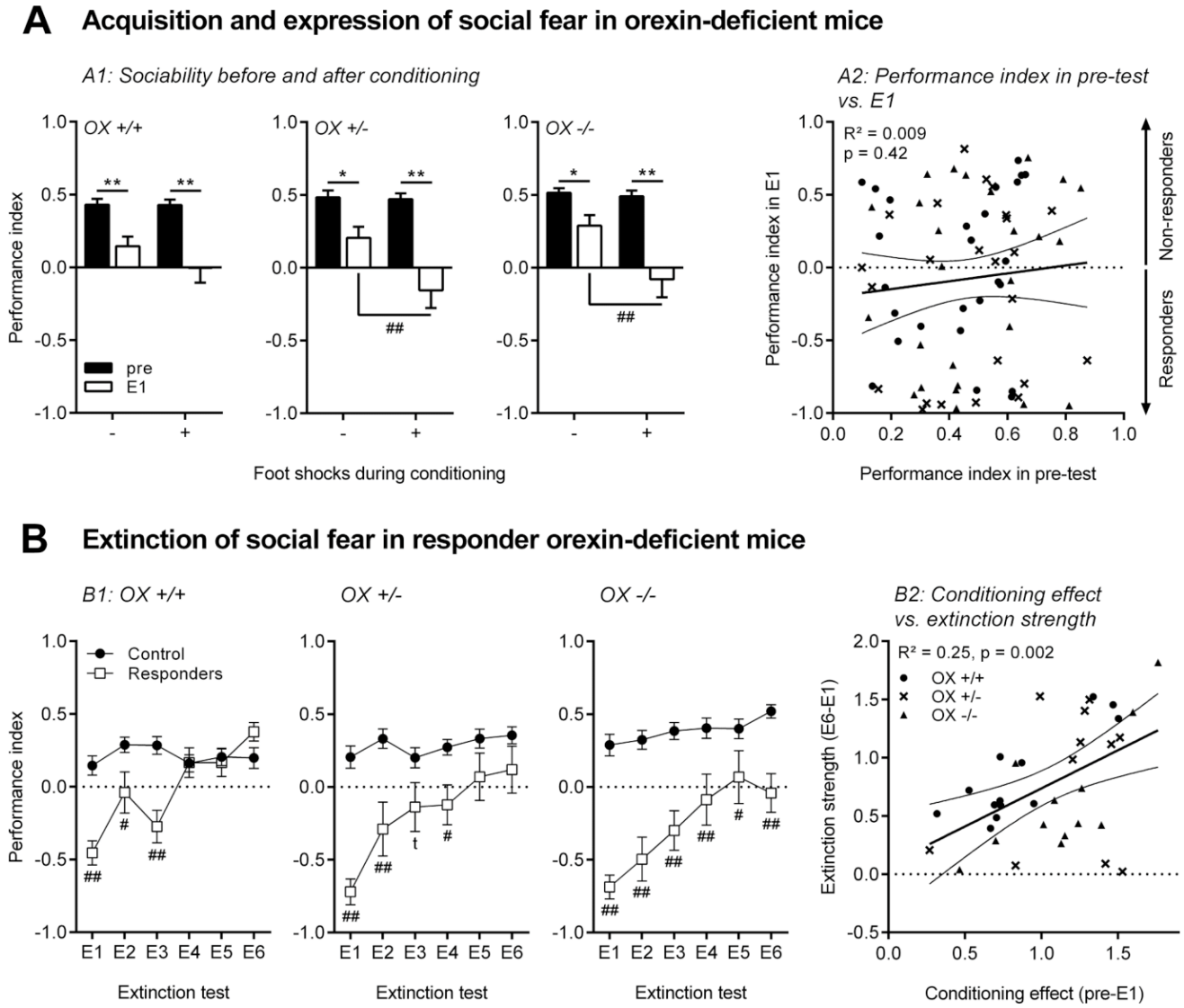

Fig. 3. Social fear conditioning in orexin-deficient mice. A) Acquisition and expression of social fear. **A1)** In all orexin genotypes, sociability was significantly reduced after conditioning (E1) compared to before (pre) in unconditioned control (-) and to a higher degree in social fear conditioned mice (+). This effect was more pronounced in OX +/− and OX −/− mice. **A2)** The sociability in the pre-test did not correlate with the sociability after conditioning (E1). Notably, approximately half of the mice of each genotype (see **B2** for symbol legends) did not acquire social fear (non-responders, E1 index ≥ 0), while the other half did (responders, E1 index < 0). **B)** Extinction of social fear. **B1)** In responder OX +/+ and OX +/− mice, the performance indices increased along the course of the extinction tests and eventually reached control level. This was not the case in OX −/− mice indicating more stable social fear memory. **B2)** The stronger the conditioning effect (pre index – E1 index) was, the better the animals extinguished the conditioned social fear (extinction strength, E6 index – E1 index). Data are presented as mean ± SEM (or individual values) and for group sizes, see text. * $p < 0.05$, ** $p < 0.01$, comparisons within groups. # $p < 0.05$, ## $p < 0.01$, t $p < 0.1$, comparisons between groups.

which may be of translational relevance. Altered orexin levels in the cerebrospinal fluid have been found in affective and anxiety disorders (e.g., Brundin et al., 2007; Johnson et al., 2010; Strawn et al., 2010), and these disorders are more prevalent in women (Kessler et al., 1994). Treatment with orexin, e.g., by nasal administration (cf. Baier et al., 2011; Weinhold et al., 2014), might be a treatment option for some of these diseases and our findings suggest that women could be more sensitive to such treatment.

In the second experiment, we addressed whether orexin deficiency affects conditioned social fear. In our SFC paradigm, approaches to an unfamiliar conspecific were punished and the acquisition, expression, and extinction of conditioned social fear were evaluated by testing the animals' sociability before and after SFC (cf. Kolodziejczyk et al., 2020). To avoid sociability deficits in female OX −/− mice already in the pre-test (as observed in experiment 1), the mice were housed individually to stimulate sociability in this experiment. Indeed, we observed that female OX −/− mice then showed normal sociability. However, after submitting the mice to SFC, we found significantly reduced sociability in all genotypes (Fig. 3A1). This conditioning effect was more pronounced in OX +/− and OX −/− mice. Both of these genotypes expressed social avoidance, indicating stronger acquisition and/or expression of conditioned social fear compared to OX +/+ mice. These differences were not confounded by changes in pain sensitivity, since we previously demonstrated that the reactivity to foot shocks of the intensity used in this study (0.4 mA) is not affected by the orexin genotype or sex (Khalil and Fendt, 2017). However, although the protocol worked well based on statistical analyses of the whole group of mice, we realized that approximately half of the mice did not express social avoidance after SFC (Fig. 3A2). Importantly, this proportion of non-responders was found in each orexin genotype and both sexes, and was not affected by the number of foot shocks received during SFC. This is in line with our previous study (Kolodziejczyk et al., 2020) in which we also observed a

substantial proportion of mice that did not display social avoidance in this paradigm. A potential explanation for this could be that non-responder mice did not react with avoidance after SFC, but expressed a more active coping strategy, such as agonistic behavior (cf. De Miguel et al., 2011). We will address this idea in future studies with more appropriate conditions to measure agonistic behavior, e.g., in a paradigm of free social interaction. Additionally, increasing the foot shock intensities (cf. Toth et al., 2013) may lead to a higher rate of responder mice. Nonetheless, although the reduction in sociability (i.e., social avoidance) failed to reach significance level in OX +/+ mice in this study, there was robust social avoidance in OX +/− and OX −/− mice. This indicated that orexin deficiency facilitates the acquisition and/or expression of conditioned social fear.

In order to analyze extinction of conditioned social fear, we grouped the mice in responders and non-responders to SFC (Fig. 3A2) and omitted the latter from subsequent analyses (cf. Kolodziejczyk et al., 2020). The orexin genotype clearly affected the extinction of conditioned social fear (Fig. 3B1). While OX +/+ mice required four extinction tests to reach the sociability level of unconditioned mice, OX −/− mice displayed significantly reduced sociability throughout all extinction tests, and OX +/− mice expressed an intermediate phenotype. These results indicated that orexin deficiency does not only lead to more pronounced conditioned social fear, but also to a more stable memory, which is more resistant to extinction.

This is the first study showing that operant conditioned fear and its extinction directed towards stimuli of high emotional content (i.e., social contact) are affected in orexin-deficient mice. Of note, these mice did not show impairments in a classical fear conditioning paradigm (Khalil and Fendt, 2017). Our findings contribute to several published studies demonstrating that the orexin system regulates emotional learning and memory. For example, genetic deletion or pharmacological blockade of the OX1R (especially in the amygdala and locus coeruleus) impaired the acquisition and expression of classical conditioned fear, and/or facilitated its extinction (Dustrude et al., 2018; Flores et al., 2014, 2017; Sears et al., 2013; Soya et al., 2013, 2017). Hence, our results do not only indicate an important role of orexin in social fear, but also that reduced orexin signaling (genetically or pharmacologically induced) differentially affects classical vs. operant conditioned fear. In agreement, clinical studies have suggested altered amygdala activity during aversive conditioning in narcolepsy patients (Khatami et al., 2007; Ponz et al., 2010). In this context, narcolepsy patients, as well as orexin-deficient mice, experience narcoleptic episodes, which are often induced by emotionally salient stimuli, with social interactions being one of the most frequent triggers (Anic-Labat et al., 1999; Chemelli et al., 1999). Surprisingly, we never observed such narcoleptic episodes in the experiments of this study. However, we previously found that running activity and food reward are the most potent triggers of narcoleptic episodes in our OX −/− mice (Durairaja and Fendt, 2020; Leibiger and Fendt, 2014).

Growing evidence from preclinical research indicates that the orexin system, via its arousal-promoting properties, serves to integrate signals of physical and psychological threats to mobilize adaptive behavioral and physiological responses (Cohen et al., 2020; Johnson et al., 2012). As such, a crucial role of orexin in mediating coping strategies to social stress has been revealed by several studies (e.g., Eacret et al., 2019; Grafe et al., 2018; Ji et al., 2019; Staton et al., 2018). Although these studies employed different experimental protocols (i.e., social defeat/rank stress), our findings comply with their general conclusion that orexin dysfunctions are associated with susceptibility to psychosocial threats and social avoidance (Ji et al., 2019; Staton et al., 2018), since we observed enhanced and prolonged conditioned social fear in orexin-deficient mice. In addition to its role in adaptation to threats, it has been proposed that orexin also promotes motivational behavior directed towards reward opportunities (Harris et al., 2005; Mahler et al., 2014). It is well established that social interactions with conspecifics are rewarding and highly motivational for rodents (Douglas et al., 2004; Panksepp et al., 2007). Hence, our findings of reduced sociability and more pronounced conditioned social fear in orexin-deficient mice support an important role of the orexin system in reward functions.

In conclusion, this study extends our knowledge about the orexin system's role in social (fear) behavior. We show that orexin deficiency in mice reduces sociability and social novelty behavior (in females) and facilitates the acquisition and/or expression of conditioned social fear, while impairing its extinction (in both sexes). These findings are in accordance with others suggesting that the orexin system serves as an integrator of motivation, affect, and emotion (Li et al., 2014; Mahler et al., 2014; Sakurai, 2014). Furthermore, our data may be of translational importance, as altered orexin signaling has been implicated in several neuropsychiatric disorders (Chen et al., 2015), and many of them are characterized by social dysfunctions and changes in emotional learning and memory.

4. Experimental procedure

4.1. Animals

Two- to four-month-old experimentally naive female and male homozygous (OX −/−) and heterozygous (OX +/−) orexin-deficient mice, and their wild-type (OX +/+) littermates were used. In these mice, the prepro-orexin gene is manipulated, which leads to a lifelong absence of both orexin A and B peptides (Chemelli et al., 1999). The mice were bred on a C57BL/6J background in our local animal facility. Genotyping was performed using a standard end-point PCR (DreamTaqTM Green PCR, Thermo Fisher Scientific, Vilnius, Lithuania). Mice were housed in single-sex and mixed-genotype groups during the whole experiment (experiment 1) or individually starting one week prior to testing and lasting until the end of the experiment (experiment 2). For housing, standard cages in a temperature- and humidity-controlled room, with a 12 h light/dark cycle (lights on at 06:00 h), were used. Mice had *ad libitum* access to water and rodent chow. Behavioral testing was performed during the light phase. All animal care and experiments were performed in compliance with international guidelines regarding the use of animals in experiments (2010/63/EU) and were approved by the local ethical committee (Az. 42502–2-1351 & 42502–3-747 UniMD).

The group sizes were as follows:

Experiment 1: OX +/+: females n = 12, males n = 14; OX +/−: females n = 12, males n = 22; OX −/−: females n = 10, males n = 13.

Experiment 2: OX +/+: females n = 11–12, males n = 14; OX +/−: females n = 10–11, males n = 12–13; OX −/−: females n = 11–12, males n = 14; per conditioning group, respectively.

4.2. Behavioral tests – Setups and procedures

4.2.1. Experiment 1: Sociability test

The sociability test was adapted from Crawley (2004). We used a setup consisting of two separated boxes (46 cm × 23 cm × 32 cm) for simultaneous testing of two individual mice. Both boxes had black opaque walls and a grey floor, and were placed inside a sound-attenuating chamber with mild illumination (22–32 lx) and a camera on the ceiling for recording the test. Two cylindrical, small wire mesh cages (diameter: 8.5 cm, height: 10 cm) for enclosing social partners were localized in two opposite corners of each box. Weighted bottles were placed on top of these cages to prevent the test mice from climbing onto them.

Before starting the behavioral test, we habituated future social partner mice to the wire mesh cages and test mice to the experimenter (2 × 5 min daily for two consecutive days, respectively). Our protocol was divided into three phases: habituation phase, sociability phase, and social novelty phase (cf. Crawley, 2004). In the habituation phase, test mice were individually placed into the center of the boxes, which contained two empty wire mesh cages in opposite corners. The mice could explore the boxes for 10 min. Immediately after that, the test mice were

shortly removed from the boxes and one of the empty cages was replaced by another cage containing a social partner (stranger 1). The following sociability phase was started directly afterward by placing the test mice back into the boxes. After 10 min, the test mice were again removed from the boxes and the empty cage was replaced by another cage with an enclosed novel social partner (stranger 2). Cages containing stranger 1 remained inside the boxes. The following social novelty phase took again 10 min and started immediately afterward by placing the test mice into the boxes. After finishing this phase, all mice were placed back to their home cages and the boxes and wire mesh cages were cleaned with water.

The locations of the wire mesh cages containing strangers 1 and 2 were alternated between subjects throughout the experiment. The social partners were always unfamiliar wild-type mice of the same sex and similar age and weight as the test mice, but descendants from different breeding lines.

4.2.2. Experiment 2: Social fear conditioning (SFC)

Our behavioral paradigm for SFC (Kolodziejczyk et al., 2020) is a combination of previously published protocols (Toth et al., 2013; Xu et al., 2019). For measuring sociability before and after SFC, we used the same setup as described above. Only for the conditioning procedure, a modified setup was employed that differed from the sociability setup regarding the following features: the box was located inside another sound-attenuating chamber and was equipped with transparent walls and a steel grid floor (bars: 4 mm diameter, distance: 8.9 mm). The floor was connected to a shock unit that allowed the delivery of electrical foot shocks of defined duration and intensity (Fear Conditioning System, TSE Systems, Bad Homburg, Germany). As for the sociability setup, two wire mesh cages were placed in opposite corners. The floors of these cages were made of plastic, i.e., the cages were electrically isolated from the floor grid.

One week prior to starting the experiment, the test mice were placed in separate holding cages and remained single-housed until the end of the experiment. On the two consecutive days before the experiment, the test mice were handled by the experimenter, while the social partner mice were habituated to the wire mesh cages for 2 × 5 min daily.

The experimental timeline is depicted in Fig. 2 and consisted of a pre-test (day 1), a conditioning test (day 3), and six consecutive expression/extinction tests (days 6 and 7). The pre-test on day 1 was used for measuring the test animals' baseline sociability. This test was identical to the first two phases (habituation and sociability) of the sociability test described above (see 4.2.1.), except that each phase lasted only 5 min, and this applied to all the following tests of this paradigm. On day 3, the conditioning was performed. In the sociability phase, the test mice were punished with a mild foot shock (0.4 mA, 1 s) for each approach to the cage containing the social partner, while approaches to the empty cage were not punished. Consequently, the number of received foot shocks was depending on the animals' approach behavior (between 1 and 5 in this study). The foot shocks were manually triggered by the experimenter who observed the mice via a monitor. After SFC, there was a break of two days without tests. We introduced this break since we previously observed generalized fear towards empty wire mesh cages when using only a 24 h break (data not shown). Next, the mice were tested six times for their sociability to measure the expression and extinction of conditioned social fear. These six tests (E1-E6), identical to the pre-test, were performed on two consecutive days (days 6 and 7) with three tests daily and breaks of ca. 3 h between the tests.

Of note, we also included an unconditioned control group, which was tested in the same way except that these mice did not receive any foot shocks during conditioning. Importantly, in all eight tests of the SFC procedure, different and unfamiliar wild-type strangers of the same sex and similar age and weight as the test animals were used. Moreover, the location of cages containing the strangers was pseudo-randomized for each test mouse.

4.3. Analyses

4.3.1. Behavioral analyses

The recorded videos were analyzed with the EthoVision XT software (Version 11.0.928, Noldus Information Technology, Wageningen, The Netherlands), which was used to track the nose-points and the body center of the test mice. Of main interest were the investigation times of the wire mesh cages. Cage investigation was defined as the presence of a test animal's noise-point in an area of ca. 3 cm around the cages. This readout was highly correlated with the investigation times manually scored by two observers (Kolodziejczyk et al., 2020). Moreover, general locomotor activity (measured as distance traveled) was assessed using the body center.

4.3.2. Data analyses

For experiment 1, only the first 5 min of the different test phases were used for the descriptive and statistical analyses. The reason for this was our observation that the investigation times during the first 5 min were much more robust and that preferences for particular cages were more pronounced. Based on this, experiment 2 was planned with 5 min phase duration (cf. Kolodziejczyk et al., 2020).

Using the investigation times, a performance index was calculated with the following formula (cage 1 is the cage with stranger 1, if applicable):

$$performance\ index = \frac{(investigation\ time\ cage\ 1) - (investigation\ time\ cage\ 2)}{(investigation\ time\ cage\ 1) + (investigation\ time\ cage\ 2)}$$

This performance index was used as a measure of cage preference (habituation phase), of sociability or social avoidance (sociability phase), or preference for social novelty (social novelty phase) of the individual mice. Dependent on the phase, a positive index indicated preference for one cage, sociability towards the first social partner (stranger 1), or no preference for the second social partner (stranger 2), respectively. An index of zero was indicative of mice showing the same investigation time close to both cages. A negative index indicated, dependent on the phase, preference for the other cage, no sociability (i.e., social avoidance) towards the first social partner (stranger 1), or preference for the second social partner (stranger 2), respectively. Of note, in the SFC experiment, mice displaying a performance index below 0.1 in the pre-test were excluded from further analyses.

Data are presented as the mean ± SEM. Normal distribution of the data was verified with the D'Agostino-Pearson normality test. Statistical analyses were based on analyses of variance (ANOVAs), if appropriate with repeated measures, and *post hoc* Holm-Sidak's multiple comparison tests. Additionally, for the sociability test, *t* tests corrected for multiple comparisons (Holm-Sidak method) were used to evaluate the investigation times at the two cages within each experimental group. Chi-squared tests were used for comparing proportions. All analyses were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, USA) or SYSTAT 13 (Systat Software Inc., San Jose, USA). Values of $p < 0.05$ were considered significant.

CRediT authorship contribution statement

Nadine Faesel: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Malgorzata H. Kolodziejczyk:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Michael Koch:** Writing - review & editing, Supervision. **Markus Fendt:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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4 Discussion

AD-TSRD represent the largest group of mental disorders in most western societies and are a significant mental health challenge of the 21st century. They are the most prevalent and economically burdensome of all psychiatric diagnoses, yet they are among the most commonly under- or misdiagnosed and undertreated in primary health care. Additionally, the limited efficacy and side-effects of the currently available treatments make matter worse. Moreover, since 2005 there have been no indications of improvement in the care and treatment of these mental disorders (Wittchen et al. 2011). The discovery of novel molecular mechanisms along with a better understanding of predisposing genetic factors and critical assessment of the interaction between genes and the environment, are some of the most crucial determinants to improve the current suboptimal treatments.

Several mechanisms have been linked, to the genesis and prognosis of AD-TSRD, such as dysfunctional brain's fear circuitry, dysregulations of the HPA axis, and predisposing factors like genetic polymorphisms in genes encoding neuropeptides. The work presented in this thesis focused on studying AD-TSRD-related endophenotype in two animal models lacking neuropeptide systems, i.e., the NPS system and the orexin system. This method is a valuable research tool to elucidate the molecular interplay that might be common between animal models and AD-TSRD patients. Hence, I measured the behavioral endophenotypes with a focus on some of the hallmarks of AD-TSRD, i.e., the overgeneralization of fear memories, dysregulation of social (fear) behavior, along with the interplay of stress hormones.

In this work, considerable insights were gained into the complex role of the NPS and orexin systems and the interaction of stress hormones in the generalization of fear memories and various social behaviors such as sociability, social novelty, and social fear. I could show that the NPS system is associated with the behavioral endophenotypes of overgeneralization and dysregulation in social (fear) behavior. Additionally, I demonstrated that the orexin system is involved in dysregulation in social (fear) behavior. My findings indicate that the interplay between the CORT and the NPS system during the consolidation of fear memories is critical for the generalization of contextual fear. Additionally, I could show that deficiency of NPSR and orexin alters social behavior under non-aversive and aversive conditions, which is in line with previously published studies. These results offer vital confirmation for supporting the importance of sex-specific differences in social behaviors and may be of translational importance by suggesting that alternation in the NPS and orexin systems influence social dysfunctions that are considered as the hallmarks of AD-TSRD.

4.1 Synergistic effects of NPSR-deficiency, CORT, and incubation time

It has been suggested that the generalization of fear memories is modulated by several intrinsic factors, i.e., genetic background (Temme et al., 2014), sex differences (Day et al., 2016; Keiser et al., 2017), stress hormone levels, and external factors, e.g., the type and intensity of aversive stimulation (Baldi et al., 2004) or early-life stress (Elliott and Richardson, 2018). Moreover, the mechanisms underlying generalization seems to be critically sensitive to the passage of time. However, it is not yet known why some individuals develop fear overgeneralization while others are resilient, suggesting that the generalization of fear can have a complex multi-dimensional mechanism.

A previous study from our laboratory attempted to understand the involvement of the NPS system in incubation-induced fear generalization by subjecting NPSR-deficient mice to a one-trial contextual fear conditioning paradigm with one retention test session (Germer et al. 2019). Although we could not observe a genotype effect on fear generalization in the mentioned paradigm, a significant increase in the plasma CORT levels of the NPSR-deficient mice at the end of the experiment was observed. This study called for further systematic investigations due to its potential caveats, such as lack of the CORT level measurements during the experiment and no individual baseline CORT level measurements. Hence in my thesis, I planned to address these points to systematically study the interplay between fear generalization, the NPS system, and the CORT. I measured the CORT levels at various stages, such as at the beginning of the experiment as the baseline, during the memory consolidation and retention, to correlate these changes to AD-TSRD-related endophenotypes in tested mice. Moreover, I included two previously described quantitative parameters to evaluate the fear memory generalization: the strength of the fear memory (revised by incubation time) and the specificity of the fear memory (revised by different contexts) (Kaouane et al. 2012; Sauerhofer et al. 2012). The new paradigm, also based on classical fear conditioning, has two retention sessions and is combined with systemic injection of the CORT during the consolidation of fear memories.

To investigate the strength of the fear memory, I subjected the same group of mice to both a recent and a remote memory test, with a gap of 1 month of incubation time. The first retention test usually enhances the fear memory in the long term. Of note, most of the earlier studies investigated the effects of either incubation time or the CORT on the generalization of fear memories used two different groups of mice (e.g., Cordero and Sandi 1998; Kaouane et al. 2012; Pollack et al. 2018; Sauerhofer et al. 2012; Siegmund and Wotjak 2007; Wiltgen and Silva 2007). However, submitting the same group of mice to two or more memory tests more adequately mimics the human cases of AD-TSRD and allows a more specific investigation of the possible interplay between the CORT and the NPS system in the development of generalized fear memory. In order to investigate the specificity of fear memory, I exposed the animals to three different contexts. Two of these contexts were very similar to each other (conditioning context A and similar context A’), and one of these was different (context B). Furthermore, I examined whether the treatment, the genotype, and the sex of the animals affected the specificity of fear memory in different contexts and the strength of this memory after incubation time. Also, I was interested in how these factors affected anxiety-like behavior, startle response, and weight gain of tested mice.

4.1.1 NPSR-deficiency, high levels of CORT, and 1 month of incubation time are the main components of fear generalization

At first, I examined whether incubation time, treatment, genotype, and sex affected the specificity of fear memory in different contexts. I observed that all treatment groups, irrespective of genotype, sex, and incubation time, showed a relatively specific fear memory to the conditioning context A compare to context A' and B (Publication 1, Figures 2 + S2, left panels; for original freezing data see Figures S1 + S3), except the group of 5 mg/kg CORT-treated NPSR −/− mice (context A vs. A'). Additionally, the strength of the fear response in a particular context after 1 month incubation period was also not affected by treatment, genotype, and sex except again in the group of NPSR −/− mice treated with 5 mg/kg CORT (Publication 1, Figures 2 + S2, right panels; for original freezing data see Figures S1 + S3). These data suggest a synergistic interplay of NPSR deficiency,

high levels of CORT, and incubation time that leads to impaired specificity along with the strength of fear memory that may mirror the maladaptive endophenotype of generalization found in AD-TSRD. Nevertheless, further work should explore the experimental design to dissect the most critical factors in the development of fear memory generalization, i.e., NPSR deficiency, incubation time, or the CORT levels. That can be achieved by measuring the generalization of fear memories in the same experimental setup lacking one or the other factor using different groups of mice. Determining the impact of memory processes activated during the first retention test should also be a very insightful experiment for future studies.

4.1.2 NPSR deficiency rescues the CORT dependent inhibition of potential safety response

In addition to measuring response to different contexts, I also measured the freezing behavior of the mice in response to a tone stimulus (T), which was previously presented during the fear conditioning session, as well as to a similar tone T' and a novel tone N (Publication 1, Figure 1D). Importantly, tone T was presented explicitly unpaired (Publication 1, Figure 1C) and did not predict the unconditioned stimuli during fear conditioning. That suggests that tone T could be learned as a safety stimulus (for review see: Kong et al. 2014), which typically requires presentation of several explicit unpairings (> 12; e.g., Khalil and Fendt 2017; Pollak et al. 2010a; Pollak et al. 2010b) as compared to two tone stimuli that were presented in the current protocol. Interestingly, in different AD-TSRD, an impaired safety response has been repeatedly reported (e.g., Jovanovic et al. 2012; Jovanovic et al. 2009; Lissek et al. 2009; Lohr et al. 2007; Norrholm et al. 2013; Sijbrandij et al. 2013) suggesting another significant involvement of NPSR signaling in AD-TSRD. Here, I observed that the CORT treatment only impaired the safety response in NPSR +/+ mice, but not in NPSR +/− and NPSR −/−. That indicates that NPSR-deficiency may have resulted in a beneficial gain of function in learned safety paradigms. This conclusion is further supported by the recent finding of our laboratory that reveals that NPSR-deficient mice have more pronounced safety learning (Kreutzmann et al. 2020). Notably, the observation that NPSR-deficiency and the CORT treatment differently interact in fear generalization and fear inhibition argues for dissociative mechanisms underlying these two phenomena.

4.1.3 Re-release of the plasma CORT levels are affected by NPSR deficiency

In addition to fear behavior, I also systematically measured the plasma CORT levels of the mice throughout the different phases of the experiment. Such detailed monitoring of the CORT during the fear conditioning paradigm has never been studied. I hypothesized that measuring the plasma CORT levels and correlating their abnormalities with the fear-related behavioral changes might help to elucidate the association between dysregulations of the HPA axis and the development of AD-TSRD (for review see: Chrousos 2009; de Kloet et al. 2005). More specifically, I wanted to address the research questions of how changes in the plasma CORT levels may be associated with the generalization of fear memories.

It has been well-established that corticosterone levels reach a peak in the plasma after 30 min of a stressful event. As expected, I observed a significant increase in the CORT levels after 30 minutes of conditioning in all experimental groups (Publication 1, Figure 3a), showing that the CORT levels robustly reflect the stressful event of conditioning. As intended, the intraperitoneal (ip) CORT injections of 2.5 mg/kg and 5mg /kg, given immediately after fear conditioning, further enhanced the plasma CORT levels. Interestingly, animals previously injected with 5 mg/kg of CORT have

significantly higher CORT levels, specifically after exposure to the conditioning context, compared to a similar or different context, suggesting the conditioning context-specific re-release of CORT (Publication 1, Figure 3C, right panel). This phenomenon was observed across all genotypes, suggesting that the high CORT levels may overshadow the involvement of the NPSR gene. However, at the lower doses of CORT (2.5 mg/kg), the conditioning context-specific re-release of CORT was observed only in NPSR +/− and NPSR −/− animals and not in the wild-type littermates suggesting that partial or complete loss of NPSR seems to be essential for this conditioning context-specific re-release of CORT (Publication 1, Figure 3C, middle and right panel). Furthermore, I did not observe this context-specific re-release of CORT in the vehicle-injected animals (Publication 1, Figure 3C, left panel), suggesting that CORT levels presented during the consolidation of fear memories might play a crucial role in the stress response of the animal during the re-exposure to the stressful event and haplodeficiency and/or complete absence of NPSR seem to regulate this process. Moreover, I did not observe these context-specific re-releases of CORT in the remote retention test after 1 month of incubation, indicating the short-term nature of such regulation (Publication 1, Figure 3d). To sum up, elevated plasma CORT levels during the consolidation of fear memories might be related to increased CORT levels in the stress response in early retention tests, and NPSR deficiency seems to affect this process.

I also observed consistently lower baseline CORT levels in all genotype and treatment groups after 1 month of incubation time, i.e., before the remote retention tests. Interestingly, it has been shown that PTSD patients also exhibit lower baseline (resting) levels of stress hormone and higher cortisol levels following exposure to trauma reminders (for review see: Pan et al. 2018). Collectively, these data are in line with previous studies and suggest decreased baseline CORT levels after a particular incubation time following trauma. That might be due to the activation of some homeostatic mechanisms controlling the plasma CORT levels. Unfortunately, I did not observe a direct significant correlation between the baselines CORT levels (BL I, BL II, and BL I/BL II ratio) and the CORT levels during the re-exposure to the conditioning context (data not shown).

Furthermore, several studies have shown a functional cross-regulation of the NPS system and the HPA axis. Interestingly, NPS seems to be released in BLA upon stress (Ebner et al. 2011) and injection of NPS in the ventricle leads to an increase in the CORT levels (Smith et al. 2006) suggest a bidirectional regulation of the NPS system and the HPA axis. Moreover, a functional variant of the functional NPSR gene (NPSR1) has been associated with higher cortisol levels in humans (Streit et al. 2017). However, I could not observe any effects of genotype on changes in the plasma CORT levels either at baselines (BL I or BL II) or after exposure to any of the context suggesting that either lack of NPSR function from the whole brain might mask the brain area-specific NPS regulations of the HPA axis or brain has developed some compensatory mechanisms due to lack of NPSR gene during the development of the brain. These results are in line with a previously published report where no difference in the CORT levels was observed in NPSR −/− mice either at the baseline or after the stress of forced swim test (Zu et al, 2010).

It would be very interesting to design further experiments to study the fear generalization in NPSR −/− animals in the one remote memory test in presence of the high level of CORT during memory consolidation. Future work should also consider continuing to systematically measure the CORT levels in mice at different phases of the experiment, as was done in this dissertation. Collectively, the data suggest that the NPS system is involved in the modulation of the CORT release during fear

memory retention by CORT injections during the consolidation of fear memories.

4.1.4 Incubation- and CORT-induced fear memory generalization in relation to anxiety-related behavior, startle response magnitudes, and body weight gain.

I submitted mice to light-dark box test and startle response test to evaluate their innate fear and to measure their sensitization, respectively (Bourin and Hascoet 2003; Golub et al. 2009; Sauerhofer et al. 2012). My data suggest that the 5 mg/kg CORT-treated NPSR-deficient mice that developed fear memory generalization did not express increased anxiety-related behavior. However, I observed specifically reduced startle response magnitudes and less body weight gain in females.

I did not observe alterations in anxiety-like behavior in any experimental groups (Publication 1, Figure 5, middle panel; Figure S5). Previous reports found that NPSR −/− showed more anxiety-like behavior in the light-dark box as compared to NPSR +/− mice and the wild-type littermates (e.g., Germer et al. 2019; Zhu et al. 2010). This different outcome can be most likely attributed to the experimental design, as in the current work, the anxiety-like behavior was measured at the very end of the experiment. Nevertheless, under current experimental conditions, impairment of the specificity and strength of the fear- memory in NPSR −/− mice did not affect their anxiety-related behavior.

Furthermore, I also tested whether NPSR deficiency affected the acoustic startle response. As previously reported by others (Fendt et al. 2011; Germer et al. 2019; Zhu et al. 2010), we observed higher startle magnitudes in male mice. While previous studies observed a decreased startle response in NPSR +/− and NPSR −/− mice, I did not observe such genotype effects within the vehicle or the 2.5 mg/kg CORT-treated groups. Interestingly, I did observe a similar decrease in the startle response but only in the group of overgeneralized NPSR −/− mice treated with the 5 mg/kg CORT (Publication 1, Figure 5C, left panel). In this group, startle magnitudes with higher startle stimulus intensities was significantly lower than for the 5 mg/kg CORT-treated NPSR +/+ and NPSR +/−. This difference in the data is potentially due to the differences in the experimental design. In the current study, I used the same animals throughout the experiment, and the startle response was performed after the fear conditioning and two retention tests with a time interval of 1 month. However, in previous studies the time points of the test and/or the number of experimental groups were different. Recently in our laboratory using two separate groups of mice, we showed lower startle response in animals 4 weeks after fear conditioning in comparison to animals tested after one week (Germer et al. 2019). As mentioned before, some authors have suggested that it is possible that the first retention test improves the specificity of the fear memory and thereby influence the fear memory and changes in the behavior of animals, probably including startle response, after the incubation time (e.g., Bueno et al. 2017; De Oliveira Alvares et al. 2013). Future experiments should include testing anxiety-like behavior and startle response both before and after the experimental timeline. In addition, abnormalities in the expression of the startle response are frequently found in patients with AD-TSRD (for review see: Beck and Catuzzi 2012). There are also several reports describing blunted startle reactivity in PTSD patients while exaggerated startle magnitudes are also documented in PTSD. In a variety of animal studies using various types of stress, such blunted startle reactivity has also been observed. Importantly, such a decline in the reactivity of the startle could not be due to the increased habituation of the startle stimuli (for review see: Beck and Catuzzi 2012). That again supports the notion that during the consolidation of fear

memory, NPSR deficiency and the elevated CORT levels contribute to behavioral changes analogous to those of patients with PTSD.

Acute or chronic stress and CORT treatment frequently result in body weight disturbances (for review see: Harris 2015; van der Valk et al. 2018). I observed a considerable increase in the body weight of all groups of mice, but surprisingly not in the 5 mg/kg CORT-treated NPSR-deficient mice, i.e., the only group exhibiting generalized fear memory and reduced startle response. This effect was, however, only observed in female mice indicating a sex-specific NPS/CORT-related hormonal imbalance that seems to affect the gain in body weight.

4.1.5 Monoaminergic signaling as a modulator of incubation- and CORT-induced fear memory generalization in NPSR-deficient mice

An increasing number of studies have found that dopamine is involved in fear memory mediated by its receptors expressed in the hippocampus, AMY, prefrontal cortex (PFC), and striatum. Mice lacking the dopamine D1 receptor (D1R) or mice treated with dopamine D2 receptor (D2R) antagonist after exposure to contextual fear conditioning showed fear memory generalization. Animals were unable to discriminate between the conditioning context and a novel context (e.g., Abraham et al. 2016; Ikegami et al. 2014; Sarinana et al. 2014), while the delivery of the D2R agonist had the opposite effect (De Bundel et al. 2016). D2R in the hippocampus may determine the extent of generalization in humans (Kahnt and Tobler 2016).

Studies related to noradrenaline (NA) showed the opposite trend suggesting that pharmacological enhancement of NA is linked with fear memory generalization (Gazarini et al. 2013). Studies with SSRI administration in rodents revealed a complex relationship between serotonin and fear memory generalization (wide range of results: increase, decrease, no differences). Furthermore, very few studies have addressed the relationship between the NPS system and monoaminergic signaling. It has been shown that NPS inhibits the release of 5-hydroxytryptaminergic (5-HT) and NA *in vivo* in mouse AMY and PFC as well as *in vitro* (e.g., Gardella et al. 2013; Raiteri et al. 2009). Moreover, the central administration of NPS enhanced extracellular levels of dopamine in a dose-dependent manner (Si et al. 2010). Interestingly, chronic treatment of rats with antipsychotic medications such as haloperidol, chlorpromazine, and olanzapine resulted in decreased expression of NPSR mRNA when administered in the striatum and hippocampus (Palasz et al. 2016; Pałasz and Rojczyk 2015). Haloperidol acts as dopamine receptor agonists, chlorpromazine works by blocking a variety of receptors in the brain, including dopamine receptors, and olanzapine binds with high affinity to the serotonin, as well as dopamine receptors.

These studies have prompted me to investigate the modulatory role of dopamine, NA, and serotonin in the generalization of fear memories in NPSR-deficient mice. Efforts of collecting tissue samples from various brain areas such as PFC, AMY, hippocampus, NA, caudate putamen, hypothalamus, thalamus, and substantia nigra are undergoing to quantify the levels of different monoamines using high-performance liquid chromatography (HPLC) analysis. Correlating levels of various monoamines with the behavioral endophenotypes and comparative analysis between different genotypes, also in naïve animals, treatment groups, and sex might reveal new insights.

4.1.6 Activation of microglia in mediating the development of fear memory generalization in NPSR-deficient mice.

Microglial cells express a variety of neuropeptide and neurotransmitter receptors, which modulate their function (for review see: Pocock and Kettenmann 2007). The last decade has witnessed a growing interest in the study of microglial activation and its modulation by neuropeptides, e.g., NPY or VIP, in the hope of developing new therapeutics for treating some neurodegenerative diseases and AD-TSRD (for review see: Carniglia et al. 2017)**.** Interestingly, no information about the role of the NPS system in the modulation of microglial function is known so far. A growing body of literature has demonstrated the crucial role of pro-inflammatory activation of microglia in mediating neuropsychiatric disorders, including AD-TSRD (e.g., Blank and Prinz 2013; Dantzer et al. 2008; Haroon et al. 2012). In an animal model of PTSD (predator exposure/psychosocial stress regimen), rats exhibited increased activation of microglia and elevated levels of pro-inflammatory cytokines in the hippocampus, a brain region critically involved in the pathophysiology of PTSD (Wilson et al. 2013). Interestingly, the immune-suppressing effects of minocycline have been shown effective in preventing PTSD-like behavior in rats exposed to predator scent stress indicating the potential use of minocycline in preventing physiological and behavioral alternations resulting from acute exposure to psychological stress (Levkovitz et al. 2014). Some clinical studies suggest that some AD-TSRD are associated with an excess of inflammatory immune activities (Gill et al. 2009; Gola et al. 2013).

In order to study the differential activation of microglia cells among brain sections of naïve NPSR +/+, NPSR +/−, and NPSR −/− mice, I have already established in our group the protocol for immunostaining for microglia activation using Iba-1 staining. Furthermore, calculating the ratio of microglia processes to cell soma size was used as a proxy method for evaluating microglia activation (Hovens et al. 2014) (Appendix, Figure 1 Appendix). As per my current working hypothesis, a group of animals that developed the generalization of fear memories might have increased activation of microglia. Future studies can be conducted to explore the molecular mechanism governing activation of microglia in mediating the development of fear memory generalization.

4.2 Role of the NPS and orexin system in social behavior

Social behavior is a complex multi-factorial mechanism involving the modulatory effects of potentially several neuropeptides. The NPS and orexin systems play an essential role in social behavior under non-aversive conditions in a sex-dependent manner. I investigated the effects of NPSR and orexin deficiency on sociability and social novelty by using a modified version of Crawley's sociability test (Crawley 2004; Moy et al. 2004). Of note, I observed that preferences for social interactions were generally stronger during the first 5 min out of 10 min long phase, indicating that these behaviors may decrease with time. Therefore, further social interaction studies should take this into consideration.

4.2.1 NPS system and social behavior

Previous studies have shown that icv NPS injections did not affect social discrimination and social preference behavior of male wild-type rats (Lukas and Neumann 2012). The work in this thesis provides the first experimental evidence of sociability and social novelty behavior of NPSR-

deficient mice. Here, I investigated the effects of NPSR deficiency on social interaction under non-aversive conditions. All mice groups showed a significant preference for the cage containing the social partner compared to the empty one, except for female NPSR +/− mice (Publication 2, Figure 2A2 & 2B2). Interestingly, haplodeficiency of NPSR resulted in reduced sociability only in females. However, the complete lack of NPSR did not show any effect, suggesting the involvement of some compensatory mechanisms. Moreover, we observed similar genotype effects in the startle magnitude (Germer et al. 2019) (but see: Fendt et al. 2011; Kolodziejczyk and Fendt 2020), although this effect was driven by male mice (Germer et al. 2019). Collectively, these data suggest compensatory mechanisms in NPSR −/− mice, which do not occur in NPSR +/− mice, and that these mechanisms may be sex-specific. Interestingly, such compensatory mechanisms were not observed in other paradigms such as spontaneous locomotor activity where NPSR −/− mice show a more robust phenotype as compared to NPSR +/− mice (Fendt et al. 2011). Moreover, neither NPSR +/− nor NPSR −/− mice show any other behavioral impairments in the paradigms of anxiety, conditioned fear, and prepulse inhibition (Fendt et al. 2011; Germer et al. 2019; Kolodziejczyk and Fendt 2020). The present data support the general advice to include heterozygous animals in the use of transgenic animals as they can help to understand the underlying either causal or compensatory mechanisms if they exist (Bailey et al. 2006; Crawley 2000).

Concerning sex-related effects of NPSR deficiency, only a few studies have been reported. Recent findings of our laboratory showed that NPSR-deficient male mice have more pronounced safety learning as compared to female mice (Kreutzmann et al. 2020). Moreover, the sex-specific association between NPSR1 variants linked with cortisol stress responses was found only in males (Streit et al. 2017). In general, very little is known on sex-related effects of NPSR deficiency, and this underlines just how important is to include both sexes in future studies, especially studies that may be of translational relevance.

Furthermore, all groups of animals showed a significant preference for the cage containing the novel social partner compared to the familiar one (Publication 2, Figure 2A3 & 2B3), except female mice. We could not derive any conclusion about the role of NPSR deficiency in the female mice as NPSR +/+ mice did not perform well in this paradigm (Publication 2, Figure 2A3 & 2B3). We currently have no clear explanation as normal social recognition has been described in female mice (Moy et al. 2004), and we clearly could observe normal behavior in the male mice as well as in the female from another mouse line (Publication 3).

In most cases, locomotor activity was not affected by the genotype, except for NPSR +/− mice of both sexes during all three phases of the sociability test (Publication 2; Figure 2C). However, since male mice displayed normal sociability and social novelty behavior, I am confident that the social impairments observed in female NPSR +/− mice were not confounded by reduced locomotor activity. It has been shown that while the reactive locomotor activity of NPSR-deficient mice might be normal (Fendt et al. 2011; Zhu et al. 2010) (but see: Duangdao et al. 2009), the spontaneous locomotor activity might be reduced (Fendt et al. 2011). Though, the effect might be more pronounced when the mice are previously been exposed to the aversive stimulus, e.g., a second exposure to the elevated plus maze (Fendt et al. 2011).

At this point, I cannot conclude whether these sex-dependent effects on sociability and social novelty were directly caused by the NPSR deficiency or by developmental compensatory mechanisms in these constitutive knockout mice, or by a combination of both. Future studies can

be designed with condition knockout mice, knockdown of these peptides using siRNA approaches, or acute interventions using specific pharmacological agents.

Overall, these results offer vital information on sex-specific differences in social behavioral endophenotypes that are a crucial component of several neuropsychiatric disorders. Hence, these findings may be of high translational importance and can contribute to elucidating the mechanisms underlying the development of AD-TSRD.

4.2.2 Orexin system and social behavior

My work described in this thesis is the first experimental evidence of sociability and social novelty behavior of orexin-deficient mice. I observed sex-specific impairments in female mice with a complete lack of orexin. These mice exhibit reduced sociability behavior (Publication 3, Figure 1A2 & B2) and a decreased preference for social novelty (Publication 3, Figure 1A3 & B3). Moreover, heterozygous female mice show impairments only in social novelty and not in sociability behavior. Interestingly, these impairments in sociability and social novelty were absent in knockouts along with heterozygous male mice, again strongly supporting the guidelines provided by Animal Research: Reporting of In Vivo Experiments (ARRIVE), which highly recommend the use of both sexes in the experiments. The rationale for the observed sex difference could also be that females often show higher trait anxiety than males (e.g., An et al. 2011; Ilse et al. 2019; Khalil and Fendt 2017). These findings are also in line with previous observations. It has been demonstrated that mice with global OX1R deficiency and mice with a localized OX2R knockdown in the BLA express reduced sociability (Abbas et al. 2015; Arendt et al. 2014). Furthermore, nasal administration of orexin A and chemogenetic activation of the orexin system were shown to ameliorate induced social memory impairments in mice (Stanojlovic et al. 2019; Yang et al. 2013). However, all previous studies were conducted in male mice and therefore did not explore sex differences.

Notably, sex-related effects of orexin deficiency were also observed in cognitive flexibility (Durairaja and Fendt 2020) panic-like anxiety, and drinking behavior (Faesel, Koch & Fendt; unpublished observation). Moreover, sexually dimorphic changes of the orexin system have been reported on the mRNA, peptide, and receptor levels at baseline conditions (Jöhren et al. 2002; Loewen et al. 2017; Taheri et al. 1999), but also concerning stress adaptation (Grafe et al. 2017a) and depression (Lu et al. 2017). Altered orexin levels in the cerebrospinal fluid have been found in AD-TSRD (e.g., Brundin et al. 2007; Johnson et al. 2010; Strawn et al. 2010), and these disorders are more prevalent in women (Kessler et al. 1994). Treatment with orexin, e.g., by nasal administration (cf. Baier et al. 2011; Weinhold et al. 2014), might be a treatment option for some of these diseases and my findings suggest that women could be more sensitive to such treatment. Collectively, these studies robustly support the sexual dimorphisms of the orexin system that may be of translational relevance.

Furthermore, the locomotor activity of the animals was not affected by the genotype or sex, except for OX −/− mice during the social novelty phase (Publication 1; Fig. 1C). However, as male mice displayed normal sociability and social novelty behavior, the association of decreased locomotor activity and observed social impairments, can be excluded. Further experiments with acute inhibition of orexin should be performed. That would give us insight into whether these sex-dependent effects on sociability and social novelty were directly caused by the orexin deficiency or developmental compensatory mechanisms specifically developed in male mice (see discussion in:

Chowdhury et al. 2019). Overall, these results offer vital confirmation for supporting the importance of sex-specific differences in social behavior. Therefore, they may be of translational importance by suggesting that alternation in the orexin systems influence social dysfunction to consider as behavioral endophenotypes relevant to the development of AD-TSRD.

4.3 Role of the NPS and orexin systems in social fear conditioning

In this thesis, I investigated the effects of NPSR and orexin deficiency on social behavior under aversive conditions by using a modified version of the SFC paradigm (see Publication 2, 2.2.2.2. and Figure 1) primarily introduced by Toth and colleagues (Toth et al. 2012; 2013). During the SFC procedure, animals were punished by electrical stimuli only when they were approaching the cage containing unfamiliar conspecific, but not to the empty cage. The main modification that I introduced in my experiments was to include a pre-test for assessing the test animals' baseline sociability. Hence, the modified paradigm allowed me to examine the role of NPSR and orexin deficiency in the acquisition, expression, and extinction of conditioned social fear by testing the animals' sociability both before and after the SFC. This additional test provides valuable information about the strength of the conditioning effect for individual mice. This pre-test consisted of the first two phases (habituation and sociability) of Crawley's sociability test. All tested mice were housed individually prior to starting the experiment to stimulate sociability and avoid sociability deficits, which were observed previously while performing sociability test in female NPSR +/− and OX −/− mice (Publication 2, Figure 2A2 & B2; and Publication 3, Figure 1A2 & B2).

This approach appears to be beneficial, and I observed that female NPSR +/− and OX −/− mice showed normal sociability behavior. Moreover, conditioned social fear and its extinction were evaluated by testing the mice's sociability, six times after conditioning.

A very similar protocol was recently published by Xu and colleagues (Xu et al. 2019). Additionally, I grouped all mice into groups of responders and non-responders, based on their performance in the first expression (extinction) test E1. Responders were the mice that show social avoidance after conditioning, while non-responders did not show social avoidance. Although the protocol worked well based on statistical analyses of the whole group of mice, I realized that approximately half of the mice did not express social avoidance after SFC (Publication 2, Fig. 4A2; and Publication 3, Fig. 3A2). Importantly, this proportion of non-responders was found in each NPSR and orexin genotype and both sexes and was not affected by the number of foot shocks received during SFC. A potential explanation for this could be that non-responder mice did not react with avoidance after SFC but expressed a more active coping strategy, such as agonistic behavior (cf. De Miguel et al. 2011). Further work needs to be done to verify the explanation for these different performances in the first expression test E1. The use of more appropriate conditions to measure agonistic behavior, e.g., in a paradigm of free social interaction, may explain that matter. Additionally, improving the rate of responder mice by increasing the foot shock intensities (cf. Toth et al. 2013) can also be implemented for future experiments.

After submitting the mice to SFC, I found significantly reduced sociability in all tested animals confirming that I socially conditioned them (Publication 2, Fig. 4A1; and Publication 3, Fig. 3A1). However, there were significant differences between the influence of NPSR and orexin deficiency on the acquisition, expression, and extinction of social fear. I discussed them in more detail below.

Nevertheless, these differences were not related to reduced sociability before SFC because the pre-test of this experiment did not show a sociability deficit in any of the tested groups. These differences were also not confounded by changes in pain sensitivity, since we previously demonstrated that the reactivity to foot shocks of the intensity used in this study (0.4mA) is not affected by the orexin genotype or sex (Khalil and Fendt 2017).

4.3.1 NPS system and social fear conditioning

Very little is known about the role of the NPS system in social behavior under aversive conditions. Seminal work in the field by Zoicas and colleagues focused primarily on icv injections of NPS. They reported that NPS reduced learned avoidance of known conspecifics induced by social defeat or unknown conspecifics induced by SFC (Zoicas et al. 2016). Few studies also show the role of NPS in aggressive behavior during social contacts. In the test, NPS injections had anti-aggressive effects on rodents' behavior depending on the high innate levels of anxiety-related behavior (Beiderbeck et al. 2014; Ruzza et al. 2015). Also, NPSR-deficient mice showed elevated levels of aggressive behavior (Ruzza et al. 2015). In humans, the association has been shown between NPSR gene polymorphism and emotional reactivity related to social stress (Kumsta et al. 2013). The analysis revealed that in response to acute psychosocial stress challenge, T/T-allele carriers showed higher emotional reactivity with simultaneous higher salivary cortisol release compare to A/T- and A/A-allele carriers that demonstrated intermediate and lower outcomes, respectively. Collectively, these data suggest the possibility that NPS might interact with the social fear circuitry by modulating the innate levels of stress-related hormones via modulating the activity of the HPA axis and thereby manifest its anti-aggressive effects.

In my experiments, heterozygous NPSR-deficient mice showed impaired and/or delayed extinction of fear without affecting the acquisition and expression of conditioned social fear. Surprisingly, homozygous NPSR-deficient mice did not affect the acquisition and expression of conditioned social fear. More interestingly, I observed a gain of function in these animals as social fear extinction was facilitated, suggesting that complete lack of NPSR might even be beneficial for these animals potentially due to the involvement of some compensatory mechanisms. One caveat of having the compensatory mechanisms is that it is not fully conclusive whether the observed differences are the result of NPSR deficiency, or the developmental compensatory mechanisms, or by a combination of both. Further experiments are required to characterize the role of NPSR using more acute treatments such as siRNA mediated knockdown or specific pharmacological inhibitions.

Involvement of the NPS system in fear extinction learning is well-documented also in models of rodents with high trait anxiety or attenuated fear extinction (e.g., Liu et al. 2017; Sartori et al. 2016; Slattery et al. 2015). That may be attributed to a profound memory-enhancing effect of NPS, i.e., activation of central NPS signaling can promote consolidation of long-term memory by interacting with noradrenergic signaling in the brain, however, it seems that it happens independent of the presence or absence of emotional content of the memory trace (e.g., Han et al. 2013; Lukas and Neumann 2012; Okamura et al. 2011). SFC data reported in this thesis are the first to show that conditioned social fear and extinction of this fear are altered in NPSR-deficient mice.

Alterations in the extinction of conditioned social fear were analyzed only based on the responders' group since extinction can be studied only in mice that express social fear (cf. Kolodziejczyk et al. 2020). NPSR genotype did not have the main effects on the extinction of conditioned social fear

(Publication 2, Figure 4B1). However, considering the pairwise comparisons between the responder and control group within the different genotypes, mild differences in NPSR +/− and NPSR −/− responder mice were observed. While NPSR +/+ mice needed four extinction tests to come back to the sociability level of unconditioned mice, NPSR +/− mice seemed to require more exposures and NPSR −/− mice fewer. NPSR +/− mice showed a tendency for lower sociability in the fourth and sixth extinction test and had significantly lower sociability in the fifth. That indicated a less pronounced extinction of conditioned social fear in these mice. In contrast, extinction of conditioned social fear was faster in NPSR −/− mice than in wild-type animals, i.e., already in the second extinction test, sociability was not different from the unconditioned mice, suggesting that NPSR −/− mice profit from NPSR deficiency. These results indicated that the extinction was impaired in heterozygous and facilitated in homozygous NPSR-deficient mice. Collectively these data suggest that complete inhibition of NPSR signaling to mimic the NPSR deficient mice may help facilitate the extinction of trauma-related fear memories. Hence, it can be an attractive therapeutic strategy for trauma-related disorders. However, the involvement of compensatory mechanism in NPSR -/- cannot be ruled out.

4.3.2 Orexin system and social fear conditioning

Here, I provide the first experimental evidence showing that operant conditioned fear and its extinction directed at stimuli of social interaction are impaired in orexin-deficient mice. I have demonstrated that orexin deficiency facilitated the acquisition and expression of conditioned social fear. OX +/− and OX −/− expressed more pronounced social avoidance resulted in an effect of conditioning as compared to OX +/+ mice, indicating the more robust acquisition and/or expression of conditioned social fear suggesting that orexin deficiency facilitated both phases (Publication 3, Fig. 3A1). Additionally, the extinction of conditioned social fear was also affected by the orexin genotype (Publication 3, Fig. 3B1). In detail, OX +/+ mice needed four extinction tests to achieve the same degree of sociability as unconditioned mice. However, OX −/− mice showed significantly decreased sociability in all extinction tests, and an intermediate phenotype was displayed by OX +/− mice. These findings showed that orexin deficiency leads not only to more pronounced conditioned social anxiety but also to a more stable and more extinction-resistant memory. Interestingly, genetic deletion or pharmacological blockade of OX1R (especially in AMY and LC) has impaired the acquisition and expression of classical conditioned fear and/or facilitated its extinction (Dustrude et al. 2018; Flores et al. 2017; Flores et al. 2014; Sears et al. 2013; Soya et al. 2013; Soya et al. 2017). These counterintuitive results can be due to several potential reasons. 1) It may be possible that orexin plays a very different role in classical conditioned fear as compared to operant conditioned fear, such as social fear. 2) Also, blocking orexin and orexin receptors may not give the same results as orexin also have another receptor (OX2R). 3) Blocking orexin receptors were acute interventions as compared to the orexin knockout mice that may have some form of developmental compensation integrated into the phenotype.

Furthermore, clinical trials in narcolepsy patients have indicated altered AMY activity during aversive conditioning (Khatami et al. 2007; Ponz et al. 2010). In this framework, narcolepsy patients and orexin-deficient mice, undergo narcoleptic episodes, frequently triggered by emotionally salient stimuli such as social interactions (Anic-Labat et al. 1999; Chemelli et al. 1999). Surprisingly, in the trials of this study, I never witnessed such narcoleptic episodes. However, I observed narcoleptic episodes in these mice, mostly in their active face (night) during rewarding activities. It has been shown that running activity and food reward in OX-/- mice are the very potent causes of narcoleptic

episodes (Durairaja and Fendt 2020; Leibiger and Fendt 2014).

Growing evidence from preclinical research suggests that the orexin system serves to incorporate signals of physical and psychological threats through its arousal-promoting properties to mobilize adaptive behavioral and physiological responses (Cohen et al. 2020; Johnson et al. 2012a). As such, multiple studies have shown that orexin plays an essential role in mediating social stress coping strategies (e.g., Eacret et al. 2019; Grafe et al. 2018; Ji et al. 2019; Staton et al. 2018). Although these studies used various experimental protocols (i.e., social defeat/rank stress), my results agree with their general hypothesis that orexin dysfunctions are correlated with psychosocial threat sensitivity and social avoidance (Ji et al. 2019; Staton et al. 2018), as I observed increased and sustained conditioned social fear in orexin-deficient mice. In addition to its role in threat adaptation, it has been suggested that orexin also promotes motivating actions towards opportunities for reward (Harris et al. 2005; Mahler et al. 2014). It is well known that social interactions are rewarding and highly motivating for rodents with conspecifics (Douglas et al. 2004; Panksepp et al. 2007). Therefore, my observations of decreased sociability and more pronounced conditioned social fear in mice with orexin deficiency support the orexin system's significant role in reward functions.

Presented data contribute to the body of information documenting that emotional learning and memory are regulated by both NPS and orexin systems and may be of translational importance due to their implication in the endophenotype of social dysfunctions, which is one of the hallmarks of several neuropsychiatric disorders (e.g., Chen et al. 2015; Dannlowski et al. 2011; Raczka et al. 2010).

4.4 Relationship between the NPS and orexin systems

Experiments performed in this thesis, along with previous work from several laboratories, have implicated NPS and orexins systems to be critically involved in AD-TSRD-related endophenotypes. Interestingly, there might be a functional overlap and cross-talk between these two systems. Hypothalamic regions where orexin neurons are located, including the LH, PFA, and DMH, are enriched with NPSRs (Clark et al. 2011). Moreover, NPS-positive cells were found to be surrounded by a dense orexin fiber network that suggests potential cross-communication between these two neuronal populations (Liu et al. 2011). Additionally, i.c.v. injection of NPS has been shown to induce fos expression in the orexinergic neurons in the LH, PFA, and DMH of rats, i.e., activate orexin neurons in these brain areas (Kallupi et al. 2010; Niimi 2006). It has also been shown that NPS is an upstream activator of hypothalamic orexin neurons in wakefulness, rewarding, stress-induced analgesia, alcohol-seeking, and alterations in food consumption behavior (e.g., Cannella et al. 2009; Cao et al. 2011; Lee et al. 2020; Niimi 2006; Zhao et al. 2012). Moreover, both these systems seem to share various other behavioral and physiological effects, such as their role in social behavior (e.g., Faesel et al. 2021; Kolodziejczyk et al. 2020; Yang et al. 2013; Zoicas et al. 2016), stress (e.g., Fendt et al. 2010; Ida et al. 2000; Johnson et al. 2012a; Jüngling et al. 2008), and regulate the physiological activity of the HPA axis (e.g., Kuru et al. 2000; Moreno et al. 2005; Reinscheid 2008; Smith et al. 2006). However, the orexin and NPS systems still represent two separate neuropeptide systems, and potential molecular mechanisms linking their functional cross-talk are still lacking.

Future experimental investigations using double knockout mice, acute perturbations affecting either of the systems and measuring the changes in another system, single-cell level investigations to

identify molecular signaling linking these two neuropeptide systems, the neuroanatomical overlap of their target neurons might shed light on this exciting area of research. The research demonstrated in this thesis will serve as an excellent base for future studies on the relation between these two neuropeptides systems, especially in the context of behavioral endophenotypes of overgeneralization and dysregulation in social (fear) behavior.

5 Limitations and outlook

Although this work provides valuable information for studying the complex role of the NPS and orexin systems and the interplay of stress hormones in fear generalization and social (fear) behavior, there are some limitations that should be addressed in future experiments. Using constitutive knockout mice is usually the most reliable scientific approach to understand the connection of a particular gene and manifested phenotype of the animals. However, such studies have one general limitation as knockout mice tend to develop compensatory mechanisms that might mask the effects related to lack of the gene. Hence, future research studies can focus on more acute perturbations done either by specific pharmacological agents or molecular tools, e.g., siRNA mediated knockdown, Crisper Cas, DREADDs.

Moreover, further work should be designed to dissect the most critical factors in the development of fear memory generalization, i.e., NPSR deficiency, incubation time, or the CORT levels. This can be achieved by measuring the generalization of fear memories in the same experimental setup lacking one or the other factor using different groups of mice. Moreover, a battery of additional behavioral paradigms should also be used to further understand the full spectrum of anxiety-like behavior and startle response both before and after the experimental timeline, to have a comprehensive understanding of behavioral outcomes.

Concerning work with the SFC paradigm, I observed a significant population of non-responder mice. More importantly, this proportion of non-responders was found in all genotypes of both NPSR and orexin deficient mice including both sexes, and was not affected by the number of foot shocks received during SFC suggesting the involvement of more general or universal phenomena. A potential explanation for this could be that non-responder mice did not react with avoidance after SFC but expressed a more active coping strategy, such as agonistic behavior (cf. De Miguel et al. 2011). Further work needs to be done to verify the explanation for these different performances in the first social fear extinction test (E1). Using more appropriate experimental conditions to measure agonistic behavior, e.g., in a paradigm of free social interaction, may help to explain that matter. Additionally, improving the rate of responder mice by increasing the foot shock intensities (cf. Toth et al. 2013) can be also implemented for future experiments. Moreover, further studies should be done to elucidate the functional relationship between neuropeptide systems and changes in levels of different monoamines along with molecular mechanisms governing microglia activation and their role in the development of AD-TSRD.

In summary, despite these limitations, I believe my work could be an excellent framework for studying the complex role of the NPS and orexin systems and the interplay of stress hormones in fear generalization and social (fear) behavior.

6 Conclusions

In conclusion, the present studies extend our knowledge about the complex role of the NPS and orexin systems and the interplay of stress hormones in fear generalization and various social behaviors, such as sociability, social novelty, and social fear. My work revealed that the 5 mg/kg CORT-treated NPSR-deficient mice developed the generalization of fear memories after one month of incubation time. These results highlight the significance of crucial elements that add to fear generalization, such as lack of the NPSR and incubation time, along with the specific CORT levels during the consolidation of fear memories. It is interesting to note that only the animals that manifested the generalization of fear memory also exhibited reduced startle response and sex-depended less body weight gain without any change in anxiety-related behavior.

My work also shows that complete loss of the NPSR gene does not affect the sociability, social novelty, and acquisition and expression of conditioned social fear behaviors. These data suggest that either the NPS system does not affect these behaviors, or these mice have developed some compensatory mechanisms to overcome the deficiency of the NPSR gene. Surprisingly, the partial loss of the NPSR gene only in the female mice reduced sociability behavior, suggesting the complex interaction of the NPSR system combined with sex-specific differences. Also, mentioned effects were specific to sociability but not to social novelty, highlights significant differences between the social behaviors paradigms. Furthermore, the extinction of social fear was differentially regulated by the lack of the NPSR gene. Partial loss of the NPSR gene leads to impaired extinction of social fear. However, complete loss of the NPSR gene facilitated the social fear extinction, suggesting that compensatory mechanism might even overcompensate the lack of NPSR, thereby leading to faster fear memories extinction.

Furthermore, my work also highlights the role of the orexin system in regulating social (fear) behavior. I observed that orexin deficiency leads to decreased sociability and preference for social novelty only in the female mice. Male mice with orexin deficiency were unaffected in these social behaviors, again highlighting the importance of sex-specific differences. Interestingly, the acquisition and the expression of the conditioned social fear were facilitated. However, the extinction of social fear was impaired in both sexes.

Overall, since overgeneralization of fear memory and reduced social behavior are behavioral endophenotypes of several neuropsychiatric disorders, my results contribute towards elucidating the mechanisms underlying the development of AD-TSRD. The presented data may help to explain why human polymorphisms in genes of the NPS and orexin systems are associated with mentioned behavioral endophenotypes and thereby also with a higher probability to develop one of AD-TSRD.

7 Abbreviations

5-HT	5-hydroxytryptaminergic
ACTH	Adrenocorticotropin
AD	Anxiety disorders
AD-TSRD	Anxiety disorders and trauma- and stressor-related disorders
AMY	Amygdala
AVP	Vasopressin
BLA	Basolateral amygdala
BZDs	Benzodiazepines
cAMP	Cyclic adenosine monophosphate
CBT	Cognitive-behavioral therapy
CeA	Central nucleus of the amygdala
CFC	Contextual fear conditioning
CGRP	Calcitonin gene-related peptide
CORT	Corticosterone
CRF or CRH	Corticotropin-releasing factor or Corticotropin-releasing hormone
CS	Conditioned stimulus
CTX	Conditioning context
D1R, D2R	Dopamine D1 receptor, Dopamine D2 receptor
DMH	Dorsomedial hypothalamus
DREADDs	Designer receptors exclusively activated by designer drugs
DSM-5	The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders
GAL	Galanin
GCs	Glucocorticoids
GPCR	G protein-coupled receptor
GRs	Glucocorticoid receptors
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High-performance liquid chromatography
Iba-1	Ionized calcium-binding adaptor protein-1
icv	Intracerebroventricular
LC	Locus coeruleus
LH	Lateral hypothalamus
MAP kinase	Mitogen-activated protein kinase
mRNA	Messenger RNA
NA	Noradrenaline
NAc	Nucleus accumbens
NPS	Neuropeptide S
NPSR	Neuropeptide S receptor
NPSR1	Neuropeptide S receptor gene
NPY	Neuropeptide Y
OXRs, OX1R, OX2R	Orexin receptors, Orexin 1 receptor, Orexin 2 receptor
OXT	Oxytocin
PFA	Perifornical area
PFC	Prefrontal cortex
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nuclei of the hypothalamus
RI test	Resident-intruder test
SA	Social anxiety
SAD	Social anxiety disorder
SAM	Stress alternatives model
SFC	Social fear conditioning

SNRIs	Selective serotonin-norepinephrine reuptake inhibitors
SP	Substance P
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
TSRD	Trauma- and stressor-related disorders
US	Unconditioned stimulus
VIP	Vasoactive intestinal peptide
Context A	Conditioning context; Publication 1
Context A'	Similar context to the conditioning context A; Publication 1
Context B	Different context than the conditioning context A; Publication 1
Tone T	Tone stimulus, which was presented during the fear conditioning session; Publication 1
Tone T'	Tone stimulus, which is similar to tone T; Publication 1
Tone N	Novel tone stimulus; Publication 1
ip	Intraperitoneal injection
BL I	Baseline CORT levels collected before the habituation phase; Publication 1
BL II	The second baseline CORT levels collected 1 day before the remote retention test (32^{nd} day); Publication 1
siRNA	Small interfering RNA
E1	First expression (extinction) test in SFC paradigm; Publication 2 & 3

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Appendix

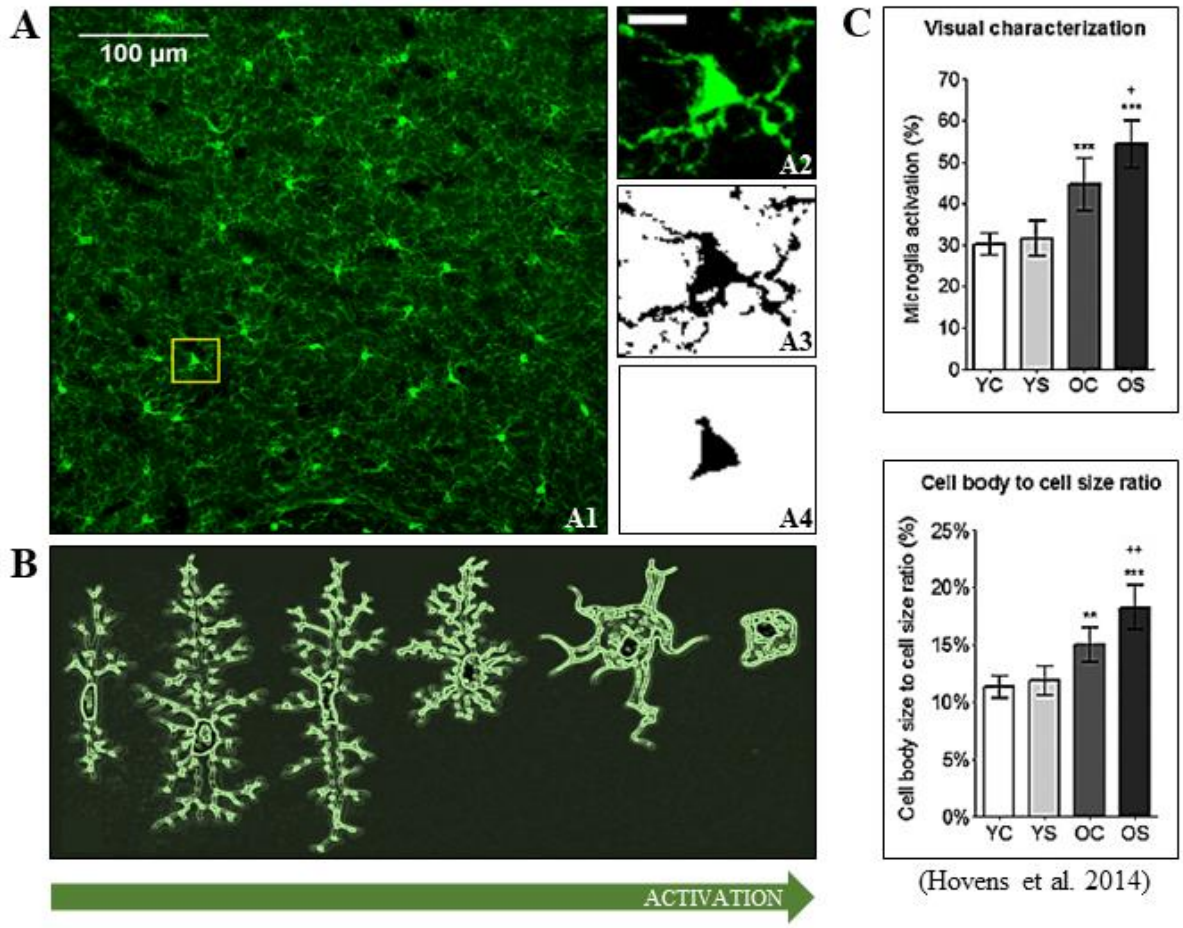

Fig. 1 Appendix. Method for evaluating microglial activation. (A) Representative image of Iba-1-positive cells and plots representing the method used for quantification of microglial activation using immunostaining for Iba-1 (Ionized calcium-binding adaptor protein-1). Iba-1 is a microglial marker that plays a role in actin-crosslinking involved in membrane ruffling of microglia, which exists in the cytoplasm of all microglia cells (e.g., Ahmed et al. 2007; Sasaki et al. 2001). A square area spanning a microglia cell was selected **(A1, A2)** and subjected to thresholding, converted to a binary signal, and automatically analyzed to count all pixels, which were darker than a background to calculate the total cell size of Iba-1-positive cells **(A3)**. Additionally, the same image was subjected to a more stringent threshold to remove all the processes to determine the total cell body size **(A4)**. The total size of dendritic processes was defined by subtracting the cell body size from the total cell size. To determine the area covered by activated microglia, the total cell size, total cell body size, and total size of dendritic processes of all microglia in the area of interest were calculated and normalized by the number of microglia in the sample area to gain the average size, cell body size, and size of dendritic processes per cell. Finally, the cell body to cell size ratio (%) was determined and utilized as an indicator of microglial activation. A higher ratio means more microglial activation. **(B)** Scheme showing different stages of microglia activation. When a threat signal is detected, microglia undergo a sudden change in morphology and function called activation (Kreutzberg 1996). Activated microglia have increased cell body size, retracted and thickening processes. Moreover, they become amoeboid cells capable of phagocytosis and start excreting cytokines (Eggen et al. 2013; Kettenmann et al. 2011; Kreutzberg 1996). **(C)** In her seminal article on the method for evaluating microglial activation, Hovens (Hovens et al. 2014) showed that the cell body to cell size ratio of microglia was strongly correlated to the visual characterization activation stage.

Ehrenerklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht.

Ich habe insbesondere nicht wissentlich:

- Ergebnisse erfunden oder widersprüchlich Ergebnisse verschwiegen,
- statistische Verfahren absichtlich missbraucht, um Daten in ungerechtfertigter Weise zu interpretieren,
- fremde Ergebnisse oder Veröffentlichungen plagiiert,
- fremde Forschungsergebnisse verzerrt wiedergegeben.

Mir ist bekannt, dass Verstöße gegen das Urheberrecht Unterlassungs- und Schadensersatzansprüche des Urhebers sowie eine strafrechtliche Ahndung durch die Strafverfolgungsbehörden begründen kann.

Ich erkläre mich damit einverstanden, dass die Arbeit ggf. mit Mitteln der elektronischen Datenverarbeitung auf Plagiate überprüft werden kann.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form als Dissertation eingereicht und ist als Ganzes auch noch nicht veröffentlicht.

Magdeburg, 20.01.2021

Malgorzata Helena Kolodziejczyk